EMBO reports

Ewing sarcoma protein promotes dissociation of poly(ADP-ribose) polymerase 1 from chromatin

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

1st Editorial Decision 12th Jul 2019

Dear Dr. Myung,

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

As you will see, all referees acknowledge that the findings are potentially interesting. However, all referees also point out that significant revisions will be required before the study can be considered for publication here. Referee 1 indeed feels that the study would be better suited to publication in a more specialized journal, as indicated in the manuscript summary table that is directly sent to the editor. However, given that all points by referees 2 and 3 are very constructive and addressable, I think that if all these points can be successfully addressed, we can offer to publish your manuscript.

I would therefore like to invite you to address all referee concerns, with a special focus on all points raised by referees 2 and 3. 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 major round of 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. Given your 6 main figures I suggest that you layout the manuscript as a full article.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

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

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure

S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, please consider to deposit primary datasets produced in this study in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study.
* Note - All links should resolve to a page where the data can be accessed. *

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

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

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

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO Reports

Referee #1:

Lee et al present an interesting investigation into the relationship between EWSR1 and PARP1. Initially they performed SILAC on isolated adipose tissue and identified several protein level differences include some that are involved in DNA repair and DNA metabolism, including a 1.5 fold increase in PARP1. They then examined sensitivity to a variety of differently acting DNA damaging agents and show EWSR1 depleted cells were more sensitive in short viability and colony forming assays. The went on to show a difference in pCHK induction. From here, the authors switch tact and look at NAD+/NADH levels in EWS-KO mBA and embryo liver. They observe a significant decrease in NAD+/NADH ratio. With this, the authors considered that PARP1 was increased in their SILAC analysis as NAD+ is required for PARP1 activity. They observed increased PARP1 expression by qRT-PCR in cells and embryonic liver with EWSR1 knockout. They observed increased PARP1 expression in EWS-KO mBA and embryo livers. They then show increased PARylation in mouse tissues and an associated increase in apoptotic cells in the brain. The authors then went on to examine amount of PARP1 and other DDR proteins on chromatin in response to damage and found an increase for a longer duration. They also find EWSR1 recruits to damage, but this is blocked by PARP1 inhibitor olaparib - of note, this has phenomenon been reported before (PMID: 26286827). The authors then performed several experiments to show that PARP1 is not leaving damaged chromatin, but the interpretations of these results raisesthe first major concern (see below). The authors did eliminate the defect as being caused by a PARG deficiency.

The authors then went on to look at PARP1:EWSR1 association and indicated that this association was dependent upon PAR and PARP1 activity (with olaparib) through EWSR1 RGG region. Again, this has been shown before (PMID: 26286827). The authors then performed deletion construct analysis of EWSR1 to map the interaction region, finding it to be the RGG repeats.

The authors went on to evaluate sensitivity of PARP1 and EWSR1 single or double knockdown and found individual depletions resulted in sensitivity while the double had no such sensitivity. However, as these are only 24 hr assays, another major concern is that there may be cytostatic effects rather than just changes in cytotoxicity (see below).

The authors then evaluated R-loops in EWSR1 cells by IF. Another major concern is that the IF figures show no nucleoli, which should have the highest signal, yet do not appear at all. How do the authors explain this? RNAseH1, RNAseA and secondary antibody alone controls should be included.

The authors then try to rescue EWSR1 ko mice with either PARP1 DKO or NAD supplementation.

The PARP1 DKO did not provide a significant rescue, though NAD supplementation seemed to improve viability. Statistics need to be provided for this result.

Finally, the authors examined PAR levels in Ewing sarcoma samples and found they were increased. Again, hyperPARP1 has been reported before for Ewing sarcoma.

Major concern:
An alternative to why PARP1 and EWSR1 are found at damage for longer and at greater amounts is because of a lack of repair, with their accumulation being secondary effects. There are several papers describing EWSR1 relocalization in response to damage, particularly to nucleoli - this possibility needs to be considered. The authors go on to assume PARP1 trapping on chromatin, however they cannot say this from these experiments, only that PARP1 is more associated with DNA - again, can be because the damage is not being repaired. However, would expect that self PARylation would remove PARP1, so is PAR activity reduced? Are NAD levels low (ratio is low as noted above)? Is this rescued by NAD supplementation? What are the absolute levels of NAD+ and NADH (not just ratio)? If the authors' scenario is correct, then why is there a decreased ration of NAD+/NADH in the absence of damage? And what happens in response to damage? Finally, the interpretation that EWSR1 is necessary for PARP1 removal from damage, suggesting an active process is again flawed. It is one possibility, however, if there is a DNA repair defect and the damage is not being repaired with the same kinetics, the same effect would be observed.

Additional concerns:
SILAC analysis - How were the brown adipocytes isolated? or were they generated from wt cells? What was their purity and how long were they expanded if isolated? Was there a difference in cell cycle profile between wildtype and EWSR1 knockout cells? What were the thresholds used to determine differentially labelled proteins? How is significance in this experiment defined? How many replicates were performed? And how was an FDR of 1% selected and utilized? How many peptides were detected and how many per "significant" protein". Though a STRING interactome is provided in Fig. 1C, a GSEA or GO should also be provided to demonstrate any significance in the findings with regard to DNA repair etc. Overall, it would appear that rather than DNA repair, DNA replication is a more significant gene set difference. A complete list of up and downregulated proteins should be provided. It is not clear where PARP1 ranked in the data overall as only 10 upregulated proteins are provided in Table S1. And then how stringent is a 1.5 fold difference?

For viability assays (Sup fig 1 A) the authors used cell titer glo assay. This is an ATP based method for assessing cell numbers, but if they are observing a metabolic change with EWSR1 loss, then there may be a discrepancy between the relationship of ATP concentration and cell number in response to damage treatment. This result should be verified with another assay.

P7, par 2, line 7: The authors state "MMS treatment significantly increased SSBs" however, as they are using alkali comet assay they cannot rule out abasic sites or DSBs. There could also be increased ssDNA regions. Better to state that there is increased damage which may indicate an increase in SSBs.

P8, par 2, line 5: the authors state "we examined whether PARP1 activation causes cell death ", but they cannot state whether PARP1 activity causes cell death, this may be the case, but here they can only ask whether there is an association. Later, the authors look at PARP1 EWSR1 dko mice, and if those brains do not show apoptosis, then they can indicate some causation.

P9, par 1, line 19: the authors state "These results indicate that EWS was

recruited to damaged DNA in a PARP1 catalytic activity-dependent manner
and promoted the dissociation of PARP1 from damaged DNA." This cannot be stated from the results provided. The authors should try another PARP1 inhibitor that causes trapping, like talozoparib and should also compare to PARP1 depletion.

P12, par 1, line 4: is the interaction between PAR and EWSR1 dependent upon DNA?

Fig 5, need to include total CHK1. Also, why does pCHK1 or gH2Ax not change irrespective of EWSR1 or PARP1 depletion? - this seems contrary to the viability findings. Treatment is only over 24 hrs - is the difference due to a cytostatic effect rather than cytotoxic? What happens to cell cycle profile?

Fig S1 E. Need to include total CHK1 to compare with pCHK1 (and in other figures).

Typographical errors
There are numerous typographical errors and grammatical errors throughout the manuscript.
Eg. p3, line 4, "challenged" should be "challenge"

Throughout nl or ul or ml should be nL, µL or mL respectively

Referee #2:

EWS (Ewing sarcoma) together with other members of the TET family of proteins are frequently translocated in human cancers. In this article, the authors observe rapid poly(ADP-ribose) (PAR) dependent accumulation of EWS at sites of DNA damage through its positively charged RGG domains. This has been reported previously, however (Altmeyer et al. 2015 Nat Commun). They also describe hypersensitivity and the accumulation of gH2AX in EWS-depleted cells in response to DNA damaging agents. The most novel finding in this work is the observation that EWS is needed for the dissociation of PARP1 from damaged DNA. However this aspect requires more detailed and robust experimental work before it is appropriate for publication.

I have following general concerns/questions:
1) The PARP1 trapping and increased levels of poly(ADP-ribose) after EWS depletion seem to be quite mild. Moreover, the extent of the effect varies from one experiment to another. Is this a specific role for EWS itself or are other FET proteins involved? In another words, would there be more PARP1 trapping on damaged DNA if the cells are depleted for all three major FET proteins (EWS, FUS, TET15)?
2) The model argues that there is more PARylation in the EWS-depleted cells because of elevated levels of PARP1 at sites of DNA damage, which they argue cannot be dissociated. The authors should consider the idea that the elevated activity arises from the elevated level of unrepaired DNA lesion. Indeed, their data suggests a level of DNA damage in EWS depleted cells as measured by alkaline comet assays (Fig S1).
3) Do the authors believe it is autoPARylated PARP1 that is dissociated by EWS? If so, what is the impact of PARG inhibition?
4) It is not clear whether the authors believe it is the hyper-PARylation, decreased NAD+ levels, or the chromatin-trapped PARP1 that cause the DDR defect and the cell death.

Specific issues:

1. The authors should correct the labelling of the supplementary figures and tables (EV vs. Figures S).

2. The word "significantly" is not used properly throughout the text (e.g. Page 9, 11, mainly when referring to WBs which are not quantified). Please use a different word in instances that do not refer to statistical significance.

3. To strength the finding that PARP1 is trapped on damaged DNA in EWS depleted cells the immunofluorescence staining after chromatin pre-extraction should be used and quantified by high-content imaging (mainly Fig. 2A, Fig. 5C, Fig. 6A).

4. For cell viability assays the same type of charts (line charts) should be used (the data format in Fig.4G and S1A don't correspond).

5. PARP1 protein levels are missing and should be measured in tissues and total cells lysates (Fig. 1D and S2). Where PARP1 chromatin binding is shown, the levels of PARP1 in soluble nucleoplasm would be useful to see as well.

6. The PAR levels in Fig. 2A and 2B need to be shown (PAR levels are shown only after H2O2 treatment). On the other hand PARP1 trapping is shown only after MMS. The conclusion will be stronger if the similar experiment are shown after H2O2 treatment.

7. In Fig. 4B control experiment should be done in EWS depleted cells.

8. In Fig. 4D and 4E the labelling of molecular size on WBs is not consistent.

9. In Fig. 4E and 4F the relative amount of PARP1 is not specified. How it wat calculated?

10. For Fig. 5D, clonogenic assays with different MMS concentrations including PARP1 KO rather than cell viability would be more convincing. Also alkaline comets would be useful to see if there are DNA breaks in DKOs. Similarly, it would be interesting to compare these data with treatment with PARP inhibitor.

11. What is the PAR level in DKO mice? (referring to Fig. 1D)

12. In Fig. S1B quantification doesn't seem to correspond the pictures.

13. In Fig. S5B the picture for EWS probe is cut off. PARP1 protein levels should be shown.

14. Why there is a difference in S9.6 positive foci per cell in EWS-KO cells in Fig. S6A vs S6B?

Referee #3:

Lee et al. present data on EWS recruitment to and its functions at DNA break sites. The novelty of this work is primarily associated with the claim that EWS, upon being recruited in a PARP1- and

poly(ADP-ribose)-dependent manner, is required to dissociate PARP1 from damaged DNA and terminate PARylation, which is an interesting finding. Other aspects, such as the PARP1- and PAR-dependent recruitment of EWS and the role of the RGG domains for the PAR interaction, are not particularly novel and these conclusions had been drawn in previous studies. It therefore seems advisable to focus the manuscript on the EWS-mediated termination of PAR signaling and its consequences for NAD+ levels and genome stability, and to further corroborate these aspects.

Specific points:

(1) The authors should try to more clearly separate between confirmatory results and new findings. For instance, that EWS is recruited to damaged DNA in a PARP1 catalytic activity-dependent manner was shown in or could be deduced from several previous reports (e.g. J Biol Chem. 2013 Aug 23;288(34):24731-41; Nucleic Acids Res. 2014 Jan;42(1):307-14; Nat Commun. 2015 Aug 19;6:8088). Also the interaction between the RGG domains and PAR chains became clear from these works (and more recently also in Cell Rep. 2019 May 7;27(6):1809-1821). While in the abstract this is less of a problem, more care should be taken in the paragraphs on page 5 bottom, page 9 (discussion of Fig. EV3C and D), page 11-13 on the contribution of the RGGs (RGGs as PAR-binding domains have been discussed in comprehensive reviews, e.g. Mol Aspects Med. 2013 Dec;34(6):1066-87, and more recently in Nucleic Acids Res. 2016 Feb 18;44(3):993-1006.).

(2) While the recruitment of EWS via the RGGs and PAR is not novel, the RGG-dependent dissociation of PARP1 is. However, how this should happen mechanistically is not clear from the current manuscript. Could the authors try to separate the recruitment of EWS from the ensuing PARP1 removal? For instance, would PARP inhibitor addition after the initial recruitment of EWS block covalent PARylation of EWS and thereby prevent EWS and PARP1 dissociation from the damaged chromatin? Or would it be possible to mutate PAR-acceptor sites in EWS and demonstrate that this abolishes PARP1 dissociation? If either of these approaches worked, this would move the mechanism of PARP1 removal beyond speculation.

(3) On a related note, the authors provide evidence that PARP1 protein and mRNA levels are increased in EWS knockout cells. It remains unclear, however, why this is the case (e.g. is EWS a transcriptional co-factor for PARP1 expression? Does it bind to the PARP1 promoter?), and how this plays into the enhanced retention of PARP1 at sites of DNA damage. It would be important to know which of the observed phenotypes is mainly driven by PARP1 up-regulation at the transcription level, or by the impaired dissociation from damaged chromatin, or by a combination of both.

(4) It should be substantiated that the observed NAD+ depletion and cell death in EWS-deficient cells is due to PARP1 hyper-activation. If true, simultaneous loss of PARP1 or PARP inhibitor treatment should rescue NAD+ levels and viability.

(5) Related to the previous point, I think it would be important to work out better whether the observed cell death upon EWS loss is due to PARP1 trapping (if this was the case, PARP inhibitor treatment would make things worse) or due to NAD+ depletion (if this was the case, PARP inhibitor treatment would make things better).

(6) The link to R-loops is very vague. To which extent do R-loops cause the observed phenotypes in EWS knockout cells? RNaseH1 over-expression is typically used to assess the specificity and functional role of R-loops. If the authors think that the elevated R-loops are relevant for the described phenotypes, such experiments would have to be included.

(7) There is quite some literature on Ewing's sarcoma cells being PARP inhibitor sensitive. These cancers carry a EWS translocation (e.g. EWS-FLI1), in which the RGGs of EWS are lost in the fusion protein. It would be interesting to discuss the studies on PARP inhibitor sensitivity of Ewing's sarcoma in the context of RGG-modulated PARP1 trapping.

(8) In Figure 2C, are the samples in the quantification graph swapped? Check also the spelling of the y-axis label.

(9) Scale bars are missing in immunohistochemistry and IF images.

(10) There are a couple of language/grammar issues that should be corrected (e.g. page 3 line 4, page 4 middle, page 10 lines 5-7).

EMBOR-2019-48676-T

Lee et al., point-by point response

Reviewer #1:

Major point:

1-1. An alternative to why PARP1 and EWSR1 are found at damage for longer and at greater amounts is because of a lack of repair, with their accumulation being secondary effects.

Response: Thank you for the comment. We also suspected the point of the reviewer regarding the uncertainty of an intrinsic requirement of EWS in DNA repair. Although we cannot completely rule out indirect effects as the reviewer point out, our functional analyses of EWS protein clearly demonstrated a specific role of EWS in DNA damage response (DDR), which operates at the early stage of DNA repair.
First, our FCS experiments showed that PARP1-GFP proteins increased diffusion time at DNA damage sites in *EWS*-KD cells (Figure 2D). These results strongly suggested that EWS directly regulates physiological function of PARP1 at DNA damage sites. Second, PARP1 protein accumulated more in *EWS*-KD cells at the DNA double strand break induced by ER-*Asi*SI restriction enzyme in ChIP assay (Figure 2E). Third, the recruitment of EWS to DNA damaged sites and the interaction between EWS with PARP1 through PARylation occurred at the early step of DDR (Figure EV3). These results strongly indicate that EWS directly function in the DDR. Furthermore, our new analysis of cell viability after treatment of PARP inhibitor showed decreased cellular viability in a dose-dependent manner in *Ews*-KO cell, while PARGi or NMN treatment did not affect cellular viability of *Ews*-KO cell (Figure 5). These results suggest that accumulation of PARP1 is the main cause of abnormal response to DNA damage in *Ews*-KO cell (New Figure 5J) (Please see the response to reviewer 3 (#4) and (#5)). Lastly, if EWS deficiency phenotypes are secondary effects, double knock out (DKO) should show a similar or worse phenotype compared to a single knockout. However, the cell viability and levels of DNA break in single knockout were partially rescued by double knockout (Figure 5 and EV5). Taken together, these results unequivocally suggest EWS functions in the DDR.

1-2. There are several papers describing EWSR1 relocalization in response to damage, particularly to nucleoli - this possibility needs to be considered. The authors go on to assume PARP1 trapping on chromatin, however they cannot say this from these experiments, only that PARP1 is more associated with DNA - again, can be because the damage is not being repaired.

Response: The paper (Paronetto *et al*. Mol Cell 2011) mentioned by the reviewer showed that relocalization of EWS protein to the nucleoli after DNA damage. Results in this paper are not relevant to our hypothesis. First, Paronetto et al used UV irradiation for DNA damage source while we used MMS, H_2O_2 and lazer-irradiation. Depending on different types of DNA damage, DDR and repair processes are quite different. Second, our study shows mainly the role of EWS at the early stage of the DDR. In contrast, Paronetto *et al*. showed EWS relocalization after 6 hours following UV irradiation, indicating that their observation was more likely the late DDR or repair process. In addition, their data interpretation are more focused on the EWS function

for alternative splicing in the nucleoli. Our micro-irradiation experiments showed EWS relocalization at the early stage of the DDR. Consistent with our data, previous study have shown that EWSR1 was relocalized at local DNA damage sites following micro-irradiation (Altmeyer *et al*. Nat Commun. 2015. 6: 8088).

1-3. However, would expect that self PARylation would remove PARP1, so is PAR activity reduced? Are NAD levels low (ratio is low as noted above)? Is this rescued by NAD supplementation?

Response: It is known that self-PARylation of PARP1 triggers dissociation of PARP1 from chromatin (Kim *et al*. Cell. 2004). We tested whether decreased NAD^+ levels could lead to reduction of self-PARylation and results in less dissociation of PARP1 from chromatin in *Ews*-KO cells. However, in our previous data, although there was decrease of NAD^+ level in *Ews*-KO cells, we observed more self-PARylation and more PARP1 in chromatin (Figure 2A and 3A).

To determine whether the supplementation of NAD^+ could rescue *Ews*-KO phenotypes as the reviewer suggested, we did several experiments and added results in the revised manuscript. We analyzed the cellular ratio of NAD^+/NADH and viability with or without NMN treatment in *Ews*-WT and -KO cells. The complementation of NMN rescued the ratio of NAD^+/NADH (Figure IA), but could not rescue the cellular viability in response to MMS treatment (Figure IB) [Figures for referees not shown.]. Taken together, these data suggested that loss of EWS induces defect in the dissociation of PARP1, hyper-PARylation, and decreases the NAD^+ level in cells. We added these results in the revised manuscript.

1-4. What are the absolute levels of NAD+ and NADH (not just ratio)? If the authors' scenario is correct, then why is there a decreased ration of NAD+/NADH in the absence of damage? And what happens in response to damage? Finally, the interpretation that EWSR1 is necessary for PARP1 removal from damage, suggesting an active process is again flawed. It is one possibility, however, if there is a DNA repair defect and the damage is not being repaired with the same kinetics, the same effect would be observed.

Response: To answer the reviewer's question, we calculated the levels of NAD^+ and NADH using NAD^+/NADH measurement kit (Abcam, ab65348). The absolute level of NAD^+ in *Ews*-WT and *Ews*-KO cell is approximately 102.92pmol/10e^6 cell and 75pmol/10e^6 cell, respectively. The absolute level of NADH in *Ews*-WT and –KO cell is approximately 37.75pmol/10e^6 and 39.83pmol/10e^6, respectively (Figure 1C). Under normal condition, PARP1 was slightly activated in *Ews*-KO cell lines (Figure 3A and B), which can reduce the cellular ratio of NAD^+/NADH in *Ews*-KO cells. Cellular NAD^+ could be reduced by various cellular processes in normal conditions, which could lead the observation we got. In response to DNA damage, PARylation became more activated in *Ews*-KO cells shown in Figure 3A and B. We additionally measured NAD^+/NADH ratio after H_2O_2 treatment. The ratio of NAD^+/NADH was more decreased in *Ews*-KO BATs following DNA damage (Figure II) [Figures for referees not shown.], which is consistent with our previous results.

Minor point:

2. SILAC analysis-How were the brown adipocyte isolated? Or were they generated from WT cells? What was their purity and how long were they expanded if isolated? Was there a difference in cell cycle profile between wildtype and EWSR1 knockout cells? What were the thresholds used to determine differentially labelled proteins? How is significance in this experiment defined? How many replicates were

performed? And how was an FDR of 1% selected and utilized? How many peptides were detected and how many per "significant" protein. Though a STRING interactome is provided in Fig. 1C, a GSEA or GO should also be provided to demonstrate any significance in the finding with regard to DNA repair etc. Overall, it would appear that rather than DNA repair, DNA replication is a more significant gene set difference. A complete list of up and downregulated proteins should be provided. It is not clear where PARP1 ranked in the data overall as only 10 upregulated proteins are provided in Table S1. And then how stringent is a 1.5-fold difference?

Response: : In our previous study, we established the stable brown adipocyte cell lines (BAT) using SV40 T antigen in *Ews*-WT and -KO mouse brown fat pad (Park *et al*. Dev Cell. 2013 26:393-404). To address whether there is a difference in cell cycle between *Ews*-WT and -KO BATs, we performed cell cycle analysis of WT and KO BATs using FACS. The results showed that the ratio of S-phase was increased in *Ews*-KO BATs compared to WT control (Figure III) [Figures for referees not shown.], which can explain why DNA replication gene is differently regulated in *Ews*-KO cells. Due to limited number of figures allowed, we present this data for reviewer's only. Two times mass-analysis were performed. Peptide identifications were accepted if they could be established at greater than 95.0% probability to achieve an FDR less than 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 1 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Al et al Anal. Chem. 2003;75(17):4646-58). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Normalization was performed iteratively (across samples and spectra) by subtracting the average ratios in log-space, and means were used for averaging. Spectra data were log-transformed, pruned of those that matched to multiple proteins, and weighted by an adaptive intensity-weighting algorithm. Out of 2611 spectra in the experiment at the given thresholds, 1134 (43%) were included in quantitation. Finally, 927 proteins were identified and quantified, with 367 proteins of that were significantly increased (greater than or equal to 1.2-fold). We attached GO analysis results in Dataset EV1. We have uploaded the complete list of up and downregulated proteins.

3. For viability assays (SF1A) the authors used cell titer glo assay. This is an ATP based method for assessing cell numbers, but if they are observing a metabolic change with EWSR1 loss, then there may be discrepancy between the relationship of ATP concentration and cell number in response to damage treatment. This results should be verified with another assay.

Response: Thanks for the comments. Since Cell-titer-Glo assay could be affected by metabolic alteration, we performed the clonogenic assay using HEK293-EWS-WT and -KO cell line after MMS (Figure IVA) and H_2O_2 (Figure IVB) [Figures for referees not shown.] treatment. Similar to our previous results, depletion of EWS resulted in sensitivity to MMS or H_2O_2 treatment in the clonogenic assay. We added these data in the revised manuscript as Figure EV1B and EV1C.

4. The authors state "MMS treatment significantly increased SSBs", however, as they are using alkaline comet assay they cannot rule out abasic sites or DSB. There could also be increased ssDNA regions. Better to state that there is increased

damage which may indicate an increase in SSBs. Remove the word 'significantly' in comet data.

Response: As suggested, we revised our new manuscript. The sentence in question has been changed to "MMS and H_2O_2 treatment induced DNA tail formation, which may indicate an increased level of SSBs in the knockout cells."

5. The authors state "we examined whether PARP1 activation causes cell death", but they cannot state whether PARP1 activity causes cell death, this may be the case, but here they can only ask whether there is an association. Later, the authors look at PARP1 EWSR1 Dko mice, and if those brains do not show apoptosis, then they can indicate some causation.

Response: Previously, many studies demonstrated that hyper-PARyation induced cellular cytotoxicity and neuronal death (Kim *et al*. Genes Dev. 2005 & Luo *et al*. Genes Dev. 2012). We looked at TUNEL signals as the reviewer suggested. We examined the TUNEL staining in *Ews*-WT, -KO, *Parp1*-KO, and DKO mouse embryo brain. Although DKO has TUNEL positive cells in the brain compared to *Ews*-WT, the number of TUNEL positive cells was lower in DKO compared to *Ews*-KO embryo brain (Figure V) [Figures for referees not shown.]. This new data could support our claim, but cannot rule out alternative possibility as the reviewer pointed besides the relationship between activation of PARP1 with apoptosis in *Ews*-KO embryo. Therefore we changed the previous sentence to "we examined if hyper-PARylation is associated with cell death in the embryonic brain of *Ews*$^{-/-}$". This phenomenon might be caused by compensation of the other PARP family protein and these data also support why DKO mouse could not be rescued after birth. The results are presented as a new Figure EV5F in the revised manuscript.

6. The authors state "these results indicate that EWS was recruited to damaged DNA in a PARP1 catalytic activity-dependent manner and promoted the dissociation of PARP1 from damaged DNA". This cannot be state from the results provided. The authors should be try another PARP1 inhibitor that cause trapping, like talazoparib and should also compare to PARP1 depletion.

Response: We agree with the reviewer. It would be important to determine whether PARP1 catalytic activity recruits EWS protein to the DNA damage sites. As the reviewer suggested, we monitored the recruitment of EWS upon talazoparib treatment in wild type and PARP1-KO cells using micro-irradiation. The treatment of talazoparib or depletion of PARP1 blocked the recruitment of EWS to microirradiated DNA damage sites (Figure VI)[Figures for referees not shown.]. Although talazoparib traps PARP1 in chromatin, talazoparib also inhibits catalytic activity of PARP1. Thus, it is still difficult to claim the recruitment of EWS in DNA damage sites was truly induced by catalytic activity or trapped PARP1. We changed our wordings in the revised manuscript "PARP1-dependent manner" and added these data as a new Figure EV3J.

7. Is the interaction between PAR and EWSR1 dependent upon DNA? (EWS and PARP1 interaction is driven by DNA?)

Response: Although our IP western method included an excessive sonication step (6 minutes at 80% power for 3/3 seconds on/off intervals in a Qsonica water bath sonicator), we cannot exclude the possibility of DNA dependency. To address this issue, we have examined the IP western with or without Benzonase treatment, which

removes DNA. Our data showed that the interaction between PARP1 and EWS was not altered with or without the Benzonase treatment (Figure VII) [Figures for referees not shown.]. We concluded that the interaction between PARP1 and EWSR1 was not affected by DNA. We stated this result in the revised manuscript with this data as Figure EV4B.

8. Fig5, need to include total CHK1. Also why does pCHK1 or gH2AX not change irrespective of EWSR1 or PARP1 depletion?-this seems contrary to the viability findings. Treatment is only over 24 hrs-is the difference due to a cytostatic effect rather than cytotoxic? What happens to cell cycle profile?

Response: Genetic mutation of EWS or PARP1 did not alter total CHK1 level (Figure EV5A and EV5B). Loss of EWS or PARP1 did not affected pCHK1 or γH2AX level without DNA damage. pCHK1 or γH2AX level was increased following treatment of MMS (Figure 5A and C). Analysis of cell cycle profile indicated that *Ews*-KO BATs increased ratio of S-phase compared to *Ews*-WT BATs (Figure III) (Please see the response to reviewer 1 (#2)). Consistently, when we measured cell proliferation kinetics in *Ews*-WT, -KO, PARP1-KO and DKO cells, *Ews*-KO cell lines had slightly increased cell proliferation speed (Figure VIII) [Figures for referees not shown.]. Therefore, we excluded the cytostatic effect in these cell lines after DNA damage.

9. Fig S1 E. Need to include total CHK1 to compare with pCHK1.

Response: As suggested, we have added total CHK1 data in the revised figures. (Figure EV1, Figure 2, Figure EV3, Figure EV4 and Figure EV5)

Reviewer #2:

Major point:

1. The PARP1 trapping and increased levels of PAR after EWS depletion seem to be quite mild. Moreover, the extent of the effect varies from one experiment to another. Is this a specific role for EWS itself or are other FET proteins involved? In another words, would there be more PARP1 trapping on damaged DNA if the cells are depleted for all three major FET proteins?

Response: Thanks for good suggestion. Although FET family proteins share the same domain and have similar functions, each member has a unique function in various cellular processes. To answer the reviewer's question regarding the role of FET family protein in PARP1 trapping, we depleted each FET family protein expression using siRNA in the HEK293 cell line. After MMS treatment (T) or MMS release for 1 hour (R), we analyzed the PARP1 trapping in the chromatin fraction by western blotting. Our results showed that depletion of EWS most dramatically increased accumulation of PARP1 in R. Triple-KD samples did not showed additive or synergetic effect for PARP1 accumulation following the releasement of MMS (Figure IX)[Figures for referees not shown.]. These results suggested that EWS is a major FET protein for dissociation of PARP1 from DNA damage sites. Though intriguing, these results are too preliminary and require careful and extensive characterization to conclusively demonstrate a role for FET family proteins. Therefore, we respectfully ask that these results are only included in the response letter to the reviewers for "peer-review purpose". We will actively pursue this line of investigation in future.

2. *The model argues that there is more PARylation in the EWS-depleted cells because of elevated levels of PARP1 at sites of DNA damage, which they argue cannot be dissociated. The authors should consider the idea that the elevated activity arises from the elevated levels of unrepaired DNA lesion. Indeed, their data suggests a level of DNA damage in EWS depleted cells as measured by alkaline comet assays (Fig S1)*.

Response: We agree with the reviewer that an increase of unrepaired DNA could induce activation of PARP1 activity. Although we cannot completely exclude the possibility of DNA damage increase in EWS depleted cells, we believe EWS has direct roles for DNA damage response (DDR) due to following reasons. The loss of EWS increased a PARP1-GFP protein diffusion time only in the DNA damage condition in our FCS data, suggesting that EWS directly regulates the physiological function of PARP1 (Figure 2D). When we induced a site-specific DNA double strand break using ER- *Asi*SI restriction enzyme, PARP1 protein was accumulated at DNA double strand break in *EWS*-KD cells. Importantly, recruitment of EWS to damaged sites occurs at very early stage of DDR (Figure 2 and EV3). These results suggested that EWS directly functions in DDR. Finally, if our findings are secondary effects, cell viability rescued by DKO cannot be achieved (Figure 5). (Please see the response to reviewer 1 (#1)). As the reviewer points out, our alkaline comet assays showed significant increase of DNA damage in the *Ews*-KO cell after DNA damage. However, in normal condition, *Ews*-KO cell did not show any differences with Ews-WT cell, indicating that there is no actual increase of unrepaired DNA in normal condition. Taken together, our results suggest that trapping of PARP1 at DNA damage sites induce elevated level of DNA damage in *Ews*-KO cell. We added description of this observation in the revised manuscript. We described these results with discussion in page 18-19.

3. Do the authors believe it is autoPARylated PARP1 that is dissociated by EWS? If so, what is the impact of PARG inhibition?

Response: Auto (or self)-PARylation of PARP1 triggers dissociation of PARP1 from chromatin (Kim *et al*. Cell. 2004). In our data, treatment of olaparib increased a synergetic accumulation of PARP1 in chromatin (Figure XA) and decreased cellular viability (Figure XB)[Figures for referees not shown.] in *Ews*-KO cell. These results strongly suggest that EWS has an essential role in the dissociation of PARylated-PARP1 from DNA damage sites.

To specifically answer the reviewer's question, we have examined the accumulation of PARP1 after PARG inhibitor treatment in *Ews*-WT and –KO cells. In EWS-WT cell, PARG depletion slightly reduced chromatin-bound PARP1 in the absence and presence of DNA damage. In contrast, there was no effect in EWS-KO cells. These data indicated that EWS regulates the dissociation of auto-PARylated PARP1.

4. It is not clear whether the authors believe it is the hyper-PARylation, decreased NAD+ level, or the chromatin-trapped PARP1 that cause the DDR defect and the cell death.

Response: To answer the question, we treated NMN, PARGi, and PARPi in Ews-WT and KO cells and monitored the cellular survival. The complementation of NMN rescued the ratio of NAD^+/NADH (Figure XIIA). However, it could not rescue the cellular viability in response to MMS treatment (Figure XIIB) [Figures for referees not shown.]. To examine whether hyper-PARylation or chromatin-trapped PARP1

causes cell death, we treated PARGi and PARPi to *Ews*-WT and -KO cell. Only PARPi sensitized *Ews*-KO cells compared to WT cells, suggesting that chromatin-bound PARP1 is a major cause of DDR defect and cell death (Figure XIIC). Loss of EWS inhibits the dissociation of PARP1 from damage chromatin. We believe the accumulated PARP1 is the major effects observed in Ews-KO. (Also see the response to reviewer 3 (#4) and (#5))

Minor point:

5. The authors should correct the labelling of the supplementary figures and tables:
Response: We thank the reviewer for finding our mistake. We corrected the labels of supplementary figures in the revised manuscript.

6. The word "significantly" is not used properly throughout the text. Please use a different word in instances that do not refer to statistical significance:
Response: We have removed the world "significantly" from places where there is no statistical significance.

7. *To strength the finding that PARP1 is trapped on damage DNA in EWS depleted cell the immunofluorescence staining after chromatin pre-extraction should be used and quantified by high-content imaging.*

Response: As the reviewer suggested, we performed the IF using chromatin pre-extraction method and the new data showed that depletion of EWS resulted in an increased PARP1 foci number following DNA damage (Figure XIIA). The result is presented as a new Figure EV3B. Furthermore, patient-derived Ewing sarcoma cells also showed an increase in the level of chromatin PARP1 after DNA damage (Figure XIIB) [Figures for referees not shown.]. This result is presented as a new Figure 6B. These results are consistent with our previous data and strongly support that EWS is essential for dissociation of PARP1 in DDR.

8. *For cell viability assays the same type of charts (line chars) should be used.*
Response: As the reviewer suggested, we have changed the chart style in Figure EV1A.

9. *PARP1 protein levels are missing and should be measured in tissues and total cells lysates (Fig 1D and S2). Where PARP1 chromatin binding is shown, the levels of PARP1 in soluble nucleoplasm would be useful to see as well.*
Response: As the reviewer suggested, we performed the immunohistochemistry and western blotting using *Ews*-WT and -KO embryo and whole cell lysates. The results showed that the depletion of EWS increased PARP1 protein level in the embryo tissues (Figure XVA) but did not affect the total amount of PARP1 protein in the BAT cell lines (Figure XVB) [Figures for referees not shown.]. While chromatin-bound PARP1 was increased upon DNA damaging agent treatment, the level of soluble PARP1 was reduced (Figure XVC). The results are presented as Figure EV2, EV3 and 2 respectively.

10. *The PAR levels in Fig 2A and B need to be shown. On the other hand PARP1 trapping is shown only after MMS. The conclusion will be stronger if the similar experiment are shown after H2O2 treatment.*

Response: We thank the reviewer for this good suggestion. We have performed the suggested experiments (Figure XVIA and B) and the results are presented in the Figure 3D and 3E. We have also examined the alkaline comet assay to confirm the level of DNA damage using H_2O_2, and our new data showed that the length of comet tail was increased in *Ews*-KO BATs after H_2O_2 treatment and also after washing out H_2O_2 (Figure XVIC) [Figures for referees not shown.]. The result is presented in the Figure EV1E.

11. In Fig. 4B control experiment should be done in EWS depleted cells. (IP western in EWS K.O. cell)

Response: We have performed the suggested experiments (Figure XVII) and the result is presented in the Figure EV4A.

13. *In Fig. 4D and 4E the labeling of molecular size on WBs is not consistent.*
Response: We thank the reviewer for finding our mistake. We have corrected them as requested.

14. In Fig 4E and 4F the relative amount of PARP1 is not specified. How it was calculated?
Response: Pull-downed PARP1 was calculated by dividing PARP1 by input PARP1 expression. Chromatin bound PARP1 was divided by chromatin H3. We added the explanation in figure legend.

15. For Fig. 5D, clonogenic assays with different MMS concentrations including PARP1 KO rather than cell viability would be more convincing. Also, alkaline comets would be useful to see if there are DNA breaks in DKO. Similary, it would be interesting to compare these data with treatment with PARP inhibitor.
Response: Thanks for good suggestions. As suggested, we have performed the alkaline comet assay and clonogenic assay using DKO cells. The clonogenic assay showed that the DKO could not rescue cellular viability after the long-term exposure to DNA damaging agents, and these results are consistent with in vivo rescue experiment, which showed partial rescue. Consistently, the alkaline comet assay showed that DNA breaks in DKO were significantly rescued compared to EWS and PARP1 single KO cells after 24 hours of DNA damaging agent treatment (Figure XVIII) [Figures for referees not shown.]. The result is presented in the Figure EV5C.

16. What is the PAR level in DKO mice? (referring to Fig. 1D)
Response: We have performed the suggested experiments to measure the PAR level in the DKO embryo. However, we did not detect the PAR bands in embryo brain samples using western blotting. Therefore, we measured the level of PAR and ratio of the NAD^+/NADH in DKO cell lines. As expected, the level of PAR was increased in *Ews*-KO cells, while DKO cells showed a clearly reduction in total level of PAR (Figure XIXA). The ratio of NAD^+/NADH also increased in the DKO cells compared to *Ews*-WT and -KO cells (Figure XIXB) [Figures for referees not shown.]. These results are presented in the Figure 5E and 5F.

17. In Fig. S1B quantification doesn't seem to correspond the pictures.

Response: As the reviewer points out, we have re-examined the clonogenic assay using HEK293-WT and EWS-KO cells and replaced the quantification results. These results are presented in the Figure EV1B.

18. In Fig. S5B the picture for EWS probe is cut off. PARP1 protein levels should be shown.
Response: The band at cutting position was non-specific band (upper panel), EWS-M7 mutant is only detectable with FLAG antibody (middle panel). We have measured the expression of PARP1 in the same experiment (Figure XXI) [Figures for referees not shown.]. This data is presented in Figure EV4D.

19. Why there is a difference in S9.6 positive foci per cell in EWS-KO cells in Fig S6A vs S6B? (R-loop signals are different)
Response: To address this issue, we have re-examined the formation of R-loops in *Ews*-KO and –KD cells. Consistent with our previous results in knock-out cell lines, EWS knock-down cells increased the formation of R-loops, which were reduced by RNase H1 over-expression (Figure XXVII) (Please see our response below to reviewer 3, #6 for details). Due to limited number of figures allowed in EMBO report, we removed this data from supplement figure and present this data for reviewer's only.

Reviewer #3:

Major point:

1. The authors should try to more clearly separate between confirmatory results and new findings. For instance, that EWS is recruited to damaged DNA in a PARP1 catalytic activity-dependent manner was shown in or could be deduced from several previous reports. Also, the interaction between the RGG domains and PAR chains became clear from these works. While in the abstract this is less of a problem, more care should be taken in the paragraphs on page 5 bottom, page 9 (discussion of Fig. EV3C and D), page 11-13 on the contribution of the RGGs.
Response: We thank the reviewer. We carefully separate confirmatory results and new findings.

2. While the recruitment of EWS via the RGGs and PAR is not novel, the RGG-dependent dissociation of PARP1 is. However, how this should happen mechanistically is not clear from the current manuscript. Could the authors try to separate the recruitment of EWS from the ensuing PARP1 removal? For instance, would PARP inhibitor addition after the initial recruitment of EWS block covalent PARyation of EWS and thereby prevent EWS and PARP1 dissociation from the damaged chromatin? Or would it be possible to mutate PAR-acceptor sites in EWS and demonstrate that this abolished PARP1 dissociation? If either of these approaches worked, this would move the mechanism of PARP1 removal beyond speculation.
Response: We thank the reviewer for suggesting good experiment. To address these questions, we have performed the IP experiments and western blotting with/without treatment of olaparib (see below). First, to address whether the recruitment of EWS after DNA damage is affected by PARylation, we have

performed the IP experiment with pre- or post-treatment of olaparib upon MMS damage as the reviewer suggested. The new results showed that treatment of pre- and post-olaparib both blocked the interaction of EWS with PARylated-PARP1 and PAR. PARylated EWS (around 90kD band) was also induced after MMS but reduced following pre- and post-olaparib treatment (Figure XXIIA) [Figures for referees not shown.].

Second, we sought to determine whether blocking of the continuous recruitment of EWS or PARylation of EWS could affect the dissociation of PARP1 and hyper-PARylation following DNA damage. We blocked the continuous recruitment of EWS and PARylation in DDR by olaparib treatment: Cells were treated with MMS for 30 minutes and incubated in fresh media with/without olaparib (Figure XXIIB). Olaparib treatment inhibited new synthesis of PARylation, but could not remove the existing synthesized PARylation. PARP1 and PARylation rapidly disappeared from chromatin bound fraction in a time-dependent manner with olaparib non-treated condition. Interestingly, PARP1 and PARylation remained longer in chromatin bound fraction even 90 minutes after washing out the MMS when olaparib was treated. This pattern was similar with *Ews*-KO cells. This data suggested that inhibition of continuous recruitment of EWS and/or PARylation of EWS would inhibit dissociation PARP1 and PAR from damaged chromatin. However, it still remains unclear whether the PARP1 dissociation were caused a single cause or multiple causes by self-PARylation, EWS recruitment, EWS PARylation or lack of PARylation of other proteins.

Third, to find the PAR-acceptor sites in EWS, we tried to identify the PAR acceptor sites in EWS by expressing various EWS mutants in HEK293-EWS-KO cell. Unfortunately, we observed PAR is attached in multiple places in EWS (Figure XXIIC).

Taken together, these findings suggested that self-PARylation of PARP1 is the initiation step of PARP1 dissociation from DNA damage. However, following other processes appear to be essential for enhancing dissociation of PARP1 (e.g. binding with EWS, PARylation of EWS and/or the other mechanisms). We described these results with discussion in page 19, line from 4 to 12.

3. On a related note, the authors provide evidence that PARP1 protein and mRNA levels are increased in EWS knockout cells. It remains unclear, however, why this is the case (e.g. is EWS a transcription co-factor for PARP1 expression? Does it bind to the PARP1 promoter?), and how this plays into the enhanced retention of PARP1 at sites of DNA damage. It would be important to know which of the observed phenotypes is mainly driven by PARP1 up-regulation at the transcription level, or by the impaired dissociation from damaged chromatin, or by a combination of both.

Response: Thanks for the comments. We thank the reviewer for suggesting to examine the precise mechanism of EWS in DDR. Since EWS is an RNA binding protein, EWS could not directly regulate the transcription of PARP1. However, there are some possibilities that EWS can control alternative splicing of PARP1 which regulates the expression of PARP1 protein, or EWS can bind with specific protein which regulates transcription of PARP1. As the reviewer suggested, we have performed the chip assay using EWS specific antibody. We found that EWS protein could bind to the promoter region of PARP1 (Figure XXIII)[Figures for referees not shown.]. However, these results require careful characterization, we respectfully ask that these results be shown to the reviewers for “peer-review purpose” only. We will pursue this regulatory mechanism in the following story.

Also, to address the effect of up-regulation of PARP1 in DDR, we overexpressed GFP-PARP1 in HEK-293 cells. The cellular viability of GFP-PARP1 over-expressed cell was not affected by MMS and H_2O_2 compared to WT cell (Figure XXIVA). As well as viability, DNA damage markers such as γH2AX and pCHK1 were also not altered in GFP-PARP1 over-expressed cells (Figure XXIVB) [Figures for referees not shown.]. These results suggested that the enhanced protein level of PARP1 would not induce abnormal DDR or trapped PARP1 in DNA damage sites. Taken together, our finding indicated observed phenotypes in *Ews*-KO cell are mainly driven by impaired dissociation of PARP1 from damaged chromatin.

4. It should be substantiated that the observed NAD depletion and cell death in EWS KO cells is due to PARP1 hyper-activation. If true, simultaneous loss of PARP1 or PARP inhibitor treatment should rescue NAD levels and viability.

Response: As suggested, we measured a NAD^+ level and cellular viability after treatment of PARP inhibitor in *Ews*-WT and -KO cell. The results showed that PARP inhibition rescued cellular NAD^+ level (Figure XXVA), but cellular viability was not rescued in *Ews*-KO cell (Figure XXVB) [Figures for referees not shown.]. These results suggested that decreased NAD^+ level is not the main cause of cell death in *Ews*-KO cell. Instead, PARP1 trapping at DNA damage sites is the major reason for cell death in *Ews*-KO cell. These results are presented in the Figure EV5E and Figure 5G, respectively.

5. Related to the previous point, I think it would be important to work out better whether the observed cell death upon EWS loss is due to PARP1 trapping (if this was the case, PARP inhibitor treatment would make things worse) or due to NAD depletion (if this was the case, PARP inhibitor treatment would make things better).

Response: We thank the reviewer for excellent suggestion. We have performed the western blot analysis and cellular viability assay with or without olaparib in *Ews*-WT and -KO cell. The results showed that treatment of olaparib induced PARP1 trapping (Figure XXVIA) and reduced cellular viability in a dose-dependent manner especially in *Ews*-KO cell (Figure XXVIB) [Figures for referees not shown.]. These results strongly suggested that PARP1 trapping on damaged DNA sites is the major reason for cell death in *Ews*-KO cell. These results are presented in the Figure 5I and 5J.

6. The link to R-loop is very vague. To which extent do R-loops cause the observed phenotypes in EWS knockout cell? RNaseH1 over-expression is typically used to assess the specificity and functional role of R-loops. If the authors think that the elevated R-loops are relevant for the described phenotypes, such experiments would have to be included.

Response: As the reviewer suggested, we have examined the formation of R-loop using RNase H1 conditional expression cell line. Consistent with our previous results in knockout cell lines, EWS knock-down cells increased the formation of R-loops, which were reduced by RNase H1 over-expression (Figure XXVII)[Figures for referees not shown.]. As shown in the new results, R-loop increase in *Ews*-KO cells suggested that loss of EWS induced genomic instability. We agree with the reviewer's comments and we wanted to show that loss of EWS increases the formation of R-loop as an indicator of genomic instability because R-loop is one of indicators for genomic instability. Due to the limited number of figures allowed in EMBO report, we removed this data from the supplemental figures and present this data for reviewer's only.

7. There is quite some literature on Ewing's sarcoma cells being PARP inhibitor sensitive. These cancers carry a EWS translocation (e.g. EWS-FLI1), in which the RGGs of EWS are lost in the fusion protein. It would be interesting to discuss the studies on PARP inhibitor sensitivity of Ewing' sarcoma in the context of RGG-modulated PARP1 trapping.

Response: We thank the reviewer for the insightful comment. We now included literatures in the discussion in page 21~22.

8. In Fig. 2C, are the samples in the quantification graph swapped? Check also the spelling of the y-axis label.

Response: We thank the reviewer for finding our mistakes. We have corrected the mistakes in the revised manuscript.

9. Scale bars are missing in immunohistochemistry and IF image

Response: Thanks for finding our mistake. As the reviewer suggested, we have added the scale bars in all revised figures.

10. There are a couple of language/grammar issues that should be corrected (e.g. page 3 line 4, page 4 middle, page 10 lines 5-7).

Response: As the reviewer suggested, we changed the places where the reviewer pointed out and asked language/grammar corrections to English speaking colleagues.

1st Revision - Editorial Decision 21st Jan 2020

Dear Dr. Myung,

Thank you for your patience while your revised manuscript was peer-reviewed at EMBO reports. We have finally received all referee comments and cross-comments that are pasted below.

I am sorry to say that the evaluation of your study is not a positive one. As you will see, while the referees acknowledge that the study is improved and that the findings are potentially novel and interesting, they also all still have a number of concerns that would have to be successfully and experimentally addressed for publication of the manuscript here. Especially referee 2 raises 2 very clear and important points, and the other referees agree (please see cross-comments below).

Given these substantial concerns, the fact that you already had a chance to significantly revise the study once, and that EMBO reports allows a single round of major revisions only, I am afraid that we cannot offer to publish the manuscript at this point. I am sorry that this decision emerges as the outcome of a lengthy review process but given that the referees are not convinced by the current set of data, I have no other option but to reject your manuscript.

However, in case you feel that you can fully address the referee concerns in a timely manner and obtain data that would considerably strengthen the message of the study, then we would have no objection to consider a new manuscript on the same topic in the near future. Please note that if you were to send a new manuscript this would be treated as a new submission rather than a revision and would be reviewed afresh, also with respect to the literature and the novelty of your findings at the time of resubmission.

At this stage, I am sorry to disappoint you, and hope that the referee comments will be helpful in your continued work in this area.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

Overall Lee et al have made an excellent effort in responding to the original critiques of this and other Reviewers' concerns. There work overall raises some interesting points. There was major point raised by the Reviewer that was not satisfactorily dealt with as it makes a major impact on the Authors' model - namely whether there is more unrepaired damage - more damage initially or a lack of DNA repair function leading to both an accumulation of basal DNA damage and altering kinetics of repair following exogenous damage. Could the absence of EWS result in a lack of repair of ssb and dsb causing the observed PAR accumulation and NADH depletion? Could a lack of effective repair cause the apparent lack of PARP1 disassociation? The authors themselves note that they have not addressed this point and without determining whether this is the case or not, the model proffered cannot be substantiated and raises questions about several of the interpretations of the results. The fact that EWS and PARP1 interact via EWSR1 RGG domains is

still a novel and interesting finding.

There are some additional minor concerns:

Abstract - The last sentence of the abstract suggest Ewing sarcoma would have genomic instability, though this is largely not the case, other than some aneuploidy. How do the authors define a genomic instability phenotype here? What is the evidence of this genomic instability and is it observed in EWSR1 knockout mouse tissues?

Pg 7 - the pCHK1 evaluated here is S345 which is usually the site considered to be activated under replication stress. Did the authors check S317?

Pg 8 - How much was the level of GFP-PARP1 overexpression? Does the GFP-tag affect PARP1 activity (association/dissociation) at damaged sites?

Pg 8 - Use of the term genome instability is an overreach here. The authors have not demonstrated genome instability thus far. What do they mean by genome instability? Damage?

Pg 12 - EV3J: It is interesting that in U2OS PARP1-KO cells, the addition of talazoparib (presumably affecting other PARP proteins since PARP1 is not there) causes such a strong relocation of EWS to nuclear bodies - are these stress granules?

Pg 12 - EV3K: I am confused by this experiment. Olaparib does not alter existing PAR chains, so in the absence of any de novo PARylation, the fact that PAR levels stay high in Olap treated cells suggests that PARP1 is trapped there, though Olaparib does not do such a great job trapping. But presumably this is already known. How does this experiment provide evidence of EWSR1 recruitment to damage in a PARP1-dependent manner? I am afraid I am not clear as to what this experiment proves.

Pg 12 - Authors use Olaparib and Talazoparib for different experiments within the same figure/section. It is important to note the difference in activity between the two and thus draw conclusions accordingly.

Pg 14 - Fig 4E: The authors claim that endogenous PARP1 interacts similarly with the EWS truncations but from the blot it appears that the second RGG motif is critical for PARP1 interaction with EWS since M4 mutant is unable to recruit PARP1 to the same extent as the others (along with M7 and M8). Second, the upstream RGG motif (M2) and the downstream RGG motif (M6) do not seem to be as important both do not dramatically lose PARP1 recruitment.

Pg 14 - Fig 4G: MMS does not appear to induce a reduction of PARP1 in chromatin fraction HEK293 under baseline conditions (first two columns of the western blot). If anything, it looks like PARP1 is increased upon damage. In order to demonstrate the desired point, the release experiment and western blots are more appropriate.

Pg 15 - Fig 5A/C: Is it possible to quantify the bands.? It is hard to tell what level the reduction in g-H2AX and pCHK1 signal really is (within standard error or significantly different).

Pg 15 - The double EWS PARP1 knockout experiments are intriguing. What type of repair is being used and are there differences in their proportional usage?

Referee #2:

The work is undoubtedly interesting, and has identified a clear connection between loss of EWS and elevated PARP activity. However, there are too many uncertainties in the manuscript as it stands, to warrant publication. I applaud the effort the authors have invested in the revisions, but two key issues remain confusing and unresolved.

First, It's still not clear to me whether the impact of EWS on PARP1 trapping reflects (i) a direct role for EWS in extracting PARP1 from chromatin or (ii) an indirect role for EWS in DNA repair i.e. the authors have not resolved this question of whether the elevated PARP in chromatin reflects increased PARP retention due to the elevated level of SSBs, to true "trapping". Both of these are interesting, but the authors need to resolve this one way or the other. The data in Fig 5 C and D suggest that PARP is binding in a toxic manner, which supports the 'direct" trapping model, because the additional deletion/depletion of PARP1 (i.e. the double KO) rescues the sensitivity of the EWS KO cells. However, there is only a minor impact on the levels of DNA damage by comet assay (Fig.EV5C) which suggests that EWS may be promoting repair (i.e. the indirect role) rather than extracting PARP1 directly. The interaction of EWS via PAR can fit with both models because it likely reflects the mechanism by which EWS is recruited to sites of DNA damage (either to promote DNA repair and/or to extract PARP1).

The second confusing issue is the mechanisms by which PARP trapping or retention in chromatin kills cells. The authors imply in the manuscript that this reflects hyperactivation and NAD depletion, and present data in the revised manuscript that supports this. However, they also state that NMN does not rescue the sensitivity, despite rescuing NAD levels. It is thus still unclear which model is true, or even which one the authors favour. Tis too, requires resolution.

Referee #3:

While the results on impaired PARP1 dissociation from damaged chromatin in EWS-deficient cells were strengthened in the revised manuscript, mechanistically it still remains unclear how EWS promotes PARP1 release, and to which extent (or whether at all) NAD+ exhaustion contributes to cell death. Also some other aspects were only partially addressed, and the manuscript would require thorough language editing to improve clarity and coherence, and to align the findings better with the existing literature.

Main points:

1) The new additions with PARGi and NMN, if properly controlled, would argue against changes in NAD+ as cause of cell death. However, on several occasions in the manuscript (including the abstract) the interpretation is unclear and slightly confusing. This is an important point and reconciling it, based on properly controlled experiments, would be essential to avoid misconceptions about the cause of cell death, i.e. NAD+ depletion may be a byproduct of hyper-activation of PARP1, but if restored NAD+ levels do not protect against cell death, cytotoxic PARP1 trapping on chromatin is the more likely cause of death in this context (see PMID 23118055, 27797957, 29992957 as reference). Similarly, the hyper-PARylation (e.g. as put in the final model, Fig. 6D) may

be a consequence of EWS deficiency, but is unlikely to cause cell death, at least not in the context of PARP inhibitor exposure (which blocks PARylation, and traps PARP1).

2) On a related note, the sensitivity of EWS-deficient cells to PARPi should be rescued by PARP1 depletion (Fig. 5J, Fig. XXV), and the relevant literature should be discussed (PARPi sensitivity of EWS-deficient and Ewing`s sarcoma cells; PARP1 trapping as mechanism of PARPi).

3) As mentioned by all reviewers, neither the PARP1- and PAR-dependent recruitment of EWS to sites of DNA damage nor the involvement of the RGG domains is novel. Previous suggestions to cite relevant related work and put the current results into context were unfortunately not, or only halfheartedly, considered. Furthermore, quite extensive language editing would be needed to improve the clarity of the manuscript.

a) Consider changing the title to "EWS-mediated regulation of PARP1 release from damaged chromatin" (or similar).
b) Please consider modifying the abstract. The first sentences seem unconnected. Further in the abstract, consider rephrasing: "Consistent with previous work, an arginine-glycine-glycine (Arg-Gly-Gly, RGG) domain-mediated interaction between EWS and poly(ADP-ribose) (PAR) chains was required to recruit EWS to sites of DNA damage, and was revealed to be essential to promote PARP1 dissociation from damaged DNA" (or similar). Please edit and correct also the subsequent sentence (grammar).
c) Previous recommendations for references should be included, e.g. PMID 23833192 shows EWS recruitment to sites of DNA damage, but is still not cited by the authors. Similarly, prior work on RGG/GAR motifs as PAR-binding motifs should be discussed and cited (PMID 23268355 and 26673700 and primary research articles referenced therein), on pages 4 top and 13 top (PAR-dependent recruitments) and on page 5 bottom (EWS and PAR-binding). Throughout page 14 (RGG-mediated EWS recruitment), page 15 top and page 18 top it should also be stated clearly that the results confirm and are consistent with prior work on RGG-mediated, PAR-dependent recruitment of EWS and related proteins (with references provided, e.g. the studies from the Lukas and Tibbetts labs).
d) Page 5 bottom, consider replacing "clearly" by "entirely"
e) Page 7 bottom, please remove "and suppress overactivation of DDR for cell survival" as this is not shown.
f) Page 12 top, please change to "... in a PARP1- and PAR-dependent manner", and check whether the Rulten et al. article should be cited in this context.
g) Page 12 bottom, 13 top, link the PARP1-dependent EWS recruitment to prior work.
h) Page 16 bottom, not clear what is meant ("per see ..."), and how it relates to the finding that NMN complementation rescues NAD+ but not viability.

The authors may want to turn to a native speaker and/or professional science editor to further improve the clarity of their manuscript and better work out the novel findings (i.e. where do they go beyond the known or expected, where do they confirm, and where do they extend prior work) and, in light of the new additions from the revision, avoid conceptual inconsistencies.

Cross-comments referee 1:

I agree with the comments by the other referees. In particular the agreement between my review and that of reviewer #2 on the difference between a problem in DNA repair caused by loss of

EWSR1 indirectly impacting PARP1i accumulation versus the direct removal. I would also agree with reviews #3 regarding the literature citations.

Cross-comments referee 3:

I also agree that it is important to discriminate between repair defects and PARP1 release (and feel that this could be best addressed by providing further insights into the mechanism of the proposed EWS-mediated PARP1 release, in parallel to assessing repair efficiency). Likewise, the role of NAD depletion is unclear and, as it stands, very confusing (my points 1 & 2 and 2nd point of reviewer 2). Merely discussing these points would not be sufficient in my view.

Point-by-point response

Referee #1:

Major concern:

Overall Lee et al have made an excellent effort in responding to the original critiques of this and other Reviewers' concerns. There work overall raises some interesting points. There was major point raised by the Reviewer that was not satisfactorily dealt with as it makes a major impact on the Authors' model - namely whether there is more unrepaired damage - more damage initially or a lack of DNA repair function leading to both an accumulation of basal DNA damage and altering kinetics of repair following exogenous damage. Could the absence of EWS result in a lack of repair of ssb and dsb causing the observed PAR accumulation and NADH depletion? Could a lack of effective repair cause the apparent lack of PARP1 disassociation? The authors themselves note that they have not addressed this point and without determining whether this is the case or not, the model proffered cannot be substantiated and raises questions about several of the interpretations of the results. The fact that EWS and PARP1 interact via EWSR1 RGG domains is still a novel and interesting finding.

Response: Thank you for positive responses. Please see our explanation for the concerns raised by the reviewer in the major criticism.

Minor concerns:

1. Abstract - The last sentence of the abstract suggest Ewing sarcoma would have genomic instability, though this is largely not the case, other than some aneuploidy. How do the authors define a genomic instability phenotype here? What is the evidence of this genomic instability and is it observed in EWSR1 knockout mouse tissues?

Response: We removed the word “genomic instability” in the abstract. We re-wrote the abstract describing our results. We wrote in the previous sentence based on several observations previously reported. Previous reports showed that loss of EWS increased the genomic instability in vitro and vivo model (J Clin Invest. 117:1314, Sci Rep. 6:32297, and Int J Cell Biol. 2013:642853). In addition, it was reported that expression of EWS-Fli1 fusion protein increased genomic instability and EWS protein regulates genomic stability mediated by transcription response to DNA damage (Nature, 555:87 and Cancer Research 2012, 7:1608).

2. Pg 7 - the pCHK1 evaluated here is S345 which is usually the site considered to be activated under replication stress. Did the authors check S317?

Response: We did not check S317 phosphorylation. As far as we know both S317 and S345 are phosphorylated by ATR in response to hydroxyurea treatment, which causes DNA replication stress. In Wilsker *et al.* paper in 2008 (PNAS, 105:20752), S317 phosphorylation occurs first and subsequent S345 follows by hydroxyurea treatment. Thus, we tested Ser345 to monitor the response to DNA damage. In many

literatures, both phosphorylations were used to check DNA damage, which links to DNA replication stresses. Thus, we checked S345 phosphorylation.

3. Pg 8 - How much was the level of GFP-PARP1 overexpression? Does the GFP-tag affect PARP1 activity (association/dissociation) at damaged sites?

Response: The level of GFP-PARP1 overexpression was more than twice compared to that of endogenous PARP1 [Figures for referees not shown.]. We measured cell viability after GFP-PARP1 overexpression and did not observe any significant difference following treatment of MMS or Hydrogen peroxide. Although we did not check the activity of GFP-PARP1 directly, there was no clear difference between non-tagged PARP1 and GFP-tagged PARP1 in the accumulation kinetics to damaged DNA. We believe there was at least no difference in the PARP1 localization (association or dissociation) at damaged sites (Fig 2D).

4. Pg 8 - Use of the term genome instability is an overreach here. The authors have not demonstrated genome instability thus far. What do they mean by genome instability? Damage?

Response: We agree with the reviewer. Since it is not relevant to what we observed, we just describe cellular survival without mentioning genome instability.

5. Pg 12 - EV3J: It is interesting that in U2OS PARP1-KO cells, the addition of talazoparib (presumably affecting other PARP proteins since PARP1 is not there) causes such a strong relocation of EWS to nuclear bodies - are these stress granules?

Response: We don't know exactly the nature of strong GFP-aggregation signals in Fig EV3J. However, FET family proteins (FUS, EWS, TAF15) are RNA-binding proteins. We suspect the strong signals may be aggregations of GFP-EWS proteins in the nuclear bodies.

6. Pg 12 - EV3K: I am confused by this experiment. Olaparib does not alter existing PAR chains, so in the absence of any de novo PARylation, the fact that PAR levels stay high in Olap treated cells suggests that PARP1 is trapped there, though Olaparib does not do such a great job trapping. But presumably this is already known. How does this experiment provide evidence of EWSR1 recruitment to damage in a PARP1-dependent manner? I am afraid I am not clear as to what this experiment proves.

Response: Since Olaparib blocks recruitment of EWS in DNA damage sites, we simply wanted to show that the reduced recruitment of EWS further accumulates PARP1 in chromatin. However, in agreement with the reviewer that this data does not provide an additional support of model, we removed it from the revised manuscript.

7. Pg 12 - Authors use Olaparib and Talazoparib for different experiments within the same figure/section. It is important to note the difference in activity between the two and thus draw conclusions accordingly.

Response: Experiments with Talazoparib was recommended in the first round of revision by referee#1. We believe it was recommended due to different trapping degree between Olaparib and Talazoparib of PARP1. Although Talazoparib is more effective for PARP1 trapping activity than Olaparib, the inhibition activity of PARylation is only about 1.5 times better (Molecular Cancer Therapeutics, 13:433). We described our results with such differences in the revised manuscript (page 12-13).

8. Pg 14 - Fig 4E: The authors claim that endogenous PARP1 interacts similarly with the EWS truncations but from the blot it appears that the second RGG motif is critical for PARP1 interaction with EWS since M4 mutant is unable to recruit PARP1 to the same extent as the others (along with M7 and M8). Second, the upstream RGG motif (M2) and the downstream RGG motif (M6) do not seem to be as important both do not dramatically lose PARP1 recruitment.

Response: We agree with the reviewer. We have revised the manuscript with results came from suggested experiments. We have replaced and rearranged figures (Fig 4F,

G, and H with M4 mutant).

9. Pg 14 - Fig 4G: MMS does not appear to induce a reduction of PARP1 in chromatin fraction HEK293 under baseline conditions (first two columns of the western blot). If anything, it looks like PARP1 is increased upon damage. In order to demonstrate the desired point, the release experiment and western blots are more appropriate.

Response: We repeated several times and presented a new blot in Fig4F and quantification of all blots in Fig4G. Figures show high level of retention of PARP1 on chromatin after MMS treatment, which was further increased in Ews-KO cells. In the same figure, we performed a new experiment with M4 mutant. Similar to previous results with M7 mutant, M4 expression could not reduce PARP1 level on chromatin unlink WT expression (Fig 4F and G).

10. Pg 15 - Fig 5A/C: Is it possible to quantify the bands.? It is hard to tell what level the reduction in g-H2AX and pCHK1 signal really is (within standard error or significantly different).

Response: We added the relative level of quantified value under the blots in Figure 5C and D.

11. Pg 15 - The double EWS PARP1 knockout experiments are intriguing. What type of repair is being used and are there differences in their proportional usage?

Response: It's an important but very difficult question. We don't know exactly what type of repair pathway(s) are activated and repair DNA damage in the DKO cell. We are currently investigating potential other DNA repair pathways which do not require PARP1 activity. It requires several knockdown of selective genes in nucleotide excision repair, homologous recombination, microhomology mediated end joining, non-homologous end joining etc. It will take a while to figure out DNA repair choice in this DKO cell line. We believe this question is beyond the scope of the current manuscript.

Referee #2:

Major concern:

The work is undoubtedly interesting, and has identified a clear connection between loss of EWS and elevated PARP activity. However, there are too many uncertainties in the manuscript as it stands, to warrant publication. I applaud the effort the authors have invested in the revisions, but two key issues remain confusing and unresolved.

First, It's still not clear to me whether the impact of EWS on PARP1 trapping reflects (i) a direct role for EWS in extracting PARP1 from chromatin or (ii) an indirect role for EWS in DNA repair i.e. the authors have not resolved this question of whether the elevated PARP in chromatin reflects increased PARP retention due to the elevated

level of SSBs, to true "trapping". Both of these are interesting, but the authors need to resolve this one way or the other. The data in Fig 5 C and D suggest that PARP is binding in a toxic manner, which supports the 'direct" trapping model, because the additional deletion/depletion of PARP1 (i.e. the double KO) rescues the sensitivity of the EWS KO cells. However, there is only a minor impact on the levels of DNA damage by comet assay (Fig.EV5C) which suggests that EWS may be promoting repair (i.e. the indirect role) rather than extracting PARP1 directly. The interaction of EWS via PAR can fit with both models because it likely reflects the mechanism by which EWS is recruited to sites of DNA damage (either to promote DNA repair and/or to extract PARP1).

Response: Thanks for good comments and we agree with the reviewer's comments. In the previous studies, PARylated PARP1 should be dissociated from DNA damage sites for proper DNA repair machinery to repair damage. We observed that PARylated PARP1 trapped in DNA damage sites in the EWS depleted cells. Thus, it will be difficult to distinguish these two events separately. We re-wrote both possibilities in the discussion of the revised manuscript.

Double depletion of PARP1 and EWS has a minor but significant rescue on the levels of DNA damage in COMET assay in double KO cells compared to EWS depleted cells. However, there was still measurable DNA damage in double KO cells (Fig EV5C). We believe this measurable DNA damage in DKO was due to activation of unknown DNA repair pathway, which rescues partially cell survival but not completely reduces DNA damage.

The second confusing issue is the mechanisms by which PARP trapping or retention in chromatin kills cells. The authors imply in the manuscript that this reflects hyperactivation and NAD depletion, and present data in the revised manuscript that supports this. However, they also state that NMN does not rescue the sensitivity, despite rescuing NAD levels. It is thus still unclear which model is true, or even which one the authors favour. Tis too, requires resolution.

Response: We agree with the reviewer. We wrote the previous manuscript to explain rescues with NAD depletion, which we favor. However, due to no rescue of sensitivity by NMN, we could not completely explain the cellular toxicity by NAD level. Since there is an alternative way for cellular toxicity, we did tone down our claim and re-wrote the manuscript.

Referee #3:

While the results on impaired PARP1 dissociation from damaged chromatin in EWS-deficient cells were strengthened in the revised manuscript, mechanistically it still remains unclear how EWS promotes PARP1 release, and to which extent (or whether at all) NAD+ exhaustion contributes to cell death. Also some other aspects were only partially addressed, and the manuscript would require thorough language editing to improve clarity and coherence, and to align the findings better with the existing

literature.

Major concern:

1) The new additions with PARGi and NMN, if properly controlled, would argue against changes in NAD+ as cause of cell death. However, on several occasions in the manuscript (including the abstract) the interpretation is unclear and slightly confusing. This is an important point and reconciling it, based on properly controlled experiments, would be essential to avoid misconceptions about the cause of cell death, i.e. NAD+ depletion may be a byproduct of hyper-activation of PARP1, but if restored NAD+ levels do not protect against cell death, cytotoxic PARP1 trapping on chromatin is the more likely cause of death in this context (see PMID 23118055, 27797957, 29992957 as reference). Similarly, the hyper-PARylation (e.g. as put in the final model, Fig. 6D) may be a consequence of EWS deficiency, but is unlikely to cause cell death, at least not in the context of PARP inhibitor exposure (which blocks PARylation, and traps PARP1).

Response: We agree with the reviewer. We revised the manuscript. We described these results more clearly in pages 16-17.

2) On a related note, the sensitivity of EWS-deficient cells to PARPi should be rescued by PARP1 depletion (Fig. 5J, Fig. XXV), and the relevant literature should be discussed (PARPi sensitivity of EWS-deficient and Ewing`s sarcoma cells; PARP1 trapping as mechanism of PARPi).

Response: Thanks for an excellent suggestion. We have performed the suggested experiment to measure cellular viability of HEK-293-WT, EWS KO, and EWS-PARP1 KO (DKO) following Olaparib treatment. The results showed a significant increase in the cellular viability following Olarparib treatment in DKO cells compared to EWS KO cells. These results suggest that trapped PARP1 in Ews-/- cells mainly causes cell death after Olaparib treatment. We now show this as a new Fig EV5F and state these results on page 17.

3) As mentioned by all reviewers, neither the PARP1- and PAR-dependent recruitment of EWS to sites of DNA damage nor the involvement of the RGG domains is novel.

Previous suggestions to cite relevant related work and put the current results into context were unfortunately not, or only halfheartedly, considered. Furthermore, quite extensive language editing would be needed to improve the clarity of the manuscript.
a) Consider changing the title to "EWS-mediated regulation of PARP1 release from damaged chromatin" (or similar).
b) Please consider modifying the abstract. The first sentences seem unconnected. Further in the abstract, consider rephrasing: "Consistent with previous work, an arginine-glycine-glycine (Arg-Gly-Gly, RGG) domain-mediated interaction between EWS and poly(ADP-ribose) (PAR) chains was required to recruit EWS to sites of DNA damage, and was revealed to be essential to promote PARP1 dissociation from damaged DNA" (or similar). Please edit and correct also the subsequent sentence (grammar).
c) Previous recommendations for references should be included, e.g. PMID 23833192: The RNA-binding protein Fused in Sarcoma functions downstream of PARP in response to DNA damage shows EWS recruitment to sites of DNA damage, but is still not cited by the authors. Similarly, prior work on RGG/GAR motifs as PAR-binding motifs should be discussed and cited (PMID 23268355: Reprogramming cellular events by PARP-binding proteins and 26673700: Readers of PARP:designed to be fit for purpose and primary research articles referenced therein), on pages 4 top and 13 top (PAR-dependent recruitments) and on page 5 bottom (EWS and PAR-binding). Throughout page 14 (RGG-mediated EWS recruitment), page 15 top and page 18 top it should also be stated clearly that the results confirm and are consistent with prior work on RGG-mediated, PAR-dependent recruitment of EWS and related proteins (with references provided, e.g. the studies from the Lukas and Tibbetts labs).
d) Page 5 bottom, consider replacing "clearly" by "entirely"
e) Page 7 bottom, please remove "and suppress overactivation of DDR for cell survival" as this is not shown.
f) Page 12 top, please change to "... in a PARP1- and PAR-dependent manner", and check whether the Rulten et al. article should be cited in this context.
g) Page 12 bottom, 13 top, link the PARP1-dependent EWS recruitment to prior work.
h) Page 16 bottom, not clear what is meant ("per see ..."), and how it relates to the finding that NMN complementation rescues NAD+ but not viability.

Response: We revised the manuscript suggested and added all references.

(a) We changed the title to “Regulation of poly(ADP-ribose) polymerase 1 chromatin dissociation by Ewing sarcoma protein”

(b) We changed the sentence to “Consistent with previous work, the arginine-glycine-glycine (Arg-Gly-Gly, RGG) domain of EWS is essential for its interaction with PAR chains, the recruitment to sites of DNA damage, and the dissociation of PARP1 from damaged DNA.”

(c) All of the reference were included in the new manuscripts in page 3, 4, 5, 12, 14, 19, and 21.

(d) We replaced “clearly” by “fully” in page 5.

(e) We removed the sentence.

(f) We changed to “PARP1- and PAR-dependent manner” and added new reference in page 12 bottom.

(g) We linked the PARP1-dependent EWS recruitment with PAR dependent interaction between EWS and PARP1 in Page 13 middle. We changed the sentence to “The fact that EWS is recruited to the DNA damage sites by PARP1- and PAR- dependent manner (Altmeyer et al., 2015, Mastrocola et al., 2013), and EWS regulate PARP1 chromatin dissociation (Fig 2) made us to investigate if PARP1 and EWS directly interact with each other.”

(H) We changed this sentence to “Thus, our results suggest that PARP1 trapped on damaged DNA sites in *Ews*$^{-/-}$ cells cause excessive damage and ensuing cell death” in page 17. In our experiments, NMN complementation rescued NAD^+ but not viability. This means that depleted NMN is not direct cause for cell death in Ews-KO cells.

The authors may want to turn to a native speaker and/or professional science editor to further improve the clarity of their manuscript and better work out the novel findings (i.e. where do they go beyond the known or expected, where do they confirm, and where do they extend prior work) and, in light of the new additions from the revision, avoid conceptual inconsistencies.

Response: We did re-write the manuscript with help of English-speaking colleagues.

Last Comments

"Regarding the possibility that the loss of EWSR1 may result in a DNA repair defect, it is notable that upon expression of EWSR1-FLI1 or depletion of EWSR1 it was previously reported that there was in fact a homologous recombination defect (see PMID: 29513652). In that report it was shown that depletion of 53BP1 rescued the homologous recombination defect. In consideration of this, in the Author's system, would 53BP1 depletion rescue the PAR accumulation that NAD supplementation failed to achieve?"

Response: We have performed the suggested experiment to confirm the role of 53BP1 in hyper-activation of PARP1. Our results showed that depletion of 53BP1 slightly increased the total level of PAR in EWS-KO cell in response to MMS. These results suggest that 53BP1 seems not to function in the PARP1 pathway when loss of EWS induced hyper-activation of PARP1 or abnormal DDR. [Figures for referees not shown.]

2nd Revision - Editorial Decision 25th Aug 2020

Dear Dr. Myung,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees that were asked to assess it. Both referees still have minor suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

I have contacted referee 2 about the removal of the in vivo data as suggested by referee 3, and referee 2 thinks that the data should be kept but more adequately discussed.

Some other changes will also be required:

- Per journal policy, "Data not shown" on page 29 needs to be removed.

- Please call the methods section "Materials and Methods"

- Please add a separate "Data Availability" section to the end of the materials and methods section with a direct URL link to the deposited data and, if necessary, login requirements.

- The Reference list needs to be moved to before the figure legends. This link shows how the manuscript should be layed out:
https://www.embopress.org/page/journal/14693178/authorguide#textformat

- Each author's contribution needs to be added into our online manuscript tracking system.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript.

- Appendix Table S1 could be changed to Table EV1, as it reports new data. EV Tables will expand when clicked in the online version of the manuscript.

- Appendix Table S2 would be better as a regular table in the method section (Table 1). This way, no Appendix file will be required. Please also correct the callouts to these tables in the manuscript text.

- The title of the Dataset EV1 needs to describe better what this dataset is.

There are several irregularities with the figures panels:

- Figs 1D and EV2C: the boxes include letters that are not called out nor mentioned in the legend. Please remove the boxes with letters or explain what these are good for.

- Fig 1D+E are missing scale bars, please add.

- Figs 5C and EV3E - the pCHK1 lanes or bands look like they are pasted on top, please explain and correct. Please send us the source data for these figure panels.

- In Fig 6A the gH2AX and H3 bands look very similar, please explain and send us the source data

for this figure panel.

- In Fig EV4D the FLAG bands look spliced, please explain and send us the source data.

I would like to suggest a few changes to the title and abstract. Please let me know whether you agree with the following and whether all sentences correctly reflect your data:

Ewing sarcoma protein promotes dissociation of poly(ADP-ribose) polymerase 1 from chromatin

Poly(ADP-ribose) polymerase 1 (PARP1) facilitates the DNA damage response (DDR). While the Ewing's sarcoma breakpoint region 1 (EWS) protein fused to FLI1 triggers sarcoma formation, the physiological function of EWS is largely unknown. Here, we investigate the physiological role of EWS in regulating PARP1. We show that EWS is required for the dissociation of PARP1 from damaged DNA. Abnormal PARP1 accumulation caused by EWS inactivation leads to excessive Poly ADP-Ribosylation (PARylation) and triggers cell death in both in vitro and in vivo models. Consistent with previous work, the arginine-glycine-glycine (RGG) domain of EWS is essential for PAR chain interaction, recruitment of EWS [OK?] to DNA damage, and PARP1 dissociation from damaged DNA. Ews and Parp1 double mutant mice do not show improved survival [OK?], but supplementation with nicotinamide mononucleotides extends Ews mutant pups' survival [It would be good to explain why this is]. Consistently, PARP1 accumulats on chromatin in Ewing's sarcoma cells expressing an EWS fusion protein that cannot interact with PARP1, and tissues derived from Ewing's sarcoma patients show increased PARylation. Taken together, our data reveal that EWS is important for removing PARP1 from damaged chromatin.

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

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

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #2:

The revised manuscript still has some lack of clarity that requires attention, in terms of textual revisions. They still do not explain key discrepancies in the data that argue different ways. For example, in Fig.5, NMN rescued the embryonic viability of Ews-/- mice, but not cellular sensitivity of Ews-/- cell lines to MMS. This argues for NAD dependent and independent mechanisms, respectively. This may be true - trapping versus NAD depletion is more likely to dominate in cultured cell lines (where replication fork collapse at trapped lesions may dominate), and/or the level of NAD rescue by NMN may not be sufficient for rescue of survival following a strong challenge with

exogenous genotoxin when compared to endogenous levels of damage in the embryos, but the authors need to clearly argue this in the discussion.

Also, the argument concerning direct or indirect suppression of PARP1 chromatin retention is still lacking clarity. It is clearer why the authors favour a direct role of EWS in promoting PARP1 dissociation, rather than indirectly via suppressing DNA damage levels (no difference in g2AX, comet breaks etc in Ews-/- cells). However, the argument concerning fluorescence spectroscopy and the fraction of PARP1 engaged at damage confuses me, because that should surely also detect the elevated PARP1 in chromatin that is key to either model? So, in summary, further textual clarification of the arguments, data, and model are required in my opinion.

Referee #3:

The revised manuscript was significantly improved with new experiments strengthening a direct role of EWS in PARP1 dissociation from DNA damage and additional data supporting elevated PARP1 trapping in EWS-deficient cells as a major cause of cell death. Pending minor revisions, I would endorse publication in EMBO Reports.

1) The in vivo NMN supplementation experiments (Fig. 5K) seem to contradict the in vitro results with NMN (Fig. 5H). Furthermore, by providing extra NAD+ through NMN supplementation it is likely that PARP1 activity is increased due to higher substrate concentration, promoting PARP1 release from chromatin through enhanced auto-PARylation, in an EWS-independent manner. The observed effects could thus be unrelated to altered mitochondria homeostasis upon NAD+ depletion. If the in vivo data (Fig. 5K and EV5G) are to be kept in the manuscript without further characterization of the underlying mechanism, enhanced PARP1 dissociation from chromatin due to NMN-fueled auto-modification should at least be discussed. Alternatively, I wonder whether the in vivo data, which show comparatively mild effects and are mechanistically not conclusive, should be removed from the manuscript.

2) In Fig. 4B, a PARP1 Western blot for input controls seems missing.

3) In the model figure (Fig. 6D) I would suggest to write "EWS KO" instead of "KO".

4) The image resolution of Fig. EV4A and EV4B seems poor. These panels should be replaced by higher resolution images.

5) Space permitted, the authors may want to refer to the studies by Smith et al. (PMID: 31566235) and Krüger et al. (PMID: 32358582) with regard to FCS/ATR-FTIR measurements of PARP1 and by Michelena et al. (PMID: 29992957) with regard to measurements of PARP1 trapping by Olaparib & Talazoparib and associated genotoxicity.

6) There are still some language issues, which should be taken care of. The abstract should be edited, e.g. the sentence "Although, the Ews and Parp1 double mutant mice were not significantly increased their survival, supplementation of the nicotinamide mononucleotide extended Ews mutant pups' survival" needs corrections (or should be left out). Additionally: "poly(ADP-ribosyl)ation" would be the correct spelling (abstract and page 3); "are not have same function" needs corrections (page 14); "a model where EWS inhibits the dissociation of PARP1" should be "... EWS **loss** inhibits ..." (discussion); "we cannot completely exclude both possibilities" should probably be rephrased (discussion); "inhibit the liquid demixing process" should probably be "excessively

stabilize the liquid demixing process" or similar (discussion).

Institute for Basic Science

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RE: EMBOR-2019-48676V3-Q

August 31, 2020

Dear Dr. Schnapp,

Thank you for handling our manuscript (EMBOR-2019-48676V3-Q) "*Regulation of Poly(ADP-ribose) polymerase 1 Chromatin Dissociation by Ewing sarcoma Protein*" We have carefully read all the comments by editors and referees. We addressed all their queries in this letter (please see our point-by-point response below). We thank editors and reviewers for their critical comments and suggestions, which greatly improved our manuscript.

Thank you for your consideration.

RESPONSES)

- Per journal policy, "Data not shown" on page 29 needs to be removed.

We removed "Data not shown" from manuscript.

- Please call the methods section "Materials and Methods"

We changed "methods" to "Materials and Methods"

- Please add a separate "Data Availability" section to the end of the materials and methods section with a direct URL link to the deposited data and, if necessary, login requirements.

We added Data Availability section to the end of the materials and methods section.

- The Reference list needs to be moved to before the figure legends. This link shows how the manuscript should be layed out: https://www.embopress.org/page/journal/14693178/authorguide#textformat

We rearranged the reference list before the figure legends.

- Each author's contribution needs to be added into our online manuscript tracking system.

We added author's contribution to online manuscript tracking system.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript.

We addressed all editor's comments.

- Appendix Table S1 could be changed to Table EV1, as it reports new data. EV Tables will expand when clicked in the online version of the manuscript.

We changed Appendix Table S1 to Table EV1.

- Appendix Table S2 would be better as a regular table in the method section (Table 1). This way, no Appendix file will be required. Please also correct the callouts to these tables in the manuscript text.

We transferred Appendix Table S2 to method section Table 1.

- The title of the Dataset EV1 needs to describe better what this dataset is.

We described title of Dataset EV1 more detail. "GO analysis" to "GO analysis of differently expressed protein between Ews WT and KO cell."

There are several irregularities with the figures panels:

- Figs 1D and EV2C: the boxes include letters that are not called out nor mentioned in the legend. Please remove the boxes with letters or explain what these are good for.

We removed the boxes with letters from Fig 1D and EV2C.

- Fig 1D+E are missing scale bars, please add.

We added scale bars to bottom of each panels.

- Figs 5C and EV3E - the pCHK1 lanes or bands look like they are pasted on top, please explain and correct. Please send us the source data for these figure panels.

This is source data of Fig 5C and EV3E. During the western blotting, we cut the membrane to see the pCHK1 and Actin, which share similar sizes. It was why it looks like blots pasted on top. Thus, we changed the blots without cut marks in the revised figure.

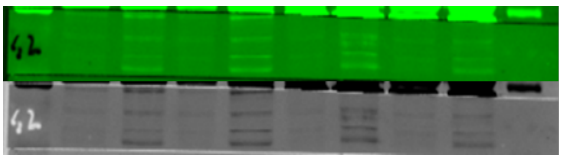


Fig 5C

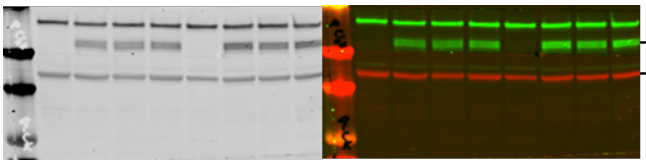
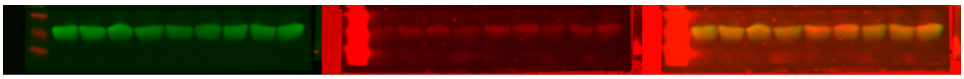


Fig. EV3E

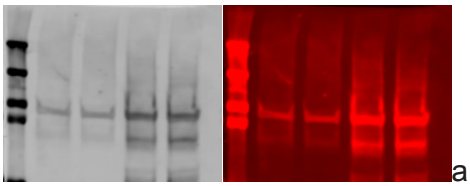
- In Fig 6A the gH2AX and H3 bands look very similar, please explain and send us the source data for this figure panel.

This is source data of Fig 6A. Because the gH2AX and H3 share very similar sizes (around 15kDa), we used different colors (depending on different host of antibodies) in Odyssey imaging system.


Rabbit H3 Mouse gH2AX MERGE

- In Fig EV4D the FLAG bands look spliced, please explain and send us the source data.

This is source data of EV4D. Although the FLAG bands look splices in this source data, it is not. We think that there is some problem during the western blotting.


anti-Flag

I would like to suggest a few changes to the title and abstract. Please let me know whether you agree with the following and whether all sentences correctly reflect your data:

Ewing sarcoma protein promotes dissociation of poly(ADP-ribose) polymerase 1 from chromatin

Poly(ADP-ribose) polymerase 1 (PARP1) facilitates the DNA damage

response (DDR). While the Ewing's sarcoma breakpoint region 1 (EWS) protein fused to FLI1 triggers sarcoma formation, the physiological function of EWS is largely unknown. Here, we investigate the physiological role of EWS in regulating PARP1. We show that EWS is required for the dissociation of PARP1 from damaged DNA. Abnormal PARP1 accumulation caused by EWS inactivation leads to excessive Poly ADP-Ribosylation (PARylation) and triggers cell death in both in vitro and in vivo models. Consistent with previous work, the arginine-glycine-glycine (RGG) domain of EWS is essential for PAR chain interaction, recruitment of EWS [OK?] to DNA damage, and PARP1 dissociation from damaged DNA. Ews and Parp1 double mutant mice do not show improved survival [OK?], but supplementation with nicotinamide mononucleotides extends Ews mutant pups' survival [It would be good to explain why this is]. Consistently, PARP1 accumulats on chromatin in Ewing's sarcoma cells expressing an EWS fusion protein that cannot interact with PARP1, and tissues derived from Ewing's sarcoma patients show increased PARylation. Taken together, our data reveal that EWS is important for removing PARP1 from damaged chromatin.

Thank you for good suggestion. We agreed your opinions and changed the title and abstract in manuscripts as you suggested.

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

We added summary, bullet points and synopsis image at the end of the revised manuscript.

Referee #2:

The revised manuscript still has some lack of clarity that requires attention, in terms of textual revisions. They still do not explain key discrepancies in the data that argue different ways. For example, in Fig.5, NMN rescued the embryonic viability of Ews-/- mice, but not cellular sensitivity of Ews-/- cell lines to MMS. This argues for NAD dependent and independent mechanisms, respectively. This may be true - trapping versus NAD

depletion is more likely to dominate in cultured cell lines (where replication fork collapse at trapped lesions may dominate), and/or the level of NAD rescue by NMN may not be sufficient for rescue of survival following a strong challenge with exogenous genotoxin when compared to endogenous levels of damage in the embryos, but the authors need to clearly argue this in the discussion.

We described this in discussion section (page 21 . line 8-16)

Also, the argument concerning direct or indirect suppression of PARP1 chromatin retention is still lacking clarity. It is clearer why the authors favour a direct role of EWS in promoting PARP1 dissociation, rather than indirectly via suppressing DNA damage levels (no difference in g2AX, comet breaks etc in Ews-/- cells). However, the argument concerning fluorescence spectroscopy and the fraction of PARP1 engaged at damage confuses me, because that should surely also detect the elevated PARP1 in chromatin that is key to either model? So, in summary, further textual clarification of the arguments, data, and model are required in my opinion.

We observed PARP1 movement at damaged DNA slowed in *EWS* null cells and caused accumulation in chromatin. Although we agree with the point of the reviewer that if there is high level of damage due to the loss of EWS, there will be more PARP1. However, with other data such as no induction of DNA damage markers, i.e. γH2AX, phospho-CHK1 and comment assay supports the direct role of EWS for PARP1 dissociation from chromatin. We explained it more clearly in Discussion (page 20, line 6-17).

Referee #3:

The revised manuscript was significantly improved with new experiments strengthening a direct role of EWS in PARP1 dissociation from DNA damage and additional data supporting elevated PARP1 trapping in EWS-deficient cells as a major cause of cell death. Pending minor revisions, I would endorse publication in EMBO Reports.

1) The in vivo NMN supplementation experiments (Fig. 5K) seem to contradict the in vitro results with NMN (Fig. 5H). Furthermore, by providing extra NAD+ through NMN supplementation it is likely that PARP1 activity is increased due to higher substrate concentration, promoting PARP1 release from chromatin through enhanced auto-PARylation, in an EWS-independent manner. The observed effects could thus be unrelated to altered mitochondria homeostasis upon NAD+ depletion. If the in vivo data (Fig. 5K and EV5G) are to be kept in the manuscript without further characterization of the underlying mechanism, enhanced PARP1 dissociation from chromatin due to NMN-fueled auto-modification should at least be discussed. Alternatively, I wonder whether the in vivo data, which

show comparatively mild effects and are mechanistically not conclusive, should be removed from the manuscript.

We described reviewer's points in the discussion section.

2) In Fig. 4B, a PARP1 Western blot for input controls seems missing.

We added PARP1 western blot for input controls in Fig 4B.

3) In the model figure (Fig. 6D) I would suggest to write "EWS KO" instead of "KO".

We changed "KO" to "EWS KO" in Fig 6D

4) The image resolution of Fig. EV4A and EV4B seems poor. These panels should be replaced by higher resolution images.

We replaced the panels to higher resolution images.

5) Space permitted, the authors may want to refer to the studies by Smith et al. (PMID: 31566235) and Krüger et al. (PMID: 32358582) with regard to FCS/ATR-FTIR measurements of PARP1 and by Michelena et al. (PMID: 29992957) with regard to measurements of PARP1 trapping by Olaparib & Talazoparib and associated genotoxicity.

We added these references to the manuscript.

*6) There are still some language issues, which should be taken care of. The abstract should be edited, e.g. the sentence "Although, the Ews and Parp1 double mutant mice were not significantly increased their survival, supplementation of the nicotinamide mononucleotide extended Ews mutant pups' survival" needs corrections (or should be left out). Additionally: "poly(ADP-ribosyl)ation" would be the correct spelling (abstract and page 3); "are not have same function" needs corrections (page 14); "a model where EWS inhibits the dissociation of PARP1" should be "...
EWS loss inhibits ..." (discussion); "we cannot completely exclude both possibilities" should probably be rephrased (discussion); "inhibit the liquid demixing process" should probably be "excessively stabilize the liquid demixing process" or similar (discussion).*

We changed the mentioned sentences.

3rd Revision - Editorial Decision 15th Sep 2020

Dr. Kyungjae Myung
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Dear Dr. Myung,

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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Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Kyungjae Myung
Journal Submitted to: EMBO Report
Manuscript Number: EMBOR-2019-48676-T

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This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs 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 and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen to satisfy the need for enough statistical power based on our experience. We performed at least three replicates, indicated in the text.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Based on our previous experience, n>25 mice per condition was chosen as a enough sample size.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Samples were randomly allocated to minimize the effects of subjective bias in all experiments.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	To minimize the effects of subjective bias, the following steps were taken:1-sample randomiation, 2-blind scoring, 3-analysis of different experiments were conducted by different co-authors, 4-IHC were conducted by computor software (image J or Zen blue).
4.b. For animal studies, include a statement about blinding even if no blinding was done	For animal histopatholgical experiments were conducted by two co-auothors.
5. For every figure, are statistical tests justified as appropriate?	Yes. Statistical tests method is mentioned at the end of the figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	Yes. One-Way or Two-way ANOVA for multiple comparison was used to compare samples and data represented as standard error of the mean (SEM).

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http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

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http://figshare.com

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Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibody catalog number were included in supplementary infromation.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Commercial cell lines and brown adipocytes were tested on a regular basis.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	The experiments were conduceted using C57BL6/J base Ews+/- (NIH) and Parp1+/- (JAX lab) congenic mouse. Throughout the experiments we used male and female mice aged from embryo day 17.5 to postnatal 3 days. Mice were housed at the animal facility in UNIST, 12 hour light-dark cycle at 23 degree with unlimited access to chow and water in SPF condition.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal procedures were approved and performed according to the guidelines provided by the Ulsan National Institute of Science and Technology's (UNIST) Institutional Animal Care and Use Committee. (Certificate No. #15-15)
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. 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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. 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.	N/A
17. 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.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The mass-spectrometry data have been desposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016145 (Username: reviewer35515@ebi.ac.uk / Password:94FGz3zA).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Done
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