EMBO reports

Dipeptidyl peptidase 9 triggers BRCA2 degradation and promotes DNA-damage repair

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Dear Dr. Geiss-Friedlander

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, the referees acknowledge that the findings are potentially interesting. However, they also raise several concerns that would need to be successfully addressed before we can consider your study for publication here.
Some of the referee concerns overlap and I list here the most crucial points:

- Better evidence needs to be provided that BRCA2 is a specific target for DPP9 and is an N-end rule target. A BRCA2 proline mutant should be included.
- The roles of BRCA2 and DPP9 in the nucleus versus the cytoplasm need to be clarified.
- The cell cycle phase with regard to protein activity needs to be determined.
- Puzzling and contrasting observations need to be clarified/discussed.

It is clear that significant revisions will be required, and the manuscript is currently a borderline case. If you decide to embark on such extensive 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. We can also discuss the revisions in a video chat, if you like.

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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.

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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. *
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
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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 {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.

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

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Yours sincerely,

Esther Schnapp, PhD

Senior Editor
EMBO reports

Referee #1:

The article from Bolgi et al. describes the HR protein BRCA2 as a new substrate for the peptidase DPP9 in response to DNA damage. The authors propose that Dpp9 targets BRCA2 in the cytoplasm, using FLNA. They state that this activity is required to control the levels of BRCA2 in the cell, via the N-end rule pathway.
While the authors write a compelling story, and while a link between DPP9 and BRCA2 would be very interesting, I find their conclusions greatly overstated. I am not convinced by the presented data, and I miss many critical controls to concur with their arguments. The authors should revisit some of their statements unless their include additional data and/or improved analysis.
- Much of the structural and biochemical data (Figures 2-3) could be explained by the presence of the proline in the peptide and the known specificity of DPP9 for such substrates, can the authors prove that the findings are somewhat specific for BRCA2? I imagine that any peptide with the MPI sequence at the N-terminus will show the presented results. In this light, these data have limited contribution to any conclusions in the manuscript. I have additional doubts about the data presented in Figure 2B: these binding curves can not be used to calculate the Kd, as they miss an upper plateau (i.e. they may not represent actual binding). Can the authors use a catalytic-dead mutant version of DPP9 as control in their biochemical experiments (Figure 2)?
- Much of the differences presented in the cellular experiments are far from convincing and the presence of a significant p value does not suffice. This is particularly evident in:
o Figure 1D, 1F: the differences are due to a very limited number of cells over the total number that is used for the quantification.
o Figure 5B, where it is unclear how can there be a significant difference at timepoint 3 and 6, but not at 24. Also, mean-signal-intensity is not a value that is used to evaluate gH2AX foci, the authors should report the number of foci per cell or foci intensity.
o Figure 4B: the top and bottom graphs should be merged to evaluate how DPP9KD +MMC relate to the WT -MMC. It's not clear why these results are shown separately but presented as strong evidence to argue that DPP9 controls BRCA2 protein stability.
- The authors draw strong conclusions about BRCA2 being a N-end rule target, but this hypothesis is not directly tested. A link with DPP9 and FLNA alone does not seem sufficient for such statement. Controls in their experiments using i.e. N-end rule pathway components could help at least strengthen this point.
- The role of FLNA: there is little to no explanation as to why FLNA should mediate the BRCA2 interaction and what are the functional consequences of this. FLNA involvement is treated as obvious, but one could argue that its presence represents a contamination. A start could be testing FLNA effects and proximity in the PLA shown in Figure 1?
- It is not clear how the expression levels of the ectopic inducible DPP9 constructs relate to the levels of endogenous DPP9. A side-by-side comparison on western blot should be shown, to evaluate if the performed experimental setup reflects the physiological situation.
- Additional comments:
o A positive control for BRCA2 interaction in their PLA experiments in Figure 1. There is a mention that the DPP9-BRCA2 spots are cytoplasmic (Discussion), can the authors provide actual statistics on this + controls?
o The authors never test a BRCA2 mutant that is simply mutated in the Proline in position 2 (i.e. in Figure 4G-H or 7 C-F).
o How does BRCA2 behavior relate to other DPP9 substrates (e.g Syk)?

Referee #2:

In the manuscript 'Dipeptidyl peptidase 9 triggers BRCA2 degradation and promotes DNA-damage repair', Bolgi et al. have described the role of DPP9 in the maintaining the stability of BRCA2 and in the subsequent HR process. However, the data presented do not sufficiently support the conclusions of the manuscript. The evidence supporting the turnover of BRCA2 by DPP9 activity are not adequate and there is lack of data for the role of N-degron pathway. DNA repair defects (g-H2Ax and Rad51 foci) in DPP9 depleted cells do not co-relate well with the effects on BRCA2 levels. Also, BRCA2 localization seems to be largely cytosolic and whether this is the reason for the observed DNA repair defects is not explained in the manuscript.

Major comments

1) The interaction of BRCA2 and DPP9 seem to be stimulated upon damage as shown by the PLA-based assay upon MMC treatment (Fig. 1C). Important validation of this damage dependent interaction is necessary.
a. Is this seen by IP as well? Fig.1B can be carried out in the presence or absence of damage. Is the interaction seen upon treatment with other DNA damaging agents like Cpt, IR or HU? Does DPP9 interact with BRCA2-PALB2-Rad51 complex or just BRCA2? A co-IP blot with Rad51 and PALB2 can be shown. Is this interaction and subsequent regulation of BRCA2 specific to S phase; wherein HR is the predominant repair pathway. Considering the hypothesis that DPP9 regulates HR, these questions should be addressed.
b. The IP experiment was done with the cytosolic DPP9 isoform. Is there any interaction with the nuclear isoform? Is this interaction increased with mutants of BRCA2 that localize mainly to the cytosol? This would support the authors' hypothesis that

DPP9 binds the soluble pool of BRCA2.
c. Finally, since the major residue of BRCA2 for these effects is proline at the second position, this can be mutated and the effects on interaction and stability of BRCA2 can be checked with this mutant. This will strengthen the data on the role of DPP9.

2) DPP8 crystals show binding to the MP dipeptide of BRCA2 (Fig.3). Is the in vitro cleavage and interaction with BRCA2 seen with DPP8 as well?

3) Fig.4 A and B. - The differences in BRCA2 and Rad51 turnover in MMC treated WT and DPP9 KD cells is not apparent in the represented blots. The authors should show better representative blots that correspond to the graphical quantitation in Fig. 4B. There seems to be a clear decrease in Rad51 levels as well in the blots (Fig.4A and 4E). Is the turnover of BRCA2/Rad51 levels also seen on chromatin?

4) DPP9 blot should be shown for Fig.4A. DPP9 KD cells don't seem to show any decrease DPP9 levels on chromatin (Supplementary Fig. 5D comparing 0h timepoints across WT and DPP9 KD). DPP9 levels also seem to increase on chromatin upon MMC treatment (Supplementary Fig. 5D). Does DPP9 get recruited to chromatin upon damage and is the turnover of BRCA2/Rad51 also happen on chromatin and the role of nuclear vs cytosolic DPP9 isoform in this should be discussed.

5) As mentioned in point 1, is this DPP9-mediated turnover of BRCA2 specific to S phase and is it seen with other DNA damaging agents? Double thymidine experiments can answer these questions. Experiments with BRCA2 proline mutants can further strengthen this data.

6) Can the degradation of BRCA2DelMP (Fig. 4H) mutant be rescued upon proteasome inhibition? Some data supporting ubiquitination of BRCA2DelMP mutant compared to the WT will strengthen the hypothesis that BRCA2 is a substrate of N-degron pathway and degraded by the proteasome.

7) Line 249- MMC-induced degradation of BRCA2 requires DPP9- This conclusion is not fully supported by the data as there is no complete restoration of BRCA2 turnover upon DPP9 KD (Fig.4A and B).

8) Increased BRCA2 turnover is associated with increased DNA damage and poor tumor prognosis (Liu et al. 2017). It seems counterintuitive that DPP9 KD should increase DNA damage given that BRCA2 levels are stabilized in these cells. This should be clarified in detail.
Supplementary Fig 4D seems to suggest an increase in g-H2Ax upon expression of DPP9 S729A mutant without damage. Is this due to BRCA2 independent effects since interaction with BRCA2 is seen mainly upon damage.

5) BRCA2-PALB2 interaction seems to be largely cytosolic instead of nuclear (Fig.6D). The authors should clarify the reason for this. The decreased Rad51 foci in Fig. 6E could be attributed to decreased Rad51 levels observed upon DPP9 KD (Fig. 4A and E).

6) Western blots for the DPP9 expression in HEK293 DPP9 KO + DPP9-S WT and S729A (Fig. 7A) should be shown. Referring to point 4, DPP9 activity would be destabilizing BRCA2 and reduced BRCA2 levels is expected to decrease Rad51 foci. The results seem to suggest the opposite effect. This is not explained sufficiently well.

7) In Fig.7 C and E, BRCA2 seems to be largely cytosolic. This seems to be especially the case in DPP9 KD cells. Is there a BRCA2 localization defect in these cells which could be responsible for reduced Rad51 loading? The authors should explain this further.
Also, from Fig.4H and I, BRCA2Del MP mutant is unstable and has a half-life of ~2-3h even without damage. How does it then sustain Rad51 loading after 24h of MMC treatment (Fig.7E)?

Minor points

1) Fig.4H The molecular weight of GFP is shown to be around 55kDa? Is this some modified GFP construct, considering the normal MW is around 28kD

2) Data in many figures throughout the paper are represented by 2 types of graphs (individual value plots and mean column plots). Since both convey the same information, this seems unnecessary.

3) Supplementary Figure 5B refers to Figure 6C and is mentioned as 5C in the legend.

Referee #3:

In the current manuscript, Bolgi et al. present a role for the dipeptidyl peptidase DPP9-dependent cleavage of BRCA2 N-terminus in promoting homologous recombination (HR). The authors identify BRCA2 as a novel substrate of DPP9, which cleaves the first 2 amino acids of BRCA2 (after an evolutionarily conserved Proline) and enhances BRCA2 turnover after DNA damage. Human cells lacking DPP9, or mouse embryonic fibroblasts expressing a catalytic inactive version of DPP9, are hypersensitive to mitomycin C, Olaparib and ionizing radiation. Consistent with this, depletion of DPP9, or expression of a BRCA2 truncation mutant that cannot be processed by DPP9, result in impaired RAD51 foci formation.

The study by Bolgi et al. is intriguing, and the manuscript concise and carefully written. My main concern regards the discrepancies between the observation of DPP9-BRCA2 interaction in the cytoplasm, and the proposed role in the nucleus. Moreover, the functional relevance of BRCA2 cleavage by DPP9 for HR should be strengthened, as suggested below.

Major points

1. In the Discussion, the authors suggest that BRCA2 N-terminus could become available for DPP9 cleavage after complexing with ssDNA and RAD51, yet the DPP9-BRCA2 PLA interaction occurs mainly in the cytoplasm. This is puzzling and should be further clarified. Can the authors elaborate more on the function of nuclear vs cytoplasmic DPP9? Which isoform is relevant for BRCA2 cleavage and DNA repair? Is the cellular distribution of FLAG-DPP9-S in DPP9 KO HeLa cells completely cytoplasmic? If so, how is DPP9 limiting BRCA2 at DNA breaks?

2. The DPP9-depleted cells should be characterized better. How is their cell cycle profile? Is the proportion of S/G2 cells as in WT cells? Also, a western blot testing whether absence of DPP9 affects total levels of RAD51 and BRCA1 should be provided.

3. On the same line as the previous comment, when assessing the number of RAD51 foci, a cell cycle marker (e.g. EdU incorporation) should be used for gating, and RAD51 foci should be counted only in EdU-positive cells.

4. If DPP9 is required to remove BRCA2 from DNA breaks and allow formation of RAD51 filaments, it would be expected that excess BRCA2 nuclear foci are present (that colocalize with gH2A.X). Can the authors test this? Immunofluorescence should be enough, with no need to perform PLA assays.

5. Can the authors expand Figure 5 including a sensitivity assay using siRNA-mediated depletion of BRCA2 and re-expression of siRNA-resistant BRCA2 1-3418 or BRCA2deltaMP 3-3418?

Minor points

1. Using a BRCA2 1-40 peptide carrying substitution of Proline to a non-cleavable amino acid (histidine?) would be a nice control for Fig. 2A.

2. Can the authors provide clearer blots to show that BRCA2 is present in DPP9 complexes?

Dear Dr. Schnapp, dear reviewers,

We thank you for handling and carefully reading this manuscript. We are submitting a revised version that addresses all the concerns of our reviewers. We hope that you will find this manuscript interesting for publishing in EMBO reports. Please see detailed answers with the clarifications and the additional experimental data below.

Sincerely

Ruth Geiss-Friedlander

Referee #1:

The article from Bolgi et al. describes the HR protein BRCA2 as a new substrate for the peptidase DPP9 in response to DNA damage. The authors propose that Dpp9 targets BRCA2 in the cytoplasm, using FLNA. They state that this activity is required to control the levels of BRCA2 in the cell, via the N-end rule pathway.
While the authors write a compelling story, and while a link between DPP9 and BRCA2 would be very interesting, I find their conclusions greatly overstated. I am not convinced by the presented data, and I miss many critical controls to concur with their arguments. The authors should revisit some of their statements unless their include additional data and/or improved analysis.

We thank this reviewer for finding this work to be a compelling story. We have addressed the concerns of this reviewer as specified below.
- Much of the structural and biochemical data (Figures 2-3) could be explained by the presence of the proline in the peptide and the known specificity of DPP9 for such substrates, can the authors prove that the findings are somewhat specific for BRCA2? I imagine that any peptide with the MPI sequence at the N-terminus will show the presented results. In this light, these data have limited contribution to any conclusions in the manuscript. I have additional doubts about the data presented in Figure 2B: these binding curves can not be used to calculate the Kd, as they miss an upper plateau (i.e. they may not represent actual binding). Can the authors use a catalytic-dead mutant version of DPP9 as control in their biochemical experiments (Figure 2)?
We have rephrased the conclusions from the SPR measurements, which we use to show a direct interaction between immobilized DPP9 and two BRCA2 peptides, but not to measure KD values. This assay complements the pull-down assays in which a BRCA2 protein fragment was immobilized and assayed for direct interaction with DPP9 in the absence or presence of a competitive inhibitor.
In this revised version we included new experimental data to compare the binding behavior of DPP9 with that of PALB2 which also binds to the N-terminus of BRCA2. By simultaneously adding DPP9 and PALB2 at a 2:9 ratio respectively, we show that PALB2 binds less efficiently to BRCA2 in the presence of DPP9 (Figure 2F).

We further addressed the question raised by this reviewer, whether indeed only the proline determines the interaction between BRCA2 N-terminus and DPP9.
Previously, we have systematically characterized the substrate specificity of DPP9 by measuring the hydrolysis of the artificial substrate GP-AMC, in the presence of a peptide in which single amino acids before and following the proline were replaced. These showed that both DPP9 and DPP8 can cleave a Pro-Ile peptide bond (Geiss-Friedlander et al, 2009).
In this revised manuscript we performed similar assays to ask whether additional residues in BRCA2, apart from the proline effect interaction with DPP9. As a positive control we measured the inhibitors effect of the Syk_{1-31} peptide. The $BRCA2_{1-40}^{P2G}$ peptide in which the proline in the second position was replaced by a glycine residue was used as a negative control, which did not compete with GP-AMC processing. Additionally, we show that a shorter 1-20 peptide competes less efficiently compared to a 1-40 BRCA2 peptide suggesting that additional residues in the 20-40 region of BRCA2 support interaction with DPP9.
Thus, we tested further peptides in which selected residues were substituted. These include also W31C and F32G which are known to interact with PALB2. We find that both the $BRCA2_{1-40}^{W31C}$

and $BRCA2_{1-40}^{F32G}$ inhibit clearly less compared to the $BRCA2_{1-40}$ peptide, whereas the substitution of E13R and I14N had only little, and no effect. In conclusion, while Pro in position 2 is critical for interaction with DPP9, this interaction is also determined by additional residues in BRCA21-40 peptide.

While BRCA2 is certainly not the only substrate of DPP9, we do see that the interaction is not constitutive, but rather induced by MMC-induced DNA damage, suggesting that it is regulated. In this version, we controlled this interaction further by testing whether it can be stimulated by an additional genotoxic agent, NCS. We find that similar to MMC, also NCS leads to a clear increase in the number of BRCA2-DPP9 PLA events. Furthermore, we find that DPP9 effects the stability of BRCA2 but not of RAD51.

- Much of the differences presented in the cellular experiments are far from convincing and the presence of a significant p value does not suffice. This is particularly evident in:
o Figure 1D, 1F: the differences are due to a very limited number of cells over the total number that is used for the quantification.

For the microscopy-based assays: PLA and IF experiments we have included at least 100 cells per condition. These are in line with standards in the field, as seen in recent papers published in high-ranking journals such as (Ahlskog *et al*, 2016; Han *et al*, 2017; Ehlén *et al*, 2020; Llorens-Agost *et al*, 2021; Lindenburg *et al*, 2021; Ambjørn *et al*, 2021; Hariharasudhan *et al*, 2022).

More importantly, to validate the reproducibility of our data we have also included graphs showing the mean PLA events of single experiments.

o Figure 5B, where it is unclear how can there be a significant difference at timepoint 3 and 6, but not at 24. Also, mean-signal-intensity is not a value that is used to evaluate gH2AX foci, the authors should report the number of foci per cell or foci intensity.
We have visualized the presence of γH2AX foci by microscopy imaging (Fig. 5A and Fig EV2B). While PLA signals were easily quantified with the DUO-LINK software, this software was not suitable for quantification of the γH2AX signals which vary in size and intensity. Thus, to quantify a large number of cells in an unbiased approach we have measured the γH2AX intensity. This allowed us to count signals from more than 1700 cells per condition per experiment. This panel shows a summary of 6 independent experiments. In Figure 5B we summarize the kinetics in which the gH2AX appear and are resolved. We find that the gki MEF DPP9 S729A cells accumulate more damage in response to NCS, but that this is resolved at 24 h reaching a similar fold increase in signal as in the WT cells. In contrast the WT cells accumulated less foci, which are resolved more rapidly.

o Figure 4B: the top and bottom graphs should be merged to evaluate how DPP9KD +MMC relate to the WT -MMC. It's not clear why these results are shown separately but presented as strong evidence to argue that DPP9 controls BRCA2 protein stability.

We have merged the graphs as suggested (Fig. 4B). We added more CHX assays from wild-type HEK 293 cells, and cells in which DPP9 CRISPRed out. These cells were then re-transfected for a stable expression of the active and inactive mutant of DPP9 (the cytosolic variant). Expression was induced by doxycycline. The results (Fig. 4D) show destabilization of BRCA2 in WT but not in the DPP9-depleted cells in response to MMC. Re-expression of active DPP9 lead to destabilization of endogenous BRCA2.

- The authors draw strong conclusions about BRCA2 being a N-end rule target, but this hypothesis is not directly tested. A link with DPP9 and FLNA alone does not seem sufficient for such statement. Controls in their experiments using i.e. N-end rule pathway components could help at least strengthen this point.

`The main determinant of an N-degron is a destabilizing Nt-residue of a protein` (Varshavsky, 2019)This pathway is defined by the differential stability of proteins based on their N-terminus. In our previous publications we have shown that DPP9 is a component of the N-degron pathway (Justa-Schuch et al, 2016; Finger et al, 2020). In this manuscript we show that the N-terminal residues Met-Pro have a stabilizing effect on BRCA2 and that DPP9 regulates BRCA2 stability. In this revised version we provide additional experimental data showing that DPP9 leads to degradation of BRCA2, while an inactive DPP9 variant does not, suggesting that it is the activity of DPP9 that regulates the stability of BRCA2. We have added additional controls with a $BRCA2_{1\text{-}1000}$P2G and P2H construct, which show lower steady state levels compared to $BRCA2_{1\text{-}1000}$ highlighting a stabilizing effect for proline in second position (Appendix Fig S4).
Non the less, we have rephrased our claims, and now state that DPP9 targets BRCA2 for degradation and highlight the stabilizing effect of the dipeptide MetPro.

- The role of FLNA: there is little to no explanation as to why FLNA should mediate the BRCA2 interaction and what are the functional consequences of this. FLNA involvement is treated as obvious, but one could argue that its presence represents a contamination. A start could be testing FLNA effects and proximity in the PLA shown in Figure 1?

Given its interactions with both DPP9 (Justa-Schuch et al, 2016) and BRCA2 (Yuan & Shen, 2001; Mondal et al, 2012; Yue et al, 2012), cells were silenced for FLNA to test whether it is relevant for the observed DPP9-BRCA2 proximity. Cell silenced for FLNA did not show an increase in the number of BRCA2-DPP9 interactions in response to MMC, implying that the DNA-damage triggered proximity of DPP9 and BRCA2 requires FLNA (Fig 1B and C, Fig EV1A, Appendix Fig S1A and B).

Since FLNA supports the MMC-induced proximity between DPP9 and BRCA2 we speculate that FLNA binds to both proteins thereby increasing their local concentration to support cleavage.

- It is not clear how the expression levels of the ectopic inducible DPP9 constructs relate to the levels of endogenous DPP9. A side-by-side comparison on western blot should be shown, to evaluate if the performed experimental setup reflects the physiological situation.
We have added immunofluorescence images showing the expression and localization of DPP9 in the HEK 293 DPP9 KO + DPP9-S^{WT} and HEK 293 DPP9 KO + DPP9-L^{WT} compared to HEK 293 DPP9 WT cells. These show that endogenous DPP9 localizes to the cytoplasm and nucleus in these cells (Appendix Fig S2 C and D), similar to our previous observation in HeLa cells. DPP9-S^{WT} is cytosolic, whereas DPP9-L^{WT} localizes mostly to the nucleus. Both constructs are over-expressed (> 5 fold) compared to the levels of the endogenous protein. Importantly, over-expression of DPP9-S^{WT} (mean 10 foci per cell) results in a better recovery as measured by the number of RAD51 foci in response to MMC compared to the number of foci in cells over-expressing DPP9-L^{WT} (mean 4 foci per cell) (Fig EV4G and H).

- Additional comments:
o A positive control for BRCA2 interaction in their PLA experiments in Figure 1. There is a mention that the DPP9-BRCA2 spots are cytoplasmic (Discussion), can the authors provide actual statistics on this + controls?
All PLAs were controlled technically (only one antibody), and by silencing a corresponding interaction partner (DPP9 or BRCA2). In this revised version we have added graphs with a differential analysis of the PLA signals showing a cytosolic and nuclear localization (Fig 1B). Statistics are also provided, as well as a calculated fold increase in response to MMC. We include new data showing the PLAs formed in the cytosol and nucleus of asynchronized cells in response to NCS (30 minutes) and after a recovery time of up to 3 h. Similar assays were performed in cells following a double thymidine block to enrich for cells in S-phase.

o The authors never test a BRCA2 mutant that is simply mutated in the Proline in position 2 (i.e. in Figure 4G-H or 7 C-F).

We have added additional controls with a $BRCA2_{1-1000}$P2G and P2H construct, which show lower steady state levels compared to $BRCA2_{1-1000}$ highlighting a stabilizing effect for proline in second position (Appendix Fig S4).

o How does BRCA2 behavior relate to other DPP9 substrates (e.g Syk)?
Very few DPP9 substrates have been characterized so far. These are mitochondrial proteins, such as AK2 and the tyrosine kinase Syk, all have been shown to be targeted for proteasomal degradation (Finger *et al*, 2020; Justa-Schuch *et al*, 2016) .

we have expanded our discussion to include also Syk:
DPP9 presents an alternative pathway that cells apply to convert otherwise stable proteins with N-terminal prolines such as AK2 and BRCA2 into substrates for proteasomal degradation. In contrast to the seemingly constitutive processing of AK2 by DPP9 (Finger et al, 2020), the interaction of BRCA2 with DPP9 is induced in response to genotoxic stress, which also promotes BRCA2 degradation. Another verified DPP9 substrate is Syk, with an alanine in second position. Similar to BRCA2, Syk degradation does not appear to be constitutive but instead is induced following stimulation of the B cell receptor, a process that relies on DPP9 activity (Justa-Schuch et al, 2016).

Referee #2:

In the manuscript 'Dipeptidyl peptidase 9 triggers BRCA2 degradation and promotes DNA-damage repair', Bolgi et al. have described the role of DPP9 in the maintaining the stability of BRCA2 and in the subsequent HR process. However, the data presented do not sufficiently support the conclusions of the manuscript. The evidence supporting the turnover of BRCA2 by DPP9 activity are not adequate and there is lack of data for the role of N-degron pathway. DNA repair defects (g-H2Ax and Rad51 foci) in DPP9 depleted cells do not co-relate well with the effects on BRCA2 levels. Also, BRCA2 localization seems to be largely cytosolic and whether this is the reason for the observed DNA repair defects is not explained in the manuscript.
We thank this referee for carefully reading this manuscript. We have addressed the concerns as described below.
Major comments

1) The interaction of BRCA2 and DPP9 seem to be stimulated upon damage as shown by the PLA-based assay upon MMC treatment (Fig. 1C). Important validation of this damage dependent interaction is necessary.
a. Is this seen by IP as well? Fig.1B can be carried out in the presence or absence of damage. Is the interaction seen upon treatment with other DNA damaging agents like Cpt, IR or HU? Does DPP9 interact with BRCA2-PALB2-Rad51 complex or just BRCA2? A co-IP blot with Rad51 and PALB2 can be shown.

In this revised version we looked more closely into the BRCA2-PALB2 interactions which are affected by DPP9. Binding assays of full length recombinant DPP9 and full length recombinant PALB2 to the $BRCA2_{1-40}$ protein fragment, suggest that DPP9 and PALB2 do not bind simultaneously to BRCA2 N-terminus. Analysis of single amino acids in a $BRCA2_{1-40}$ peptide suggest that W31C and F32G which are known to bind to PALB2, also influence binding to DPP9 (Fig 2F-H). Finally, PLAs in the absence of DPP9 show more interactions between BRCA2 and PALB2 (Fig 6C and D). Taken together, our data suggest that DPP9 does not stably interact with a BRCA2-PALB2 complex but instead limits the interaction between BRCA2 and PALB2.

Is this interaction and subsequent regulation of BRCA2 specific to S phase; wherein HR is the predominant repair pathway. Considering the hypothesis that DPP9 regulates HR, these questions should be addressed.

Thank you for this question. In this revised version we have extended our observations by inducing DNA DSBs with an additional source - the radiomimetic drug Neocarzinostatin (NCS) (30 minutes, 250 ng / mL). NCS was then removed to allow recovery. We have then performed

PLAs both directly after NCS removal (time 0 h), and at different recovery times. To test whether the interaction occurs in S-phase, we enriched for cells in S-phase by double thymidine blocks, before addition of the NCS.

`*To extend our observations, cells were treated with another DSBs causing agent, the radiomimetic drug Neocarzinostatin (NCS). Similarly, NCS-treated cells presented more DPP9-BRCA2 PLA signals compared to mock-treated cells, with most of these events in the cytosol (Fig 1D and E, Fig EV1B). The fold increase of DPP9-BRCA2 PLA events in response to NCS was even higher in cells that were first arrested by double thymidine blocks and treated with NCS following release form the second block, validating a close proximity between DPP9 and BRCA2 in S phase (Fig 1D and E, Fig EV1B).*`

b. The IP experiment was done with the cytosolic DPP9 isoform. Is there any interaction with the nuclear isoform? Is this interaction increased with mutants of BRCA2 that localize mainly to the cytosol? This would support the authors' hypothesis that DPP9 binds the soluble pool of BRCA2.

In this revised version we added graphs with a differential analysis of the BRCA2-DPP9 PLA signals. We find PLA signals both in the cytosol and in the nucleus. We have also analysed the fold increase in the number of events in both compartments. We note a similar fold increase in both compartments in response to MMC and NCS. The absolute number of PLA events was higher in the cytosol than the number of events that show a nuclear localization (Fig 1B-D). These findings are similar in asynchronysed and in synchronysed (double thymidine block) cells. Thus, we do not find strong evidence for a preferred DPP9-BRCA2 interaction in the nucleus.

To functionally test for the role of DPP9 in the cytosol, CHX chase experiments were performed in HEK 293, HEK 293 DPP9 KO, HEK 293 DPP9 KO + DPP9-S^{WT} and HEK 293 DPP9 KO + DPP9-S^{S729A} (Fig 4C and D). Of note similar to our previous findings in HeLa WT cells (Justa-Schuch *et al*, 2014)DPP9-S^{WT} shows a cytosolic localization in the HEK 293 DPP9 KO + DPP9-S^{WT} (Appendix Fig S2). In line with the results in HeLa WT and HeLa DPP9 KD cells, CHX chase assays show degradation of BRCA2 in HEK 293 but not in HEK 293 DPP9 KO cells in response to MMC. Importantly, degradation of BRCA2 was restored by the induced expression of DPP9-S^{WT} but not the enzymatically inactive DPP9-S^{S729A}. Thus, the cytosolic variant of DPP9 functionally regulates BRCA2 turn-over.

Further support for the role of the cytosolic DPP9 variant is provided by analysis of RAD51 foci in HEK 293, HEK 293 DPP9 KO + DPP9-S^{WT} and HEK 293 DPP9 KO + DPP9-L^{WT}, showing a better recovery with the cytosolic DPP9 variant.

c. Finally, since the major residue of BRCA2 for these effects is proline at the second position, this can be mutated and the effects on interaction and stability of BRCA2 can be checked with this mutant. This will strengthen the data on the role of DPP9.

To address this question, we constructed two $BRCA2_{1\text{-}1000}$ mutants: P2G, P2H. Both showed reduced steady state levels compared to $BRCA2_{1\text{-}1000}$, further implying a stabilizing role for the N-terminal Pro_2 (Appendix Fig S4A).

2) DPP8 crystals show binding to the MP dipeptide of BRCA2 (Fig.3). Is the in vitro cleavage and interaction with BRCA2 seen with DPP8 as well?
Yes, processing of the peptide is seen in vitro by both DPP8 and DPP9. For clarification: crystals of DPP8 and DPP9 were soaked with the $BRCA2_{1\text{-}40}$ peptide, resulting in cleavage of the peptide within the crystal. The dipeptide in the active site of DPP8 and DPP9 is a result of binding of the full-length peptide followed by hydrolysis of the peptide.

3) Fig.4 A and B. - The differences in BRCA2 and Rad51 turnover in MMC treated WT and DPP9 KD cells is not apparent in the represented blots. The authors should show better representative blots that correspond to the graphical quantitation in Fig. 4B. There seems to be a clear

decrease in Rad51 levels as well in the blots (Fig.4A and 4E). Is the turnover of BRCA2/Rad51 levels also seen on chromatin?
We replaced these blots as requested. Our quantifications do not point to a clear effect of DPP9 on the overall stability of RAD51.

4) DPP9 blot should be shown for Fig.4A. DPP9 KD cells don't seem to show any decrease DPP9 levels on chromatin (Supplementary Fig. 5D comparing 0h timepoints across WT and DPP9 KD). DPP9 levels also seem to increase on chromatin upon MMC treatment (Supplementary Fig. 5D). Does DPP9 get recruited to chromatin upon damage and is the turnover of BRCA2/Rad51 also happen on chromatin and the role of nuclear vs cytosolic DPP9 isoform in this should be discussed.

We have included western blots of DPP9 in the HeLa DPP9 KD cells.
We do see DPP9 on chromatin, however we do not detect a reproducible increase in the binding of DPP9 to chromatic fractions in response to DNA damage. Since we can restore the DPP9-induced degradation of BRCA2 and RAD51 foci formation by the over-expression of DPP9-S, our data supports the notion that the interaction and DPP9-mediated degradation of BRCA2 in response to DNA damage is carried out by the cytoplasmic variant of DPP9 (Fig 4C and D, Fig 7A and B, Fig EV4F and G, Appendix Fig S2D).

5) As mentioned in point 1, is this DPP9-mediated turnover of BRCA2 specific to S phase and is it seen with other DNA damaging agents? Double thymidine experiments can answer these questions. Experiments with BRCA2 proline mutants can further strengthen this data.

BRCA2 was previously shown to undergo ubiquitin-proteasome mediated degradation in S-phase (Liu *et al*, 2017). As suggested, we enriched for cells in S-phase using a double thymidine block. We clearly see an increase in the number of BRCA2-DPP9 PLA events in cells following a double thymidine block, compared to asynchronized cells. More importantly, cells in S-phase show a greater fold increase in the number of events compared to asynchronized cells. Additionally, the interaction between DPP9 and BRCA2 is initiated not only by MMC-induced damage but also following exposure to NCS. Removal of NCS show that the interaction is transient (Fig 1D and E, FigEV1B). Thus, we can conclude that the interaction between BRCA2 and DPP9 is enriched in S-phase.

We constructed two $BRCA2_{1\text{-}1000}$ mutants in which proline was replaced by a glycine and a histidine. Both show lower steady state levels suggesting that proline has a stabilizing effect on BRCA2 (Appendix Fig S4A).

6) Can the degradation of BRCA2DelMP (Fig. 4H) mutant be rescued upon proteasome inhibition? Some data supporting ubiquitination of BRCA2DelMP mutant compared to the WT will strengthen the hypothesis that BRCA2 is a substrate of N-degron pathway and degraded by the proteasome.
In this revision we have included mutations of the Pro_2 to test the importance of this residue for the steady state levels of BRCA2. We rephrased our claims, and state that DPP9 targets BRCA2 for degradation, and highlight the importance of the Pro_2 for BRCA2 stability.

7) Line 249- MMC-induced degradation of BRCA2 requires DPP9- This conclusion is not fully supported by the data as there is no complete restoration of BRCA2 turnover upon DPP9 KD (Fig.4A and B).
Fig. 4B shows an overlay of all conditions. These show that the turn-over rate of BRCA2 is similar in HeLa WT – MMC, HeLa WT + MMC+MG132 and HeLa DPP9 KD + MMC. Nontheless, we have mildened this conclusion.

8) Increased BRCA2 turnover is associated with increased DNA damage and poor tumor prognosis (Liu et al. 2017). It seems counterintuitive that DPP9 KD should increase DNA damage given that BRCA2 levels are stabilized in these cells. This should be clarified in detail.

Indeed low BRCA2 levels impair HR. However, Liu et al have also shown that :"USP21 is overexpressed in hepatocellular carcinoma, where it promotes BRCA2 stability and inversely correlates with patient survival". We suggest that the levels of BRCA2 a carefully monitored and fine-tuned in cells, probably by factors that promote degradation (DPP9) and factors that favour stabilization (Usp21). We propose that deregulation in BRCA2 levels would lead to unproductive interactions, for example, an incorrect stoichiometry between BRCA2 and RAD51.

Supplementary Fig 4D seems to suggest an increase in g-H2Ax upon expression of DPP9 S729A mutant without damage. Is this due to BRCA2 independent effects since interaction with BRCA2 is seen mainly upon damage.

Indeed we observe a slight (but not-significant) increase in gH2AX levels in the gki MEF DPP9^{S729A} cells also in the absence of DNA damage. This increase possibly reflects accumulation of DNA damage in response to endogenous conditions such as replication stress or oxidative stress. Quantification and statistical analysis suggest that this is not a significant difference.

5) BRCA2-PALB2 interaction seems to be largely cytosolic instead of nuclear (Fig.6D). The authors should clarify the reason for this. The decreased Rad51 foci in Fig. 6E could be attributed to decreased Rad51 levels observed upon DPP9 KD (Fig. 4A and E).

Indeed, to our surprise, we observe PLA signals both in the cytoplasm and in the nucleus. Differential analysis of these PLAs suggest that the majority of these interactions are in the cytoplasm (Fig 1B-D, Fig EV1A and B). Further support for a role of DPP9 in the cytosol is found in the pulse-chase experiments in which DPP9-S was re-expressed in HEK 293 DPP9 KO cells, and in the RAD51 foci assays which are recovered by the re-expression of the active cytosolic DPP9 (DPP9-S).
We note that also Usp21 was shown to de-ubiquitinate BRCA2 in the cytoplasm and not in the nucleus (Liu *et al*, 2017)Thus, the results of this manuscript are in line with previous publications. We propose that the role of DPP9-mediated degradation is to control the cellular concentration of BRCA2, rather than remove BRCA2 from the chromatin. High levels of BRCA2 or BRC4 have been linked previously to defects in HR, possible due to titrating out RAD51 (so that the proffered ratio between BRCA2 and RAD51 is not established). Additionally, we provide more data suggesting that DPP9 limits the interaction of PALB2 and BRCA2, and a higher cytosolic localization of the BRCA2-PALB2 complex in the absence of DPP9 (Fig 2F-H, Fig 6D and E, Fig EV1D). Thus, DPP9 may regulate the interaction between PALB2-BRCA2, which may ensure the formation of productive complexes (Fig. We have discussed this more thoroughly in this version.

6) Western blots for the DPP9 expression in HEK293 DPP9 KO + DPP9-S WT and S729A (Fig. 7A) should be shown.

Western blots showing the Dox-induced over-expression of DPP9 is shown in Fig 4C, Appendix Fig S2A. Immunofluorescence images show the cytosolic localization of DPP9-S and the predominantly nuclear localization of DPP9-L.

Referring to point 4, DPP9 activity would be destabilizing BRCA2 and reduced BRCA2 levels is expected to decrease Rad51 foci. The results seem to suggest the opposite effect. This is not explained sufficiently well.

We have discussed this in more detail:
How the processing of the BRCA2 N-terminus promotes DNA repair is currently unknown. That lower BRCA2 levels impair HR is well known, however, also higher BRCA2 levels impair HR, and for example USP21 which is known to stabilize BRCA2 is overexpressed in hepatocellular

carcinoma, where it inversely correlates with patient survival (Magwood et al, 2012; Liu et al, 2017). A possible scenario is that the cellular levels of BRCA2 are tightly regulated and fine-tuned to allow productive molecular interactions of BRCA2 with PALB2 and RAD51. Consistent with the higher levels of BRCA2 in DPP9-deficient cells, more PALB2-BRCA2 interactions are observed in these cells, suggesting that DPP9 limits this interaction by lowering the cellular concentration of BRCA2. DPP9 may additionally limit this interaction by competing with PALB2 for interaction with BRCA2. Nonetheless, despite the increase in the BRCA2-PALB2 association, DPP9-depleted cells show a decrease in the colocalization of BRCA2 with γH2AX which locates to sites of DNA damage. Consistently DPP9-depleted cells accumulate fewer RAD51 foci, a phenotype that can be partially restored by the over-expression of active DPP9-S^{WT}, less efficiently by DPP9-L^{WT}, but not by the enzymatically inactive DPP9-S^{S720A} mutant. Additionally, expression of the BRCA2ΔMP truncation mutant results in a better recovery of RAD51 foci in DPP9 KD cells silenced for BRCA2, compared to expression of the WT BRCA2 construct. Since both constructs compensate for BRCA2 silencing in cells expressing endogenous levels of DPP9, these results imply that DPP9 promotes the formation of RAD51 foci by modifying the N-terminus of BRCA2. Similarly, fewer RAD51 foci were reported for cell expressing an excess of BRCA2 or the BRC4 repeat (Magwood *et al*, 2012; Chen *et al*, 1999; Abe & Branzei, 2014)*. The interaction between BRCA2 and RAD51 involves eight BRC repeats in the centre of BRCA2 and an additional domain in the C-terminus (CTD) allowing BRCA2 to bind multiple copies of RAD51* (Davies *et al*, 2001; Davies & Pellegrini, 2007; Pellegrini *et al*, 2002; Esashi *et al*, 2007; Galkin *et al*, 2005; Esashi *et al*, 2005)*. Different studies estimate a ratio of one BRCA2 monomer binding simultaneously at least 5-7 molecules of RAD51* (Yang *et al*, 2005; Liu *et al*, 2010; Jensen *et al*, 2010; Shahid *et al*, 2014; Sidhu *et al*, 2020)*. In vitro studies suggest that binding to multiple monomers of RAD51 to BRCA2 provides a rapid mechanism for nucleating the RAD51 filaments on the ssDNA and promoting their growth* (Thorslund *et al*, 2010; Jensen *et al*, 2010; Liu *et al*, 2010; Carreira & Kowalczykowski, 2011; Shahid *et al*, 2014; Sánchez *et al*, 2017)*. Thus, the DNA-damage induced degradation of BRCA2 that we and other observe* (Liu *et al*, 2017; Schoenfeld *et al*, 2004) *may serve to establish an optimal stoichiometric ratio between BRCA2 and RAD51 for repair. In this scenario, DPP9 modulates efficient repair by fine-tuning the cellular concentration of BRCA2.*
The interaction between RAD51 and BRCA2 is cell cycle dependent (Ayoub et al, 2009), through the CDK-mediated phosphorylation of BRCA2 C-terminus at S3291 (Esashi et al, 2005) and ubiquitination of RAD51 which interfere with the interaction (Luo et al, 2016). Fine-tuning the protein levels of BRCA2 by DPP9 present an additional layer of regulation cells apply to ensure efficient repair by HR.

7) In Fig.7 C and E, BRCA2 seems to be largely cytosolic. This seems to be especially the case in DPP9 KD cells. Is there a BRCA2 localization defect in these cells which could be responsible for reduced Rad51 loading? The authors should explain this further.

To address this question, we looked into the intra-nuclear colocalization of BRCA2 to γH2AX.We observe fewer BRCA2-γH2AX PLA events in HeLa DPP9 KD cells suggesting defects in targeting of BRCA2 to site of damage despite the increased interactions with PALB2 (Fig. 6E and F, Fig EV1E). We propose that deregulation of the BRCA2:PALB2 interactions lead to defects in correct localization of BRCA2.

Also, from Fig.4H and I, BRCA2Del MP mutant is unstable and has a half-life of ~2-3h even without damage. How does it then sustain Rad51 loading after 24h of MMC treatment (Fig.7E)?
One possible explanation is that the lower concentration of BRCA2 is sufficient for repair, whereas over-expression (as DPP9-depleted cells cannot process BRCA2) impairs repair, due to irregular stoichiometry between DPP9-PALB2 and ARD51.

Minor points

1) Fig.4H The molecular weight of GFP is shown to be around 55kDa? Is this some modified GFP construct, considering the normal MW is around 28kD

This is a double GFP construct.

2) Data in many figures throughout the paper are represented by 2 types of graphs (individual value plots and mean column plots). Since both convey the same information, this seems unnecessary.
We used both figures in order to show single cell results as well as the reproducibility of the biological repetitions.

3) Supplementary Figure 5B refers to Figure 6C and is mentioned as 5C in the legend.
This has been corrected, thank you

Referee #3:

In the current manuscript, Bolgi et al. present a role for the dipeptidyl peptidase DPP9-dependent cleavage of BRCA2 N-terminus in promoting homologous recombination (HR). The authors identify BRCA2 as a novel substrate of DPP9, which cleaves the first 2 amino acids of BRCA2 (after an evolutionarily conserved Proline) and enhances BRCA2 turnover after DNA damage. Human cells lacking DPP9, or mouse embryonic fibroblasts expressing a catalytic inactive version of DPP9, are hypersensitive to mitomycin C, Olaparib and ionizing radiation. Consistent with this, depletion of DPP9, or expression of a BRCA2 truncation mutant that cannot be processed by DPP9, result in impaired RAD51 foci formation.

The study by Bolgi et al. is intriguing, and the manuscript concise and carefully written. My main concern regards the discrepancies between the observation of DPP9-BRCA2 interaction in the cytoplasm, and the proposed role in the nucleus. Moreover, the functional relevance of BRCA2 cleavage by DPP9 for HR should be strengthened, as suggested below.
We are pleased that this reviewer finds this study to be intriguing. Please find below a detailed response to the concerns of this reviewer.

Major points

1. In the Discussion, the authors suggest that BRCA2 N-terminus could become available for DPP9 cleavage after complexing with ssDNA and RAD51, yet the DPP9-BRCA2 PLA interaction occurs mainly in the cytoplasm. This is puzzling and should be further clarified. Can the authors elaborate more on the function of nuclear vs cytoplasmic DPP9? Which isoform is relevant for BRCA2 cleavage and DNA repair? Is the cellular distribution of FLAG-DPP9-S in DPP9 KO HeLa cells completely cytoplasmic? If so, how is DPP9 limiting BRCA2 at DNA breaks?

It has been previously reported that either DSS1 or ssDNA alone could disrupt BRCA2 multimers *in vitro*, and that both stabilize the monomeric form (Le *et al*, 2020). Similar to DPP9, also DSS1 localizes both to the nucleus and to the cytoplasm (Wei *et al*, 2003). Indeed the majority of the DPP9-BRCA2 PLA interactions appear in the cytoplasm (Fig 1B-D). Furthermore, in this current version, we report that the overexpression of the cytosolic variant DPP9-S leads to destabilization of BRCA2, while the over-expression of the inactive variant does not (Fig 4C and D). Additionally, we show that re-expression of DPP9-S supports the formation of RAD51 foci, while the overexpression of DPP9-L is less efficient (Figure EV4G and H). We provide microscopy images showing the cellular localizations of these overexpression systems (Appendix Fig S2D), where FLAG-DPP9-S is detected in the cytoplasm, while DPP9-L is mostly in the nucleus. Thus, our results suggest that it the cytosolic form of DPP9 that regulates the cellular concentration of BRCA2 pool and thereby reduces the relative concentration of BRCA2 compared to RAD51, facilitating an optimal ratio between these two proteins for efficient nucleation of RAD51 to the DNA break sites.

2. The DPP9-depleted cells should be characterized better. How is their cell cycle profile? Is the

proportion of S/G2 cells as in WT cells? Also, a western blot testing whether absence of DPP9 affects total levels of RAD51 and BRCA1 should be provided.
In this version, we added further characterization of the HeLa DPP9 KD cells. We provided the cell cycle profiles in Fig EV2A and B. With a double thymidine block, we have chased the cell population through the respective cell cycle stages and could detect no statistical difference between the two cell lines (Figure EV2B, Analysis was done on at least 3 independent experiments). We have reported the effect of reduced DPP9 levels, transient silencing of DPP9, DPP9 knock-outs and the over-expression of active and inactive FLAG-DPP9-S in DPP9-KO cells on RAD51 stability (Figure 4A-C). We could not observe a statistical difference that was caused by the absence of DPP9.

3. On the same line as the previous comment, when assessing the number of RAD51 foci, a cell cycle marker (e.g. EdU incorporation) should be used for gating, and RAD51 foci should be counted only in EdU-positive cells.
In this version, we have investigated the number of RAD51 foci in EdU positive HeLa WT and DPP9 KD cells (Figure EV4B and C, Appendix Figure S1G). The number of RAD51 foci we detected were comparable to our previously reported values, furthermore, we could reproduce the loss of RAD51 foci phenotype in DPP9 deficient cells. Furthermore, analysis of asynchronized cells show a similar cell cycle profile of HeLa WT and HeLa DPP9 KD cells.

4. If DPP9 is required to remove BRCA2 from DNA breaks and allow formation of RAD51 filaments, it would be expected that excess BRCA2 nuclear foci are present (that colocalize with gH2A.X). Can the authors test this? Immunofluorescence should be enough, with no need to perform PLA assays.

Our results support a cleavage of BRCA2 by DPP9 in the cytosol and not at sites of DNA breaks. This is based on PLAs (Fig 1B-E) and over-expression studies using DPP9-S which localizes to the cytoplasm (Appendix figure S2D) and can restore BRCA2 degradation and RAD51 foci formation (Fig 4C and D, Fig 6G). In this version, we have included PLA assays investigating BRCA2 and γH2AX (Figure 6E and F, Figure EV1E, Appendix Figure S1G). From three independent experiments, we could show that the depletion of DPP9 results in fewer BRCA2-γH2AX PLA events, suggesting a mislocalization of BRCA2, despite the increase in interactions between BRCA2-PALB2 (Fig. (Fig 6C and D). Thus, we suggest that the N-terminal processing of BRCA2 by DPP9, does not facilitate the removal of BRCA2 from the sites of DNA breaks. Instead, we propose that DPP9 is critical for regulation the cellular concentration of BRCA2 and thus the relative ratio between BRCA2 and its interaction partners PALB2 and Rad51.
We argue that the BRCA2 levels in healthy cells are tightly regulated for efficient repair and localization and therefore expect that a surplus of BRCA2 in DPP9 deficient cells, would result in mislocalization of BRCA2. Over-expression of BRCA2 has been correlated with defects in HR, and linked to poor prognosis.

5. Can the authors expand Figure 5 including a sensitivity assay using siRNA-mediated depletion of BRCA2 and re-expression of siRNA-resistant BRCA2 1-3418 or BRCA2deltaMP 3-3418?

Thank you for this question. We planned the generation of BRCA2 KO cell lines where we could overexpress BRCA2 1-3418 or BRCA2 3-3418, however this could not be achieved given the time constraints of the revision and global supply chain problems.

Minor points

1. Using a BRCA2 1-40 peptide carrying substitution of Proline to a non-cleavable amino acid (histidine?) would be a nice control for Fig. 2A.

Thank you for this suggestion. In this revised version we have tested the importance of the proline for binding to DPP9. As a read out we measured hydrolysis of GP-AMC, an artificial DPP9 substrate. Inhibition of GP-AMC cleavage suggest competition with a BRCA2 1-40 peptide

for binding to DPP9. These assays show an importance for Pro in second position for binding to DPP9 (Fig 2G and H).

2. Can the authors provide clearer blots to show that BRCA2 is present in DPP9 complexes?
Thanks for the suggestion, we have now replaced all the blots in Figure 4 to represent the effects we report more clearly.

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Dear Dr. Geiss-Friedlander,

Thank you for the submission of your revised manuscript to our editorial offices. I have received the reports from the three referees that were asked to re-evaluate your study, you will find again below. As we have already discussed, referee #2 is satisfied by the revisions. Referees #1 and #3 have remaining concerns and suggestions to improve the manuscript that would need to be addressed in a final revision.

After the pre-decision consultation with you and going through the preliminary point-by-point-response you sent, we feel that the remaining concerns of the referees will be adequately addressed by the suggested revisions. I thus invite you to revise the manuscript as proposed in your revision plan. Please also provide a detailed final p-b-p-response to the remaining concerns of the referees.

Moreover, I have these editorial requests:

- Please reduce the number of keywords to five and mention these only once (below the abstract).

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

- Please order the manuscript sections like this:
Title page - Abstract - Key Words - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Author contributions - 'Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- Per journal policy, we do not allow 'data not shown', which is stated three times in the manuscript (page 32). All data referred to in the paper should be displayed in the main or Expanded View figures, or an Appendix. Thus, please add these data (or change the text accordingly if these data are not central to the study). See:
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- It seems separate callouts for panels Fig. EV2A and B, EV5A and B, and for Appendix Fig. S4A are missing. Pleas check and make sure that all figure panels are called out separately and sequentially.

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- Please name the 'crystallographic table' 'Table 1' and call it out with this name. Or, if you feel it is not necessary to show this in the main manuscript, move it to the Appendix (as Appendix Table S1 - please change the callouts).

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (for main, EV and Appendix figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. There are several bar or line diagrams (with n>2) without statistical testing (e.g. 2H, 4F, EV1).

- Please add scale bars of similar style and thickness to all the microscopic images (also those in the Appendix), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, for most images the scale bars that are too thin. Please check.

- Please upload the information in the 'Reagents and Tools table' separately and remove this from the manuscript text. I have attached templates for that in word or excel format. Please upload the filled in table to the manuscript tracking system as a 'Reagent Table' file. Then remove the tables and their legends from the final manuscript file. The example linked below shows how the table will display in the published article and includes examples of the type of information that should be provided for the different categories of reagents and tools. Please list your reagents/tools using the categories provided in the template and do not add additional subheadings to the table. Reagents/tools that do not fit in any of the specific categories can be listed under "Other":
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- As they are significantly cropped, please provide the source data for the entire Western blots shown in the manuscript

(including EV figures and Appendix figures). The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure (main and EV) and one file for the Appendix figures.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

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

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

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

All the best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have included new experiments to this revised version, and I acknowledge that the current version contains a large collection of data from biochemical, structural and cellular approaches. However, I still have concerns about the validity of the overall conclusions and the final model, drawn by the authors. Focusing on the most important points:

- The BRCA2 proline mutants were an important point, which has been addressed by the authors with Supplemental Figure S4A. It is not clear why the authors chose to test this in the context of a construct that only contained 1000 aminoacids of BRCA2, rather than a full-length construct. A lack of stability of this 1-1000 constructs is not informative on the role of the Pro in the full-length functional context. As pointed out by reviewers, how such mutation (in the full-length protein) would behave in i.e. Figure 7C or 7E, etc is a critical question for the validity of the story.
Of note, Supp figure S4A is not referred to nor discussed in the revised manuscript.

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- The final model that DPP9 fine tunes the level of BRCA2 (in the cytoplasm) in relation to RAD51/PALB2 to control proper nucleation of RAD51 during HR (in the nucleus) is very speculative, based on the presented data.

Minor points:
- N-end rule / N-degron should be removed from the keywords.

Referee #2:

The authors have addressed the majority of reviewer concerns. I am satisfied with the rigor of the study and validation of results. Very little has been done in this area and their findings on N-terminal processing of BRCA2 will be of interest

---
Referee #3:

The authors have partly addressed we raised - surprisingly, simple experiments such as checking BRCA1 total levels in DPP9 KO cells (Point 2) and providing clearer blots for the immunoprecipitation experiments in Fig. 1F (Minor Point 2) were not provided but they should have been. Directly assessing the contribution of BRCA2 cleavage to olaparib resistance (Point 5) by

re-expressing an siRNA-resistant version of BRCA2, as we suggested since the authors already have the siRNA depletion system set up, is also missing. They have however satisfactorily addressed that the RAD51 foci formation / HR defect in DPP9 KO cells is not due to cell cycle defects. We also note that the present version of the manuscript is less carefully prepared than the original submission, with some figures seemingly mis-labelled. These errors made the evaluation more difficult (see Fig. 4D, 6D for example).

Dear Dr. Geiss-Friedlander,

Thank you for the submission of your revised manuscript to our editorial offices. I have received the reports from the three referees that were asked to re-evaluate your study, you will find again below. As we have already discussed, referee #2 is satisfied by the revisions. Referees #1 and #3 have remaining concerns and suggestions to improve the manuscript that would need to be addressed in a final revision.

After the pre-decision consultation with you and going through the preliminary point-by-point-response you sent, we feel that the remaining concerns of the referees will be adequately addressed by the suggested revisions. I thus invite you to revise the manuscript as proposed in your revision plan. Please also provide a detailed final p-b-p-response to the remaining concerns of the referees.

Dear Dr. Breiling, thank you for your pre-decision consultation and for the invitation to revise this manuscript. We gladly submit a revised version and a detailed point by point response which addresses the remaining concerns of the referees as well as the editorial requests.

Moreover, I have these editorial requests:

- Please reduce the number of keywords to five and mention these only once (below the abstract).

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interestsand update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

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Appendix. Thus, please add these data (or change the text accordingly if these data are not central to the study). See: http://embor.embopress.org/authorguide#unpublisheddata

We have addressed all the above points

- It seems separate callouts for panels Fig. EV2A and B, EV5A and B, and for Appendix Fig. S4A are missing. Pleas check and make sure that all figure panels are called out separately and sequentially.

Thank you for pointing this out. All panels are called out separately in this version. We have rearranged the Appendix and the EV figures to better fit the structure of the manuscript.

- Appendix Fig S5 has only one panel. It thus does not need the "A" panel label. Please remove this and update the callouts.

These have all been corrected. We have reorganized the EV and Appendix figures.

- Please name the 'crystallographic table' 'Table 1' and call it out with this name. Or, if you feel it is not necessary to show this in the main manuscript, move it to the Appendix (as Appendix Table S1 - please change the callouts).

The table is now labelled as Appendix Table S1.

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The number of independent experiments is clearly labelled for each panel in each figure, including the differentiation between biological and technical repetitions. Error bar, mean and SEM are also clearly defined in each figure legend. We have added complete statistical analysis.

- Please add scale bars of similar style and thickness to all the microscopic images (also those in the Appendix), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, for most images the scale bars that are too thin. Please check.
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- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please

provide your final manuscript file with track changes, in order that we can see any modifications done.
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These are provided.

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

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

All the best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have included new experiments to this revised version, and I acknowledge that the current version contains a large collection of data from biochemical, structural and cellular approaches.
Thank you for acknowledging the additional experimental data that was added to this manuscript to address the concerns that were raised on the first submitted version.

However, I still have concerns about the validity of the overall conclusions and the final model, drawn by the authors. Focusing on the most important points:
- The BRCA2 proline mutants were an important point, which has been addressed by the authors with Supplemental Figure S4A. It is not clear why the authors chose to test this in the context of a construct that only

contained 1000 aminoacids of BRCA2, rather than a full-length construct. A lack of stability of this 1-1000 constructs is not informative on the role of the Pro in the full-length functional context. As pointed out by reviewers, how such mutation (in the full-length protein) would behave in i.e. Figure 7C or 7E, etc is a critical question for the validity of the story.
Of note, Supp figure S4A is not referred to nor discussed in the revised manuscript.

We are surprised by this critique. We have shown beyond doubt that DPP9 activity determines the stability of endogenous BRCA2.

We then raised the question whether Met-Pro effect the stability of BRCA2. Thus, in the first submitted version we have performed CHX chase experiments on two constructs. In both cases we have used the ubiquitin fusion technique to define the N-terminus. This approach was established by Alexander Varshavsky and is standard in the N-degron field.
Due to its large size (384 kDa), the use of BRCA2 fragments is common in the BRCA2 field. Consistently, we also used fragments of BRCA2, in this case the N-terminal 1000 amino acids of BRCA2.

The control construct $BRCA2_{1\text{-}1000}$, was turned over at a rate similar to endogenous BRCA2 in the absence of MMC. The second construct $BRCA2\Delta MP_{3\text{-}1000}$ that lacks the two N-terminal residues Met-Pro was considerably less stable than $BRCA2_{1\text{-}1000}$, demonstrating that the dipeptide Met-Pro has a stabilizing effect on BRCA2.
Thus, the 1-1000 construct was very useful for studying the effect of the N-terminus on BRCA2 stability and was not criticized in the first revision round. In this version for correct comparison, we have again used a 1-1000 amino-acid construct of BRCA2, and mutated the Pro in position 2. We have added a reference to this panel (Appendix Figure S4B) within the main text.

- The localization of the BRCA2-DPP9 interaction has not been clarified. Immunoprecipitation +/- damage, and in the cytoplasmic vs nuclear fractions have not been added. These were important suggestions from the referees. The new PLA data analysis does not shed new light.

We respectfully disagree.
To cite the comments that we received for the first submitted version:

Reviewer 1: … "There is a mention that the DPP9-BRCA2 spots are cytoplasmic (Discussion), can the authors provide actual statistics on this + controls? "

As reviewer 1 wrote, this was not a major point for our work, it was an observation that we reported in the discussion. As requested, we have provided statistical data for the localization of the BRCA2-DPP9 PLA events in both compartments, showing the number of events was higher in the cytosol. Additionally, we state the fold increase in the PLA number in response to DNA damage.

Reviewer 2: "The IP experiment was done with the cytosolic DPP9 isoform. Is there any interaction with the nuclear isoform? Is this interaction increased with mutants of BRCA2 that localize mainly to the cytosol? This would support the authors' hypothesis that DPP9 binds the soluble pool of BRCA2."

We need to stress that interactions between an enzyme and its substrates are dynamic. Therefore, for interaction / close proximity studies we have a great preference to the PLA approach, which is extremely well-suited for detection of highly dynamic interactions. We control these experiments not only technically (one antibody only), but also by silencing the corresponding partners.

Most importantly: while PLAs do not provide evidence for direct interactions, neither do co-immunoprecipitation assays. Even more so, co-immunoprecipitation assays can provide an incorrect picture, as it may lead to dissociation of complexes due to detergent and salt conditions, or alternatively enrich for non-specific binders if the buffer conditions are too mild. To perform an immunoprecipitation of nuclear and cytosolic fractions – different buffer conditions would be required. Therefore, we do favour the PLA as an indication for a close proximity between proteins, thanks to the less destructive nature of the experiment which allows for a visualization of the close-proximities in the native state of the cells at the sites where the interaction occurs. The use of PLAs for studying BRCA2 interactions is not uncommon in the field, see a recent example in a paper published in EMBO (Sessa *et al*, 2021). We stress here that we do not exclude that DPP9 and BRCA2 co-localize to the nucleus, we do however document the presence of more PLA BRCA2-DPP9 events in the cytosol. We show that a fold increase in response to DNA damage occurs both in the nucleus and in the

cytosol, using MMC and NCS as DNA damaging agents. We also show more interactions in cells that were synchronized to S-phase.

Using the PLAs we investigate the close proximity between endogenous DPP9 and endogenous BRCA2. To test for direct interactions, we have used recombinant components which we assayed in pull-down assays, SPR and crystal structures. We confirmed *in vitro* cleavage of BRCA2 N-terminal peptide by mass spectrometry and in crystals. We further mapped residues in BRCA2 N-terminus, apart from the proline to be important for binding.

Moreover, in this revised version we applied different approaches to functionally test whether the cytosolic variant of DPP9 can rescue the MMC-induced degradation of BRCA2 and the formation of RAD51 foci. To test the roles of DPP9 in the nucleus and in the cytoplasm, we took advantage of the fact that cells express 2 variants of DPP9, which have alternative translation initiation sites. The two variants only differ in the presence of an N-terminal extension in DPP9-L which includes a nuclear localization signal (Justa-Schuch *et al*, 2014). We have established cell lines in which the endogenous DPP9 was first CRISPRed out, and then transfected for stable integration of a doxycycline-inducible expression of either the DPP9-L or DPP9-S. Immunofluorescence imaging demonstrate the nuclear-only localization of DPP9-L, and the exclusion of DPP9-S from the nucleus. We show that over-expression of DPP9-S in these cells restores the MMC-induced destabilization of BRCA2. Additionally, over-expression of DPP9-S rescues the RAD51 foci formation, the effect of DPP9-L was significantly lower.
Thus, our results show beyond doubt that DPP9 activity functionally affects BRCA2 stability and RAD51 foci formation, and that this is rescued by the cytosol-localized variant of DPP9.

- The final model that DPP9 fine tunes the level of BRCA2 (in the cytoplasm) in relation to RAD51/PALB2 to control proper nucleation of RAD51 during HR (in the nucleus) is very speculative, based on the presented data.
We have simplified the model in the synopsis.

Previous publications have shown that BRCA2 undergoes degradation in response to DNA damage, and that USP21 which localizes to the cytoplasm stabilizes BRCA2. Over-expression of BRCA2 is toxic to cells, and high USP21 expression (which stabilizes BRCA2) is linked to cancer progression. In vitro assays showed that functional RAD51 filaments

require a sub-stoichiometric molarity ratio of BRCA2 to RAD51 (all points are in the discussion, including the references).

In this manuscript we show that:

- DPP9 activity is critical for the previously described MMC-induced, accelerated degradation of BRCA2. This can be carried out by DPP9-S.
- DPP9 and PALB2 compete for interaction with BRCA2.
- DPP9 depleted cells show various defects in DSB-repair: HR-recombination, hyper-sensitivity to genotoxic agents, accumulation of gH2AX, fewer RAD51 foci.
- Since RAD51 is a direct down-stream binding partner of BRCA2, we also performed rescue experiments to reverse the loss of RAD51 foci formation in DPP9 deficient cells by expressing BRCA2 full-length variants that contain either the processed or the un-processed BRCA2 N-terminus.

Based on these findings we discuss the following model, in which we speculate that DPP9 fine-tunes the cellular levels of BRCA2 to ensure a proper interactome.

Future work is aimed to address and characterize this model in depth and map potential other novel substrates of DPP9 in the DNA damage response area, however this, we believe, would be out of the scope of this current work.

Minor points:
- N-end rule / N-degron should be removed from the keywords.
If this is a critical point, we would remove this keyword. However, we would prefer not to do this, since we would like to increase the visibility of this manuscript to the N/C degron community, to highlight DPP9 as a parallel to the Pro-N degron pathway.
DPP9 is an amino-peptidase which modifies the N-termini of its substrates. As we have previously shown, DPP9 plays an upstream role in converting the stable proteins Syk and AK2 to substrates of the N-degron pathway. Here we add BRCA2 to this short list of characterized DPP9 substrates.

Referee #2:

The authors have addressed the majority of reviewer concerns. I am satisfied with the rigor of the study and validation of results. Very little has been done in this area and their findings on N-terminal processing of BRCA2 will be of interest

Thank you so much, for highlighting the experimental quality and novelty of our findings.

Referee #3:

The authors have partly addressed we raised - surprisingly, simple experiments such as checking BRCA1 total levels in DPP9 KO cells (Point 2) and providing clearer blots for the immunoprecipitation experiments in Fig. 1F (Minor Point 2) were not provided but they should have been.

We apologize for this. This question was part of a larger point:

„2. The DPP9-depleted cells should be characterized better. How is their cell cycle profile? Is the proportion of S/G2 cells as in WT cells? Also, a western blot testing whether absence of DPP9 affects total levels of RAD51 and BRCA1 should be provided.“

As this referee states (see below) we have clearly shown that the observed HR defects in DPP9 depleted cells is not due to cell cycle defects, which we perceived as the major concern of this referee. We have also shown the RAD51 levels (in figure 4). We have over-looked the request for BRCA1 blots. These are included in this version, in EV2 for HeLa cells and Appendix Figure S2 for HEK293 cells

Minor point 2: immunoprecipitations of endogenous BRCA2 have always been challenging, mostly due to the large size of BRCA2. In many publications BRCA2 has been N- and C-terminally tagged for the immunoprecipitation, here we co-immunoprecipitated the endogenous untagged BRCA2. The quality of our gels is similar to that of others in the field as published in Oncogene in 2016 and in EMBO in 2021 (Chalermrujinanant *et al*, 2016; Sessa *et al*, 2021).

Directly assessing the contribution of BRCA2 cleavage to olaparib resistance (Point 5) by re-expressing an siRNA-resistant version of BRCA2, as we suggested since the authors already have the siRNA depletion system set up, is also missing.

„Can the authors expand Figure 5 including a sensitivity assay using siRNA-mediated depletion of BRCA2 and re-expression of siRNA-resistant BRCA2 1-3418 or BRCA2deltaMP 3-3418?“

We had technical difficulties to asses this question with transiently silenced cells, since it requires exposure of cells to Olaparib for 72 h in contrast to the 24 h MMC treatment for the RAD51 foci assay.
We observe an optimal level of siRNA depletion in 72h, while the transient re-expression of the siRNA resistant BRCA2 was initiated 24h after the siRNA treatment and reached optimal levels 48h after transient transfection of the re-expression plasmids, after which the depletion effect of the siRNA and the re-expression of siRNA resistant BRCA2. Our current system is not suitable for this experiment and introduces toxicity due to the harsh nature of the different treatments that the cells must endure. This experimental setup would work best with a stable cell-line system that has the different BRCA2 variants integrated. However, we have not completed the generation of these cell-lines.

We, therefore, concentrated on the recovery of RAD51 foci in the HeLa WT and HeLa DPP9 KD cells, in which cells were less stressed. We also think that this is a more direct measurement of BRCA2 activity, compared to the survival assays which are indirect.

They have however satisfactorily addressed that the RAD51 foci formation / HR defect in DPP9 KO cells is not due to cell cycle defects.
Thank you.

We also note that the present version of the manuscript is less carefully prepared than the original submission, with some figures seemingly mis-labelled. These errors made the evaluation more difficult (see Fig. 4D, 6D for example).
Thank you very much for picking up these points. We have corrected these errors in this version.

Dr. Ruth Geiss-Friedlander
Albert-Ludwigs-Universität Freiburg
Institut für Molekulare Medizin und Zellforschung
Stefan-Meier-Str. 17
Freiburg D-79104
Germany

Dear Ruth,

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.

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

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Abridged guidelines for figures

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The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods and Key Resources Table

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	Reagents and Tools table

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	Reagents and Tools table

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

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	microscopes used from the Lighthouse Core Facility (materials and methods, Acknowledgments)

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Material and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legend and Materials and methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legend
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legend

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.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	materials and methods
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Yes	materials and methods
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	references