53BP1-mediated recruitment of RASSF1A to ribosomal DNA breaks promotes local ATM signaling

Stavroula Tsaridou, Georgia Velimezi, Frances Willenbrock, Maria Chatzifrangkeskou, Waheba Elsayed, Andreas Panagopoulos, Dimitris Karamitros, Vassilis Gorgoulis, Zoi Lygerou, Vassilis Roukos, Eric O'Neill, and Dafni-Eleftheria Pefani

DOI: 10.15252/embr.202154483

Corresponding author(s): Dafni-Eleftheria Pefani (dpefani@upatras.gr) , Eric O'Neill (eric.oneill@oncology.ox.ac.uk)

**Review Timeline:**

| Event                        | Date       |
|------------------------------|------------|
| Submission Date              | 9th Dec 21 |
| Editorial Decision           | 20th Jan 22|
| Revision Received            | 18th Apr 22|
| Editorial Decision           | 26th May 22|
| Revision Received            | 30th May 22|
| Accepted                     | 3rd Jun 22 |

*Editor: Esther Schnapp*

**Transaction Report:**

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

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

As you will see, none of the referees is very enthusiastic about the study. They point out that important controls are missing, and that the data are not sufficiently convincing or conclusive. This is a borderline case, and we can only proceed with the manuscript here if the data can be significantly strengthened.

The most important concern is that it remains unclear whether RASSF1A localization and its role in repair are specific to rDNA loci. The referees further point out that several important controls are missing, that it is counterintuitive that 53BP1 promotes HR repair, that it should be demonstrated whether loss of BRCA1 impacts 53BP1 localization to nucleolar caps, that it should be shown whether loss of RPA and RAD51 binding to nucleolar caps is a result of RASSF1A depletion-induced ATM signaling, and that it remains unclear why RASSF1A depletion increases rDNA copy numbers. I think that all referee concerns would need to be successfully addressed in order to proceed with the study here. If you like, we could also discuss the revisions in a video chat.

If you decide to embark on such revisions, I would like to invite you to revise your manuscript with the understanding that the referee concerns 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 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 (22nd Apr 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

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

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.

3) 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>

- Additionally, 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.

4) 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.

5) 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
6) 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>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/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 & Method (see also 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. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also 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

10) Regarding data quantification (see Figure Legends: https://www.embopress.org/page/journal/14693178/authorguide#figureformat)

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} 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.
- Please also include scale bars in all microscopy images.

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

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."
I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The manuscript entitled "53BP1 mediated recruitment of RASSF1A at ribosomal DNA breaks promotes local ATM signaling" by Tsaridou and colleagues documents the finding that RASSF1A is recruited to DNA damage-induced foci. The authors conclude the RASSF1A is recruited to DSBs by 53BP1, but it is not convincingly demonstrated that the RASSF1A-53BP1 interaction is direct. RASSF1A depleted cells are sensitive to IR and accumulate persistent DSBs and extranumeral rDNA copies although a potential mechanism for the latter observation is not clear. The authors promote the idea that RASSF1A is particularly important for nucleolar DNA repair. However, I am not convinced that RASSF1A foci are restricted or even enriched in nucleoli (Figure 1E). At the end of the day, there are too many loose ends to support the model in Figure 6 and publication of the manuscript in its current state.

Major concerns:
1. What is the evidence for the specificity of the RASSF1A antibody in immunostaining? How do cells stain after knockdown or knockout of RASSF1A?
2. Figure 3H and EV4N: The is no mention of DNAse treatment in the methods section. Hence, it is possible that the interaction is bridged by DNA. Please repeat with Dnase treatment. Same problem with 3I and 3J.
3. Fig EV4E, EV4F and EV4G: Please show the western blot to document successful knockdown of 53BP1.
4. Fig 4H: Please show the western blot to document successful knockdown of RASSF1A and 53BP1.
5. Page 13: I don't find the data on RPA1 foci after RASSF1A knockdown in Figure 5C.
6. What is the phenotype and localization of a mutant of RASSF1A lacking the SARAH domain?

Minor corrections:
1. Figure 1A and Discussion: The authors previous reported that RASSF1A was found in the nucleolus independent of the presence of damage (Pefani et al, 2018). This seems to contradict Figure 1A, where RASSF1A appears to be mostly excluded from the nucleolus.
2. Page 12, top: I don’t quite follow the logical of the conclusion “recruitment is independent of the source of the lesion or site of damage” from the first half of that sentence. Perhaps rephrase?
3. Figure EV5E: the I-PpoI should fully span the last 6 columns?
4. Bottom of page 12: EV4G written twice.
5. It is somewhat confusing that the text does not follow the order of those figures.
6. Page 13, bottom: change “we persistent” to "with persistent".
7. Figure 5A, legend: What is the time from siRNA transfection to I-PpoI mRNA transfection?
8. Figure 5D: How do the authors explain that the number of rDNA increases in RASSF1A depleted cells?
9. Page 16: After the sentence “Further analysis showed that RASSF1A gets phosphorylated upon rDNA DSB formation by ATM at Serine 131” please provide the appropriate reference (Hamilton et al, 2009).
10. Page 16, middle: What does “establishment of RASSF1A” mean?
11. Page 16, middle: What is meant by “stability of the recombinase”? The wording suggests that RAD51 is degraded, while the referenced paper reported a defect in RAD51 foci. Please rephrase or clarify.
12. Page 17, top: Please define "nucleolar signal establishment" and "RASSF1A signal".

References

Hamilton G, Yee KS, Scrace S, O'Neill E (2009) ATM regulates a RASSF1A-dependent DNA damage response. Curr Biol 19: 2020-2025

Pefani DE, Tognoli ML, Pirincci Ercan D, Gorgoulis V, O'Neill E (2018) MST2 kinase suppresses rDNA transcription in response to DNA damage by phosphorylating nucleolar histone H2B. EMBO J 37

Referee #2:
This article by Tsaridou et. al. reports that RASSF1A is recruited to sites of ribosomal double-strand break. The authors present compelling evidence that this localization is mediated in an ATM- and 53BP1-dependent manner. Authors suggest that this recruitment promotes HR-dependent repair, but this reviewer believes more evidence and clarity is needed, see below. Overall, this manuscript advances our understanding of how RASSF1A contributes to genomic integrity. However, there are a few caveats and missing controls that I believe are important to address before acceptance of this manuscript.

Major Points:

Fig.1: Authors want to emphasize that RASSF1A is mostly recruited to rDNA breaks, but there is no direct evidence to support this finding. There are various nuclease directed systems that are widely used in the field, such as mCherry-LacI-FokI nuclease or TRF-FokI nuclease. It's imperative that authors use these systems to examine if RASSF1A localization and its role in repair are specific to rDNA locus.

Fig.4C-D: Role of 53BP1 in negatively regulating HR is well established in the field. In this context, it is counterintuitive why 53BP1-dependent RASSF1A recruitment would promote HR repair. Also does the loss of other anti-resection proteins like Shieldin or Rev7 result in a similar phenotype?

On a similar note, authors report that loss of BRCA1 does not impact RASSF1A recruitment to nucleolar caps (Fig. EV4K and EV4L). It is well established that BRCA1 loss increases 53BP1 at breaks. Does loss of BRCA1 impact 53BP1 localization to the nucleolar caps? Addressing this is critical to examine if 53BP1 contributes to regulating resection at rDNA breaks.

Fig.5:

#1. It is unclear why in cell-based experiments authors have examined 18S and 28S copy number but in patient analysis they have examined 45S rDNA copy number.

#2 Authors demonstrate that loss of RASSF1A impacts rDNA copy number and compromised viability upon I-Ppol induction. It remains unclear if this phenotype is owing to HR-mediated repair or the transcription regulation function of RASSF1A. Examining these phenotypes in siMST2 and si53BP1 conditions would perhaps help address this issue.

#3 Does loss of RASSF1A lead to reduced viability when breaks are directed to other genomic loci? Using mCherry-LacI-FokI nuclease or TRF-FokI nuclease would be helpful.

Minor points:

• Fig1D: Expand NCL
• Fig1E: um should be µM.
• Pg.4 Expand MEOH
• Methods: Please include the dilutions of antibody used.
• Include the reference for Baldock et al, 2015 (PMID:26628370) and Kleiner et al, 2015 (PMID: 26344695) when discussing a role of 53BP1 in heterochromatin repair.

Referee #3:

In this study Tsaridou et al., describe a follow up role of RASSF1A in nucleolar integrity. The results are very well documented and the paper is well written. Nevertheless I have some issues with the interpretation of the results and some minor comments on the Figures that the authors should take care before publication.

-Interpretation:
The authors interpret the lack of RPA and RAD51 binding to nucleolar caps as a result of the RSSF1A depletion on ATM signaling. Do they have any direct evidence that this is indeed the case? How does the RSSF1A mutant which does not bind 53BP1 behave under these conditions? Is it possible that this is due to the partial effect on the formation of nucleolar caps?

-In Figure 5D the authors observe not only a rescue of loss of nucleolar repeats but some amplification? Is this something consistent and could be due to elevated SSA between the repeats?

Minor comments:
-In many images the authors use a line to help visualise the distance between foci. The presence of the line does not allow to clearly see the signal below and I would recommend that they remove it.
Many of the images seem saturated and the authors should check the saturation levels. In other instances the authors use only merged images and not the individual channels. I recommend that they provide single channels for clarity.
We would like to thank the reviewers for the constructive comments and criticism. In our revised manuscript we have addressed their comments and we now provide all the requested data that further support our model. Please find below a point-by-point reply to the raised comments:

Referee #1:

The manuscript entitled “53BP1 mediated recruitment of RASSF1A at ribosomal DNA breaks promotes local ATM signaling” by Tsaridou and colleagues documents the finding that RASSF1A is recruited to DNA damage-induced foci. The authors conclude the RASSF1A is recruited to DSBs by 53BP1, but it is not convincingly demonstrated that the RASSF1A-53BP1 interaction is direct. RASSF1A depleted cells are sensitive to IR and accumulate persistent DSBs and extranumeral rDNA copies although a potential mechanism for the latter observation is not clear. The authors promote the idea that RASSF1A is particularly important for nucleolar DNA repair. However, I am not convinced that RASSF1A foci are restricted or even enriched in nucleoli (Figure 1E). At the end of the day, there are too many loose ends to support the model in Figure 6 and publication of the manuscript in its current state.

To further clarify whether there is preferential recruitment of RASSF1A at a subset of breaks, we have employed a reporter cell system to induce breaks in a cassette that is randomly introduced to two different chromosomal loci (Fig 1H and 1I). We employed a Lac-repressor/operator-tethering system which consist of approximately 10 kb tandem arrays of the Lac operator (LacO) sequence adjacent to an I-Scel endonuclease site stably integrated into human U2OS cells at two random chromosomal locations. Exogenous expression of I-Scel results in break induction in the vicinity of the LacO repeats. Lac Repressor (LacR) fused with a fluorescent protein is exogenously expressed to mark the LacO sites (Burgess et al, 2014). In this reporter system, we find increased occupancy of 53BP1 at the lacO arrays upon break induction by I-Scel. On the other hand, RASSF1A is not found at DSBs generated by I-Scel, indicating that chromatin context may be important for recruitment of the scaffold. In a time-course following ionizing radiation (Fig 1A) we consistently observe that RASSF1A is only recruited to a subset of breaks, and not due to delayed recruitment kinetics. We present evidence that a location of these sites are rDNA breaks (Fig 1F, 1J, EV2E and EV2F). Given that rDNA is an emerging fragile site and our previous observations that RASSF1A is involved in the nucleolar DNA damage response (Pefani et al, 2018) we decided to focus on the characterization of RASSF1A function in rDNA break repair. However, as highlighted by this reviewer our data indicates that rDNA loci are not the only recruitment sites of the scaffold, we have carefully edited our text to make clear that rDNA breaks are not the only break recruitment break sites we identify. However, the results of the reporter system indicate that recruitment specificity exists, and chromatin context could play a role in that as discussed in page 20.
In our revised MS we also highlight that in the interaction analysis between RASSF1A and 53BP1 benzonase was added in the cell lysis buffer to make sure that co-immunoprecipitation did not result from bridging DNA. Our colocalization analysis (Fig 3A, 3B and EV2C) and loss of RASSF1A recruitment upon 53BP1 depletion (Fig 3C and 3D) are also indicative of a functional relationship between 53BP1 and RASSF1A. In our revised MS we have also included data that shows that loss of ATM-pS1981 evident upon 53BP1 and RASSF1A depletion is rescued upon re-expression of full length RASSF1A but not from re-expression of a deletion mutant that lacks the SARAH domain, necessary for interaction with 53BP1 (Fig 4O and 4P). We have also extended our analysis for the impact of ATM inhibition in DNA end resection and Homologous Recombination at nucleolar caps and find that RASSF1A depletion phenocopies loss of ATM signaling further supporting a role of the scaffold in the establishment of active ATM signaling at nucleolar caps (Fig 5A, 5B, EV4R and EV4S). We also further discuss our findings on rDNA copy number discrepancies upon deletion of RASSF1A and provide further explanations.

Please find below a point-by-point reply in the critique raised by Reviewer 1:

**Major concerns:**

1. **What is the evidence for the specificity of the RASSF1A antibody in immunostaining? How do cells stain after knockdown or knockout of RASSF1A?**

In Fig EV4J we show images stained with RASSF1A (Atlas, HPA040735) antibody of siRNA control treated and cells treated for two different siRNAs against RASSF1A after l-Ppol mRNA transfection. The signal generated at the nucleolar caps by the RASSF1A antibody is significantly reduced in cells treated with both siRNAs. We have also validated the antibody in western blot for lysates from control and siRASSF1A treated cells (EV4K and EV4Q). Please note that the efficacy of the siRNAs has also been validated in Western Blot with the RASSF1A (3F3, Santa Cruz sc-585770) antibody. Please also note that in immunoprecipitation experiments where RASSF1A (Atlas, HPA040735) or RASSF1A (3F3, Santa Cruz sc-585770) antibodies were used to pull down endogenous RASSF1A, at western blot analysis a band at the expected molecular weight, was detected in the RASSF1A (Atlas, HPA040735) IPs and RASSF1A (3F3, Santa Cruz sc-585770) IPs using RASSF1A (Atlas, HPA040735) antibody. Please see example below:
3. **Figure 3H and EV4N**: The is no mention of DNAse treatment in the methods section. Hence, it is possible that the interaction is bridged by DNA. Please repeat with Dnase treatment. Same problem with 3I and 3J.

We apologize for not providing this information in our methods section. Immunoprecipitation lysis buffer contains 750 U/ml Benzonase. We have now included this information in the description of Immunoprecipitation protocol in method section. We have also provided more details on sonication and centrifuging steps of the Immunoprecipitation protocol followed in this study.

3. **Fig EV4E, EV4F and EV4G**: Please show the western blot to document successful knockdown of 53BP1.

Requested blots are shown in EV3I, EV3K.

4. **Fig 4H**: Please show the western blot to document successful knockdown of RASSF1A and 53BP1.

Requested blot is shown in EV4Q.

5. **Page 13**: I don't find the data on RPA1 foci after RASSF1A knockdown in Figure 5C.

The correct Figure would be 4C in the original MS, we apologize for this error in editing. Please find data on RPA positive caps upon knock down of RASSF1A in Fig 5C of the revised MS. Please note that in the revised MS we have extended our analysis on ssDNA and we also provide data for native BrdU staining upon siRASSF1A in Fig 5J and 5K. We also provide data to assess DNA end resection and RAD51 recruitment at nucleolar caps upon inhibition of ATM and 53BP1 depletion (Fig 5A, 5B, 5H, 5I, 5J and 5K).
6. What is the phenotype and localization of a mutant of RASSF1A lacking the SARAH domain?

In our revised MS we have included localization analysis of the RASSF1A mutant that lacks the SARAH domain and assessed nucleolar ATM-pS1981 upon re-expression of full length and SARAH domain deleted RASSF1A constructs in cells depleted for RASSF1A (Fig 4M, 4N, 4O and 4P). We find limited presence of the SARAH domain deletion mutant in the chromatin bound fraction upon induction of rDNA damage (Fig 4N) and reduced ability to re-establish ATM-pS1981 in siRASSF1A treated (Fig 4O and 4P).

Minor corrections:

1. **Figure 1A and Discussion:** The authors previously reported that RASSF1A was found in the nucleolus independent of the presence of damage (Pefani et al, 2018). This seems to contradict Figure 1A, where RASSF1A appears to be mostly excluded from the nucleolus. We hypothesize that this discrepancy is due to the different methodologies used in the two studies. Please note that when we increase exposure during imaging, we detect RASSF1A signal in the nucleolar interior, however this results in saturating the signal at the nucleus/cytoplasm. Please see example below.

2. **Page 12, top:** I don’t quite follow the logical of the conclusion ”recruitment is independent of the source of the lesion or site of damage” from the first half of that sentence. Perhaps rephrase? We have rephrased to “Inhibition of ATM signaling or depletion of the 53BP1 adaptor also resulted in reduced RASSF1A foci formation in irradiated cells, indicating that RASSF1A recruitment dependency on the ATM-53BP1 axis is independent of the source of damage”.

3. **Figure EV5E:** the I-PpoI should fully span the last 6 columns? Corrected.

4. **Bottom of page 12:** EV4G written twice.
5. It is somewhat confusing that the text does not follow the order of their figures.
We have changed the order of the figures to make sure we follow the order in the text. At certain occasions we refer to figure panels that were presented earlier in the MS if they additionally support experimental conclusions that are presented later in the text.

6. Page 13, bottom: change "we persistent" to "with persistent".
Corrected.

7. Figure 5A, legend: What is the time from siRNA transfection to I-Ppol mRNA transfection?
Noted in the Figure legend.

8. Figure 5D: How do the authors explain that the number of rDNA increases in RASSF1A depleted cells?

Please note that this is not a robust statistically significant increase. Please find below the P values for each primer set and siR1A that derive from 3 biological replicates.

| Primer set | Conditions                  | P value |
|------------|-----------------------------|---------|
| #1         | CON siLUC vs I-Ppol siR1A_1 | 0.14    |
| #1         | CON siLUC vs I-Ppol siR1A_2 | 0.24    |
| #2         | CON siLUC vs I-Ppol siR1A_1 | 0.06    |
| #2         | CON siLUC vs I-Ppol siR1A_2 | 0.02    |

However, we do recognize the trend. Several factors could contribute to this trend, including the impact of endogenous rDNA damage prior to I-Ppol induction or the previously described role of RASSF1A in regulating ssDNA stability at stalled replication forks (Pefani et al., 2014). In page 23 we highlight the observed trend and offer potential explanation.

9. Page 16: After the sentence "Further analysis showed that RASSF1A gets phosphorylated upon rDNA DSB formation by ATM at Serine 131" please provide the appropriate reference (Hamilton et al, 2009).

Reference added.
10. Page 16, middle: What does "establishment of RASSF1A" mean?

Changed to "recruitment".

11. Page 16, middle: What is meant by "stability of the recombinase"? The wording suggests that RAD51 is degraded, while the referenced paper reported a defect in RAD51 foci. Please rephrase or clarify.

Changed to "decreased formation of RAD51 nucleofilaments at nucleolar caps" to avoid confusion.

12. Page 17, top: Please define "nucleolar signal establishment" and "RASSF1A signal".

Changed to "facilitating the concentration of DNA repair factors" and to "antibody signal in immunofluorescence experiments".

Referee #2:

This article by Tsaridou et. al. reports that RASSF1A is recruited to sites of ribosomal double-strand break. The authors present compelling evidence that this localization is mediated in an ATM- and 53BP1-dependent manner. Authors suggest that this recruitment promotes HR-dependent repair, but this reviewer believes more evidence and clarity is needed, see below. Overall, this manuscript advances our understanding of how RASSF1A contributes to genomic integrity. However, there are a few caveats and missing controls that I believe are important to address before acceptance of this manuscript.

In our revised MS we provide additional experimental data using a reporter system suggested by the reviewer to assess RASSF1A recruitment at DSBs targeted at other sites than rDNA. We have also performed a series of experiments to assess rDNA end resection upon depletion of 53BP1 or downstream anti-resection factors known to co-operated with 53BP1 and compare the resection phenotype observed in 53BP1 depleted cells with RASSF1A depletion or ATM signal inhibition.

Please find below a point-by-point reply in the critique raised by Reviewer 2:

Major Points:

Fig.1: Authors want to emphasize that RASSF1A is mostly recruited to rDNA breaks, but there is no direct evidence to support this finding. There are various nuclease directed systems that are widely used in the
field, such as mCherry-LacI-FokI nuclease or TRF-FokI nuclease. It's imperative that authors use these systems to examine if RASSF1A localization and its role in repair are specific to rDNA locus.

We employed a Lac-repressor/operator-tethering system which consist of approximately 10 kb tandem arrays of the Lac operator (LacO) sequence adjacent to an I-SceI endonuclease site stably integrated into human U2OS cells at two random different chromosomal locations (Burgess et al., 2014). Exogenous expression of I-SceI results in break induction in the vicinity of the LacO repeats. In this system, we observed increased 53BP1 accumulation at the I-SceI containing arrays (marked by LacR-mcherry) upon expression of GFP-I-SceI (Fig 1H and 1I), however recruitment of RASSF1A at the arrays was not detected under these conditions (Fig 1H an 1I). These findings are in agreement with RASSF1A being recruited in a subset of breaks possibly dictated by the chromatin environment.

Please note that we do observe RASSF1A recruitment at sites other than rDNA breaks (Fig 1A), we have altered our text to make sure that we are not reporting exclusive recruitment at rDNA sites. However, data derived from a time-course following ionizing radiation (Fig 1A) shows consistently RASSF1A in a subset of breaks in agreement with the data derived from the LacR-I-SceI reporter system, indicating that chromatin context may be important for recruitment the recruitment of the scaffold.

Given that rDNA is an emerging fragile site and our previous observations that RASSF1A is involved in the nucleolar DNA damage response (Pefani et al., 2018) we decided to focus on the characterization of RASSF1A function in the rDNA break repair.

*Fig.4C-D: Role of 53BP1 in negatively regulating HR is well established in the field. In this context, it is counterintuitive why 53BP1-dependent RASSF1A recruitment would promote HR repair. Also does the loss of other anti-resestation proteins like Shieldin or Rev7 result in a similar phenotype?*

This is a very intriguing point. 53BP1 has a well-established role as an anti-resestation protein. We therefore examined RPA nucleolar cap formation (Fig 5H and 5I) and ssDNA accumulation (Fig 5J and 5K) at nucleolar caps of damaged nucleoli upon depletion of 53BP1. Despite decreased ATM-pS1981 at nucleolar caps of 53BP1 depleted cells (Fig 4C and 4D) we observed increased ssDNA accumulation (Fig 5J and 5K). Depletion of REV7 also results in robust RPA foci formation at nucleolar caps, whilst ATM-pS1981 establishment did not get affected (Fig EV5A and EV5B). This data indicates that ATM signal establishment at nucleolar caps could be independent of the 53BP1-RIF1-REV7-Shieldin axis that regulates end resestation at the sites of damage.

We and others have shown that ATM inhibition leads to reduced RPA foci formation at nucleolar caps indicative of limited DNA end resestation (Korsholm et al, 2019; Mooser et al, 2020) (Fig 5A, 5B, EV4R and EV4S). We show here that depletion of RASSF1A results in reduced ATM-pS1981 (Fig 4E and 4G) and phenocopies ATM inhibition that results in reduced establishment of RPA and RAD51 at nucleolar caps (Fig 5A, 5B, 5D and 5F).
Despite necessity for ATM signal establishment at nucleolar caps, depletion of the upstream regulator 53BP1 does not recapitulate the phenotype of ATM inhibition or RASSF1A depletion in DNA end resection. This data indicates that depletion of 53BP1, an upstream regulator of the rDNA damage response, establishes a different landscape during rDNA repair and when depleted concentrated ATM signaling becomes dispensable for DNA end resection.

We have also previously reported that RASSF1A protects stalled replication forks from extended resection via BRCA2 stabilization of RAD51 foci (Pefani et al, 2014). Therefore, we cannot exclude that perturbed regulation of RAD51 nucleofilament stability in the absence of RASSF1A also affects ssDNA stability at rDNA damage sites.

We have included a paragraph (pages 22-23) where we discuss our findings and highlight how 53BP1 focused studies could shed light on the significance of 53BP1 recruitment at nucleolar caps, a site that previous studies have shown that NHEJ does not take place (Harding et al, 2015; van Sluis & McStay, 2015).

In future 53BP1 focused studies would be interesting to clarify how 53BP1 balances end resection and ATM signal activation at rDNA sites and potentially regulate choice between HR and alternative end joining pathways, as has been previously described in response to ionizing radiation (Ochs et al. 2016). Despite appreciating the significance of understanding the function of upstream regulator 53BP1 at nucleolar caps, we feel that this goes beyond the scope of this study which aims to characterise RASSF1A recruitment and role at the sites of damage.

On a similar note, authors report that loss of BRCA1 does not impact RASSF1A recruitment to nucleolar caps (Fig. EV4K and EV4L). It is well established that BRCA1 loss increases 53BP1 at breaks. Does loss of BRCA1 impact 53BP1 localization to the nucleolar caps? Addressing this is critical to examine if 53BP1 contributes to regulating resection at rDNA breaks.

In Figures EV4G, EV4H and EV4I we have assessed the impact of siBRCA1 in 53BP1 recruitment. We did not observe a difference in the number of cells with positive 53BP1 nucleolar caps in agreement with previous observations that the 53BP1 is present at all damaged nucleoli independent of cell cycle stage (van Sluis & McStay, 2015), however siBRCA1 cells show increased 53BP1 nuclear intensity indicating increased recruitment in agreement with data presented with IR-induced damage. Please see above our insight on 53BP1 as an upstream and multifunctional regulator of the nucleolar DNA damage response.

Fig.5:

#1. It is unclear why in cell-based experiments authors have examined 18S and 28S copy number but in patient analysis they have examined 45S rDNA copy number.
We have now included a schematic representation of the 45S rDNA repeat to avoid confusion (Fig 6D). Please note that the 45S rDNA repeat unit encodes a 45S rRNA precursor, transcribed by RNA polymerase I, which is processed to form the 18S, 5.8S and 28S rRNAs. In our in-cell analysis for rDNA copy number we designed two primer sets that bind to the 45S rDNA repeat at the sites marked in Fig 6D. One primer set binds within the genomic region that after processing will result in 18S rRNA and the other one within the genomic region that after processing will result in 18S rRNA. To avoid confusion, we also changed to primer sets #1 and #2 instead of 18S and 28S.

Authors demonstrate that loss of RASSF1A impacts rDNA copy number and compromised viability upon I-Ppol induction. It remains unclear if this phenotype is owing to HR-mediated repair or the transcription regulation function of RASSF1A. Examining these phenotypes in siMST2 and si53BP1 conditions would perhaps help address this issue.

Data for rDNA copy number following rDNA repair for cells depleted for siMST2 is presented in Fig EV5H. Data for cell viability after exposure to rDNA damage in siMST2 and si53BP1 treated cells are shown in Fig EV5F. In agreement with our previous report (Pefani et al., 2018) we find reduced cell viability of cells depleted for MST2 kinase, potentially due to inadequate Pol I inhibition during rDNA repair. Please note that due to significantly compromised cell viability in si53BP1 treated cells in response to rDNA breaks (Fig EV5F), we were unable to collect adequate numbers of cells 96 hours post rDNA break induction to perform rDNA copy number analysis.

Does loss of RASSF1A lead to reduced viability when breaks are directed to other genomic loci? Using mCherry-LacI-FokI nuclease or TRF-FokI nuclease would be helpful.

We used the LacO-I-SceI system to assess whether induction of a break site within the integrated loci could result in reduced cell viability and the impact of RASSF1A depletion. Please note that we did not observe compromised cell viability upon expression of the I-SceI endonuclease for induction of DSBs in either siLUC or siRASSF1A treated cells. Therefore, we cannot draw conclusions on whether retained viability upon DSB formation in the LacO-I-SceI system is due to tolerable levels of damage or redundancy of RASSF1A for repair under these conditions.
Clonogenic survival assay of U2OS cells with stable integration of a Lac operator (LacO) sequence adjacent to an I-SceI endonuclease site transfected with Lac Repressor (LacR)-mcherry and GFP or GFP-I-SceI-NLS. Survival ratio GFP/I-SceI-GFP in each siRNA condition is presented.

Minor points:

• **Fig1D: Expand NCL**
  Due to space limitation, we have kept the acronym. We explain abbreviations in the Figure legend.

• **Fig1E: um should be μM.**
  Corrected.

• **Pg.4 Expand MEOH**
  Corrected.

• **Methods: Please include the dilutions of antibody used.**
  Antibody dilutions added.

• **Include the reference for Baldock et al, 2015 (PMID:26628370) and Kleiner et al, 2015 (PMID: 26344695) when discussing a role of 53BP1 in heterochromatin repair.**
  References are now included.

Referee #3:

In this study Tsaridou et al., describe a follow up role of RASSF1A in nucleolar integrity. The results are very well documented and the paper is well written. Nevertheless I have some issues with the interpretation of the results and some minor comments on the Figures that the authors should take care before publication.

In the revised version of our MS we have included additional analysis of ATM inhibition to assess the impact on RPA and RAD51 establishment at nucleolar caps of damaged nucleoli (Fig 5A and 56B, EV4R and
EV4S) and discuss our findings in more detail. We also provide experimental data to assess recruitment and function of the RASSF1A mutant that lacks the SARAH domain and does not interact with 53BP1 (Fig 4M, 4N, 4O and 4P). We also discuss potential involvement of alternative end joining pathways in rDNA repair and the impact they may have in rDNA copy number upon rDNA break repair.

Please find below a point-by-point reply in the critique raised by Reviewer 3:

-Interpretation:
The authors interpret the lack of RPA and RAD51 binding to nucleolar caps as a result of the RASSF1A depletion on ATM signaling. Do they have any direct evidence that this is indeed the case?
Previous studies have shown that ATM inhibition results in limited RPA recruitment at nucleolar caps (Korsholm et al., 2019; Mooser et al., 2020). We also confirm that upon ATM inhibition there is a significant decrease in RPA and RAD51 establishment at nucleolar caps of damaged nucleoli (Fig 6A and 6B, EV4R and EV4S). In agreement with RASSF1A involved in ATM signal regulation we find that depletion of RASSF1A results in decreased ATM and ATM-pS1981 and downstream KAP1-pS821 at nucleolar caps of damaged nucleoli (Fig 4E, 4F, 4G and EV4O and EV4P). Given limited establishment of ATM-pS1981 at nucleolar caps of siRASSF1A treated cells and the fact that RASSF1A depletion phenocopies the lack of RPA and RAD51 recruitment at nucleolar caps, we propose a working hypothesis where RASSF1A contributes to the establishment of local ATM signaling upon rDNA break formation. We discuss these findings in detail in pages 22-23.

How does the RASSF1A mutant which does not bind 53BP1 behave under these conditions?
In our revised MS we have included localization analysis of the RASSF1A mutant that lacks the SARAH domain and assessed nucleolar ATM-pS1981 upon re-expression of full length and SARAH domain deleted RASSF1A constructs in cells depleted for RASSF1A (Fig 4M, 4N, 4O and 4P). We find limited presence of the SARAH domain deletion mutant in the chromatin bound fraction upon induction of rDNA damage (Fig 4N) and reduced ability to re-establish ATM-pS1981 in siRASSF1A treated (Fig 4O and 4P).

Is it possible that this is due to the partial effect on the formation of nucleolar caps?
To assess whether partial segregation in RASSF1A depleted cells would affect exposure of rDNA in nucleolar periphery we have performed rDNA ImmnoFISH using an rDNA specific probe and stained for UBF, a marker for nucleolar segregation (Fig 4I). We observed robust mobilization of rDNA in the nucleolar periphery (Fig 4I) despite defective nucleolar segregation based on UBF staining (Fig 4I, EV4M and EVN). Moreover, knockdown of RASSF1A did not impact on 53BP1 establishment at nucleolar caps (Fig 4K and 4L), indicating that there is a platform for DNA repair factor binding in RASSF1A depleted cells.
In Figure 5D the authors observe not only a rescue of loss of nucleolar repeats but some amplification? Is this something consistent and could be due to elevated SSA between the repeats?

Please note that there is no robust statistically significant increase. Please find below the P values that derive from 3 biological replicates.

| Primer set | conditions            | P value |
|------------|-----------------------|---------|
| #1         | CON siLUC vs I-Ppol siR1A_1 | 0.14    |
| #1         | CON siLUC vs I-Ppol siR1A_2 | 0.24    |
| #2         | CON siLUC vs I-Ppol siR1A_1 | 0.06    |
| #2         | CON siLUC vs I-Ppol siR1A_2 | 0.02    |

However, we do recognize the trend. Several factors could contribute to this trend, including the impact of endogenous rDNA damage prior to I-Ppol induction or the previously described role of RASSF1A in regulating ssDNA stability at stalled replication forks (Pefani et al., 2014). In page 23 we have included a short paragraph to highlight the trend and offer potential explanation.

In the discussion session we now discuss the potential contribution of alternative end joining pathways in rDNA repair and rDNA copy number (pages 22-23). Given the repetitive nature of the rDNA loci alternative end joining pathways (SSA, MMEJ) could contribute to break repair and rDNA copy number discrepancies. Previous studies have reported RAD52 recruitment at nucleolar caps (van Sluis & McStay, 2015) indicating that SSA may contribute to rDNA break repair. Future studies focused on alternative and joining pathways in rDNA repair could give further insight on how rDNA repair is organized and how rDNA copy number variation arises.

Minor comments:

-In many images the authors use a line to help visualise the distance between foci. The presence of the line does not allow to clearly see the signal below and I would recommend that they remove it.

Please note that the transparent line presented in single channel images is used to illustrate the area the fluorescent intensity is measured to create the fluorescent intensity profiles to show colocalization. Therefore, we believe is important to show where the data shown in intensity profiles derive from. We have now made sure that merged images do not contain any lines and individual channel images also contain other foci (at nucleolar caps) where signal is not covered by the transparent line.
Many of the images seem saturated and the authors should check the saturation levels. Saturation levels checked and corrected in several cases.

In other instances the authors use only merged images and not the individual channels. I recommend that they provide single channels for clarity. where merged images do not provide clear information on the individual channel staining (eg. Individual images for Fig 1C-E provided in EV1D).

References:
Burgess RC, Burman B, Kruhlak MJ, Misteli T (2014) Activation of DNA damage response signaling by condensed chromatin. Cell Rep 9: 1703-1717
Harding SM, Boiarsky JA, Greenberg RA (2015) ATM Dependent Silencing Links Nucleolar Chromatin Reorganization to DNA Damage Recognition. Cell Rep 13: 251-259
Korsholm LM, Gal Z, Lin L, Quevedo O, Ahmad DA, Dulina E, Luo Y, Bartek J, Larsen DH (2019) Double-strand breaks in ribosomal RNA genes activate a distinct signaling and chromatin response to facilitate nucleolar restructuring and repair. Nucleic Acids Res 47: 8019-8035
Mooser C, Symeonidou IE, Leimbacher PA, Ribeiro A, Shorrocks AK, Jungmichel S, Larsen SC, Knechtle K, Jasrotia A, Zurbriggen D et al (2020) Treacle controls the nucleolar response to rDNA breaks via TOPBP1 recruitment and ATR activation. Nat Commun 11: 123
Pefani DE, Latusek R, Pires I, Grawenda AM, Yee KS, Hamilton G, van der Weyden L, Esashi F, Hammond EM, O’Neill E (2014) RASSF1A-LATS1 signalling stabilizes replication forks by restricting CDK2-mediated phosphorylation of BRCA2. Nat Cell Biol 16: 962-971, 961-968
Pefani DE, Tognoli ML, Pirincci Ercan D, Gorgoulis V, O’Neill E (2018) MST2 kinase suppresses rDNA transcription in response to DNA damage by phosphorylating nucleolar histone H2B. EMBO J 37
van Sluis M, McStay B (2015) A localized nucleolar DNA damage response facilitates recruitment of the homology-directed repair machinery independent of cell cycle stage. Genes Dev 29: 1151-1163
Dear Prof. Pefani,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees, and I am happy to say that all support its publication now. Referee 1 still has a few minor suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

A few other editorial requests will also need to be addressed:

- Please add up to 5 keywords to the manuscript file.
- Please correct the conflict of interest subheading to "Disclosure and Competing Interest Statement"
- Please include the funding information in the Acknowledgement section.
- The Materials & Methods section should follow after the Discussion section.

I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript (if you have not already done so).

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the final manuscript.

I would like to suggest a few minor changes to the title and abstract. Please let me know whether you agree with the following:

53BP1-mediated recruitment of RASSF1A to ribosomal DNA breaks promotes local ATM signaling

DNA lesions occur across the genome and constitute a threat to cell viability; however, damage at specific genomic loci has a relatively greater impact on overall genome stability. The ribosomal RNA gene repeats (rDNA) are emerging fragile sites. Recent progress in understanding how the rDNA damage response is organized has highlighted a key role of adaptor proteins. Here we show that the scaffold tumor suppressor RASSF1A is recruited to rDNA breaks. RASSF1A recruitment to double strand breaks is mediated by 53BP1 and depends on RASSF1A phosphorylation at Serine 131 by ATM kinase. Employing targeted rDNA damage, we uncover that RASSF1A recruitment promotes local ATM signaling. RASSF1A silencing, a common epigenetic event during malignant transformation, results in persistent breaks, rDNA copy number alterations and decreased cell viability. Overall, we identify a novel role for RASSF1A at rDNA break sites, provide mechanistic insight into how the DNA damage response is organized in a chromatin context, and provide further evidence for how silencing of the RASSF1A tumor suppressor contributes to genome instability.

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

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The revised manuscript entitled "53BP1 mediated recruitment of RASSF1A at ribosomal DNA breaks promotes local ATM signaling" by Tsaridou and colleagues provides a comprehensive analysis of the role of RASSF1A in the response to rDNA damage. RASSF1A knockdown leads protection of rDNA copy number, reduced resection, reduced RPA-phosphorylation, reduced repair and survival. Interestingly, impairment of RASSF1A recruitment to rDNA damage by 53BP1 knockdown does not affect nucleolar segregation or pol I inhibition, while RASSF1A knockdown impair pol I inhibition and nucleolar segregation. This suggests that RASSF1A mediates pol I inhibition and nucleolar segregation independent of its recruitment to nucleolar caps, indicating that two pools of RASSF1A exist in the nucleolus. This is an interesting study, which increases our understanding of the complexity of rDNA damage repair. The authors have addressed my comments to the original manuscript well.
I suggest the following minor corrections to the revised manuscript:
1. Lower panel in Figure 1H: The GFP and mCherry channels are misaligned, so that it appears that one of the lacO arrays is in the nucleolus.
2. It is not clear from the methods or legend, which cells were used to induce DSBs by Cas9 in Figure EV2E.
3. Page 14, line 16: Fig EV4J and EV4K do not examine Pol I transcriptional inhibition. Please correct figure reference.
4. Page 16, line 17: change "ImmnoFISH" to "ImmunoFISH".
5. Page 19: change "affects" to "correlates with".
6. The Discussion could be more concise and to the point.
7. The manuscript could benefit from correction of English grammar throughout.

Referee #2:
The new data included by the authors, particularly the use of LacR-Scel to demonstrate the specificity of RASSF1A to certain chromatin context, have addressed the major concerns of this reviewer. Authors have also clarified that the role of 53BP1 at the rDNA break site seems to be different from its anti-resection role in regulating HR. Other critiques have also been appropriately addressed. I look forward to seeing this work in-print!

Referee #3:
The authors have taken seriously the referees comments and satisfied the concern. I recommend publication.
The authors have addressed all minor editorial requests
Dear Prof. Pefani,

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

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

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

Best regards,

Esther

Esther Schnapp, PhD  
Senior Editor  
EMBO reports

THINGS TO DO NOW:

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: [https://www.embopress.org/pb-assets/embo-site/er_apc.pdf](https://www.embopress.org/pb-assets/embo-site/er_apc.pdf)

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2021-54483V3 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.
Reporting Checklist for Life Science Articles (updated January 2023)

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: 10.11222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- A specific description of the experimental system investigated (e.g., 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 entities that are being measured.
- An explicit mention of the biological and chemical entities 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 value = x but not P value < x;
  - Definition of ‘center values’ as median or average;
  - Definition of error bars as s.d. or s.e.m.

Materials

Newly Created Materials

Information Included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?

Information in which section is the information available?

Information included in the manuscript?
**Study protocol**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Not Applicable | (Methods and/or Tools, Materials and Methods, Figures, Data Availability, Legend) |

- If study protocol has been pre-registered, provide DOI in the manuscript.
- For clinical trials, provide the trial registration number OR cite DOI.
- Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

**Laboratory protocol**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Not Applicable | (Methods and/or Tools, Materials and Methods, Figures, Data Availability, Legend) |

- Provide DOI OR other detailed if external detailed step-by-step protocols are available.

**Experimental study design and statistics**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Yes | (Methods and/or Tools, Materials and Methods, Figures, Data Availability, Legend) |

- Include a statement about sample size estimate even if no statistical/methods were used.
- Include a statement about blinding even if no blinding was done.
- Describe inclusion/exclusion criteria even 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 addition or intentional exclusion and provide justification.
- The investigators were not blinded.

**Sample definition and in-laboratory replication**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Yes | (Methods and/or Tools, Materials and Methods, Figures, Data Availability, Legend) |

- In the figure legends: state number of times the experiment was replicated in laboratory.
- In the figure legends: define whether data describe technical or biological replicates.

**Ethics**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Not Applicable | (Methods and/or Tools, Materials and Methods, Figures, Data Availability, Legend) |

- Studies involving human participants: State details of authority granting ethics approval (IRB or equivalent committee), provide reference number for approval.
- 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 Helsinki Declaration.
- Studies involving human participants: For publication of patient photos, include a statement confirming that consent to publish was obtained.
- Studies involving experimental animals: State details of authority granting ethics approval (IRB or equivalent committee(s)), provide reference number for approval.
- Studies involving specimen and field samples: State if consent permits obtained, provide details of authority approving study; if none were required, explain why.

**Dual Use Research of Concern (DURC)**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Not Applicable | (Methods and/or Tools, Materials and Methods, Figures, Data Availability, Legend) |

- Could your study fall under dual use research restrictions? Please check biosafety documents and list of select agents and toxins (CDC https://www.selectagents.gov/index.html).
- If you used a select agent, is the security level of the list appropriate and reported in the manuscript?
- 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?

**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.

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Not Applicable | (Methods and/or Tools, Materials and Methods, Figures, Data Availability, Legend) |

- Adherence to community standards:
  - If relevant guidelines or checklists (e.g., IMPC, IBC, ARRIVE, PRISMA) have been followed or provided.
  - For marker expression studies, we recommend that you follow the (RE)MARK reporting guidelines (see link at top right).
  - For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link at top right) and submit the CONSORT checklist (see link at top right) with your submission. See author guidelines, under Reporting Guidelines.

**Data Availability**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Not Applicable | (Methods and/or Tools, Materials and Methods, Figures, Data Availability, Legend) |

- Have primary datasets been deposited according to the journal’s guidelines (see “Data Depositor” section) and the respective accession numbers provided in the Data Availability Section?
- Have human clinical and genomic datasets deposited in a public-access-controlled repository in accordance to ethical obligations to the patients and the applicable consent agreements?
- Are computational models that are central and integral to study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?
- If publicly available data were reused, provide the respective data citations in the reference list?