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NELF-E is recruited to DNA double-strand break sites to promote transcriptional repression and repair

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 07 September 2016

Thank you for the submission of your research manuscript to our journal. We have now received the enclosed reports on it.

As you will see, all referees acknowledge that the findings are potentially very interesting. However, they also point out that significant revisions are required, that important controls are missing, and that the relevance of NELF-E-mediated transcriptional repression for genome stability remains unclear.
I read through the referee comments and think that all of them should be addressed. Please let me know if you think that any of the concerns cannot or would not need to be addressed experimentally, and we can discuss this further.

Given the overall very constructive comments, we would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of 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.

REFEREE REPORTS

Referee #1:

Awwad and Ayoub identify invovlement of the NELF-E protein in the DNA damage response, specifically in transcriptional silencing downstream of double strand breaks (DSBs). The authors show convincingly that NELF-E localizes to UV-microbeam and nuclease induced DSBs through its N-terminal LZ domain. This is mediated through interaction with PARylated proteins, though PARylation of NELF-E itself is dispensable. Despite early data in the manuscript pertaining to transcriptional silencing and the title of the manuscript the authors do little to close the circle between NELF-E, PARylation and silencing. This leaves their model incomplete and the picture a bit murky. There are also a few instances where controls are missing and where data analysis does not follow convention. For these reasons, in the opinion of this reviewer, the manuscript is not currently suitable for publication in EMBO Rep.

Specific comments:

Figure 1:
The effect of NELF-E depletion is modest. How does this compare to previously described modifiers of this response, such as ATM? As this authors note, no other reported factor has led to reversal of silencing to the extent of ATM. It is important to make this comparison. It would also be useful to perform epistatic analyses to determine how NELF-E compares with other known components of transcriptional silencing (e.g. ENL, PBAF, etc.).

Figure 2:
Note the title of the manuscript suggests that NELF-E's main role is in transcriptional silencing. This area of the manuscript must be strengthened considerably.

(b) Where are the controls? What does the MS2-YFP locus look like when there is no damage present? What about when there is damage with control siRNA? Is this the same experiment as in Figure 1C? The controls should be performed simultaneously to give an appropriate picture of how NELF and these mutants impact the response.

With respect to the above point the YFP-MS2 point is much less obvious in this panel than in the earlier ones. The rescue by the mutants is even less evident. It would be useful to perform an intensity analysis of the MS2 spots and quantify the transcripts by qRT-PCR. How does this compare to the previous factors in silencing, especially ATM?

The authors suggest that DSIF subunits may be involved in transcriptional silencing. Have they tested this? As it stands a single member of the NELF complex is explored in transcriptional silencing. What about the other members that localize to DSBs (i.e. NELF-A)? The introduction suggests that active NELF also has B and C/D members, why are these not recruited? Are they required for silencing? Is there some form of sub-complex involved?

Why do the del(RRM) mutants only rescue ~60% of the transfected cells? If this is the region required for silencing in other contexts through what mechanism does NELF-E contribute to silencing at DSBs?

Figure 3: This is an excellent system for exploring DSBs. The authors would be well served to better characterize the system given its novelty. At the very least the presentation of ChIP data needs to be improved significantly.

-Is NELF required for Cas9 dependent DSB silencing at this locus?

3F: This is not typically the way ChIP data is presented. They should be normalized (with a standard curve) to the input DNA and fraction of input is presented. Although these data look consistent with what the authors state, one cannot exclude the possibility that there were simply different amounts of DNA present in these samples at the outset. Normalizing to the mock IgG control is insufficient.

-The authors state several times that to their knowledge, NELF is the first factor that shows differential recruitment to DSBs within transcriptionally active regions. This is an incorrect statement. Several reports have shown increased HR factor recruitment to transcriptionally active regions (Tang et al. NSMB 2013 PMID:23377543; and Aymard et al. NSMB 2014 PMID: 24658350). This should be noted and the claim of NELF as the first removed.

Figure 4D: Is this mislabeled as MS2-YFP?

Does PARPi impact transcriptional silencing? This would add to cohesiveness of the model.

I would suggest improving the text with respect to Figure 4. Although I gather that NELF-E ADP-ribosylation itself is not required for its recruitment and that PAR moieties induced by DDR on other proteins are required, this point is confusing as written.

Figure 5: In Figure 3 the authors show that NELF-E recruitment requires active transcription. DRB blocks transcriptional elongation but does not impact NELF-E localization. The authors need to revise the interpretation of the earlier data if they now suggest it isn't active transcription but rather the presence of RNA PolII, active or not.

Referee #2:

Awwad and Ayoub demonstrate that the transcriptional repressor negative elongation factor NELF-E is required for switching off transcription nearby DNA double-strand breaks (DSBs). The authors show that NELF-E is recruited to DNA break sites in a poly(ADP-ribose) (PAR) and PARP1-dependent manner and provide evidence that NELF-E recruitment occurs preferentially at DSBs induced upstream of RNA polymerase II bound, transcriptionally active genes. Both the PAR- and PARP1-dependent recruitment of NELF-E as well as its repressive activity is found to be important for DSB-induced transcriptional repression, suggesting that NELF-E acts locally to switch off genes close to DSB sites. Overall, this is an interesting and timely manuscript, which addresses the important aspect how DNA damage response signals are transmitted from DSBs to the transcription machinery in order to coordinate DSB repair with transcriptional repression. While the authors convincingly demonstrate that NELF-E plays a hitherto unknown role in this process, the relevance of NELF-E-mediated transcriptional repression for maintenance of genome stability is less well developed, and certain claims about the mechanism of DSB-induced transcriptional repression via NELF-E function would benefit from consolidation as outlined below.

Major points:

1) As the authors point out in the introduction, transcription activity is rapidly and transiently paused in response to DNA damage to eliminate the production of abnormal transcripts and to avoid deleterious collisions between transcription and repair machineries. It would be good to test directly whether conditions, in which the NELF-E mediated transcriptional repression is impaired, would indeed lead to such deleterious consequences and undermine genome stability.

2) Related to the previous point, would PARP inhibition or PARP1 knockdown also lead to impaired transcriptional repression as expected in light of the abolished NELF-E recruitment? And would this in turn result in similar consequences for DSB repair and genome stability? Testing the effect of PARP inhibition or PARP1 knockdown on DSB-induced repression would be particularly important since both perturbations were recently shown to promote (rather than inhibit) NELF-E function on Pol II in the absence of DSBs (Gibson et al. 2016).

3) In light of some of the previous publications discussed in the manuscript (e.g. Kruhlak et al.; Shanbhag et al.; Pankotai et al.), the relative contribution of DDR kinase signaling (primarily ATM and DNA-PK) versus PAR signaling should be assessed in the system the authors use to measure DSB-induced transcriptional repression. Experiments to control for the inhibition should be included, and potential differences to other previously used systems should be discussed.

4) How is the interaction between the N-terminal region of NELF-E and PAR mediated? Does it contain any known PAR-binding motif? While a complete dissection of the interaction mechanism

might be beyond the scope of this manuscript, this aspect should at least be discussed based on the amino acid sequence of the N-terminal region and in the context of know PAR interaction mechanisms.

5) The authors note that DSIF and NELF co-operate to silence the activity of Pol II. It should be easy to test whether DSIF depletion would also impair transcriptional repression in response to DSBs.

6) In Fig. 1 DSBs induction in control and NELF-E-depleted cells was validated by H2AX immunostaining. However, in the representative cells shown in panel 1C H2AX levels seem lower in NELF-E siRNA transfected cells. It would be important to clarify that the induced damage is the same in both conditions, e.g. by quantifying the H2AX intensities in the cherry-TA-ER foci of all analyzed cells. Similarly, for laser-microirradiation experiments it would be good to show that the generated damage is comparable between conditions (e.g. by H2AX co-staining for selected time-points). In Supplementary Fig. 6 a H2AX co-staining would provide the missing control that the ATM inhibitor worked under these conditions.

7) In Fig. 3 the authors employ a system to monitor the accumulation of endogenous NELF-E upstream the A20 gene in its active and inactive transcriptional states (with and without TNF respectively). By ChIP the authors show that NELF-E shows a 20-fold increase at sequences surrounding DSB sites of transcriptionally active A20 gene, compared to a 5-fold increase when A20 is silent. It would be important to show that the expression of NELF-E is not affected by TNF , and the observed increase in NELF-E binding is due to the transcriptional activation of A20 gene and not because of increased levels of NELF-E.

8) For ChIP analyses, the occupancy of NELF-E and H2AX was normalized to IgG control. It seems more appropriate to present percentages of input from representative experiments (rather than normalizing to a background control) and include the unrelated IgG antibody separately.

9) In Fig. 4b,c it would be nice to include representative images and a quantification of the recruitment kinetics (as was done in Fig. 4a). In Fig 4d the NELF-E panels seem to be missing and the MS2 signal seems to be in conflict with the data shown in Fig. 1.

10) Most of the experiments were performed with U2OS cells, while some experiments were performed in MCF7 cells. No explanation is given for this change of cell line. For example, in Supplementary Fig. 1, how NELF-E knockdown affects NELF-A and NELF-B protein expression is shown in MCF7 cells infected with shRNAs different from the siRNAs used throughout the manuscript. The authors suggest that "alleviation of transcription repression following DSB is likely due to disruption of the entire NELF complex rather than sole depletion of NELF-E subunit", however, it would be important to show that NELF-E knockdown disrupts the integrity of NELF complex in U2OS-TRE-I-Sce 19 cells using the same siRNAs applied for the analysis of DSB-induced transcription silencing. In Supplementary Figure 4 is not clear whether U2OS cells or MCF7 cells were used.

11) The alpha-amanitin experiment should be better controlled; at the very least, DNA damage induction and DDR signaling should be monitored by H2AX staining after laser microirradiation, and PARP1 levels and PAR induction should also be tested under these conditions.

12) Markers of molecular weight should be included on western blot images.

Minor points:

1) In the section "Preferential accumulation of endogenous NELF-E at DSB nearby transcriptionally active genes" the following phrase is twice used: "To the best of our knowledge, NELF-E is the first example of protein that shows differential recruitment to DSB nearby transcriptionally active rather than inactive genes."

2) Panels 3b and 3c could be combined.

3) Fig. 3e could be moved to the supplementary information. If the data represent a western blot

after IP (rather than a ChIP experiment), this should be clarified and described better in the legend.

Referee #3:

In this study, Awwad and Ayoub uncover a novel role for the negative elongation factor NELF in blocking transcription of genes nearby DNA double-strand breaks (DSBs) in human cells. They focus most of the study on NELF-E subunit, showing that it is recruited to DSBs in the promoters of active genes in a poly-ADP-ribose- and RNA polymerase II-dependent manner. They also provide evidence that both NELF-E recruitment to DSBs and its repressive activity are necessary for turning off transcription.

This is a very nice piece of work, which brings a series of new and interesting findings. It is not entirely clear though how NELF connects with pathways previously involved in shutting off transcription near DSBs. Furthermore, while the results provided are generally convincing, some important controls are missing, which should be included to improve the manuscript before it can be accepted for publication in EMBO Reports.

Major comments:

1- Fig.1: gammaH2AX spots seem smaller upon siNELF-E. The authors should check whether DSB induction is affected by siNELF-E.
Does NELF-E siRNA similarly impair transcription inhibition at laser damage?

2- The expression of the NELF-E mutants used in Fig.2 and S3 should be controlled by WB to verify that they run at the expected size, are expressed at comparable levels and do not show much degradation.

3- The authors observe the recruitment to DSBs of NELF-A and NELF-E subunits only, suggesting that NELF is not recruited as a whole complex yet still functional for transcription inhibition. The authors should discuss this point.

4- Fig. 3a-c: MS2-YFP should be included as a control for transcription activation

5- Fig.4: Given that the transcriptional status of the region close to the damage is critical for NELF-E recruitment, the authors should assess the effect of PARP inhibitor and siPARP1 on transcription.

6- Fig. 4e-f: Inputs should be shown in the IP experiments and a control for IR treatment (like gammaH2AX) should be included.

7- Fig. 4f: the EQ mutant should be examined along side wild-type NELF-E to confirm that this mutant displays impaired PARylation

8- The authors show that NELF-E N-terminal region binds PAR in vitro. Does it contain any of the known PAR binding motifs?
Different NELF-E mutants are used in Fig. 2 and Fig. 4 to assess NELF-E recruitment to DSBs in vivo and PAR binding in vitro, respectively. To connect the two types of analyses, similar mutants should be used in both assays. The authors should check the recruitment of NELF-E Nt and Ct fragments to laser damage and/or use the LZ delta mutant in the PAR binding assay.

9- The authors cannot claim that "NELF-E is the first example of protein that shows differential recruitment to DSB nearby transcriptionally active rather than inactive genes" (this sentence appears twice on p.7). See for example work by the Legube group showing that HR factors are preferentially recruited to active chromatin (Aymard et al., NSMB 2014).

10- It would be important to examine whether cell treatment with PARP inhibitor or siPARP1 can recapitulate the effect of siNELF-E in the system used in this study for transcription inhibition nearby I-SceI cut.

11- Fig. S6: the efficiency of ATM inhibition should be controlled.

12- The authors could discuss more how NELF may connect with previous factors/pathways involved in shutting off transcription near DSBs.

Minor points:

- Introduction p.3: the authors should cite work by the Mailand group on H1 being a ubiquitylation target of RNF8. They should also cite work by the Miller group when mentioning transcription repression via NuRD at DNA damage sites.

- p.5 : it should read "shRNA of NELF-E concomitantly disrupts"

-Fig. 3: it would help if the authors could indicate + and - I-SceI on panels b and c and also the position of the DSB on panel f

-Fig. 4d: it should read NELF-E instead of YFP-MS2

-Fig. S4: the legend says MCF7 cells and U2OS cells so it is not clear which cells were used for this experiment.

1st Revision - authors' response 18 December 2016

Thank you for your letter dated September 7th 2016, inviting us to resubmit a revised version of this manuscript after addressing the reviewers' comments. With this letter comes the revised paper. You will see that we added substantial amount of new experimental data addressing the issues raised by the three reviewers. The revised manuscript includes 7 large main figures and 11 supplementary figures. In summary, we provided a significant amount of additional experimental work to fully address the reviewers' concerns. We believe that our manuscript is now of high standard and is suitable for publication in EMBO Reports.

Response to the reviewers of Awwad et al., EMBOR-2016-43191V1

We thank the three reviewers for their thorough evaluation and constructive criticisms of the manuscript. Indeed, some of their suggestions prompted us to carry out experiments that we might not otherwise have done. After careful scrutiny of the comments raised by the reviewers, we focused our experimental efforts to address all the points raised by the reviewers. This document includes:

1) Summary of the main new findings of the revised manuscript.
2) Point-to-point responses to the reviewers' comments.
3) An appendix addressing the reviewers' comment regarding the potential role of the DSIF complex in DNA damage-induced transcription repression (data not included in the Peer Review Process File).

1) Summary of the main new data presented in the revised manuscript:

We provided two new lines of evidence that further strengthen the role of NELF-E in DNA damage-induced transcription repression.

(i) We showed that NELF-E contributes to global transcription shutdown in response to IR using CLICK-IT methodology (*New Figure 1A, B*).

(ii) We showed that the expression level of A20 gene after CRISP-Cas9-induced DSB is significantly higher in NELF-E depleted cells in comparison to control cells (*New Figure 3E-G*).

- We included new experiments demonstrating the NELF-E promotes homology-directed repair and non-homologous end joining of double-strand breaks in cells (*New Figure 7*).
- We showed the effect of ATM and PARP1 inhibition on the expression of MS2 gene before and after DSB induction (*New Figure 4H and New Supplementary Figure 2)*.

- We showed that the intensity of γH2AX is not affected by NELF-E depletion (*New Figure 1F*).
- We determined the effect of α-amanitin on the induction of γH2AX, PARP1 and PAR accumulation at DNA damage sites (*New Supplementary Figure 11A,B).*
- We confirmed the efficacy of ATM inhibitor using CtIP protein as a positive control (*New Supplementary Figure 9B)*.
- We measured the PARylation level of NELF-E-EQ mutant (*New Supplementary Figure 10).*
- We demonstrated that the protein level of NELF-E is not affected by TNFα treatment (*New Supplementary Figure 8).*
- We showed the recruitment of the C-terminal and the N-terminal of NELF-E to laser microirradiated sites (*New Supplementary Figure 4A)* and added western blots showing the expression of NELF-E mutants *(New Supplementary Figure 4B and New Supplementary Figure 5).*
- We showed that NELF-E depletion leads to degradation of NELF-A and NELF-B in U2OS-19 cells *(New Supplementary Figure 1D).*
- We demonstrated that the N-terminal region of NELF-E contains a putative PAR-binding motif *(New Figure 4D).*
- We reanalyzed the ChIP data and added all the missing controls requested by the reviewers (please see *Revised Figures 2B, 3D, 5A, 5B*).
- We discussed all the points raised by the reviewers.

2) Point-to-point responses to the reviewers' comments (responses in italics).

Referee #1:

Awwad and Ayoub identify involvement of the NELF-E protein in the DNA damage response, specifically in transcriptional silencing downstream of double strand breaks (DSBs). The authors show convincingly that NELF-E localizes to UV-microbeam and nuclease induced DSBs through its N-terminal LZ domain. This is mediated through interaction with PARylated proteins, though PARylation of NELF-E itself is dispensable. Despite early data in the manuscript pertaining to transcriptional silencing and the title of the manuscript the authors do little to close the circle between NELF-E, PARylation and silencing. This leaves their model incomplete and the picture a bit murky. There are also a few instances where controls are missing and where data analysis does not follow convention. For these reasons, in the opinion of this reviewer, the manuscript is not currently suitable for publication in EMBO Rep.

Specific comments:

Figure 1: The effect of NELF-E depletion is modest.

We observed that ~70% of NELF-E depleted cells show expression of MS2 gene in the presence of DSBs, compared to ~5% of cells transfected with control siRNA. We believe that this is big and significant difference. The fact that not all NELF-E depleted cells show expression of MS2 gene in the presence of DSBs suggest that other factors contribute to transcription repression after damage. In this regard, we would like to draw the reviewer's attention that the revised manuscript includes additional two lines of evidence that further substantiated the contribution of NELF-E to DNA damage-induced transcription repression. We showed that control cells exhibit ~40% reduction in transcription activity at 30min after IR. On the other hand, NELF-E deficient cells show milder reduction in transcription activity after IR (~5%) comparing to mock cells ***(New Figure 1A, B)****. Moreover, we tested the effect of NELF-E on A20 expression after DSB induction using CRISPR-Cas9 endonuclease. Results show that the expression level of A20 gene after CRISP-Cas9-induced DSB is significantly higher in NELF-E depleted cells in comparison to control cells* ***(New Figure 3E-G).***

How does this compare to previously described modifiers of this response, such as ATM? As this authors note, no other reported factor has led to reversal of silencing to the extent of ATM. It is important to make this comparison.

We agree with the reviewer's comment and accordingly we measured the effect of ATM inhibition on the transcription of MS2 in the presence of DSBs. Toward this end, DSBs were induced in U2OS-TRE-I-Sce-19 cells (by co-transfecting the cells with pCherry-tTA-ER; pYFP-MS2 and pCMV-NLS-I-SceI plasmids) pretreated with ATM inhibitor. As shown in ***New Supplementary Figure 2****, pharmacological inhibition of ATM abrogated transcription silencing of MS2 following DSB induction in 90% of the cells. This result is in agreement with published data implicating ATM in shutting down transcription after DSB induction [1-3]. Notably, the number of cells that show expression of MS2 gene in the presence of DSBs in ATM inhibited cells is comparable to NELF-E depleted cells* ***(New Supplementary Figure 2)****.*

It would also be useful to perform epistatic analyses to determine how NELF-E compares with other known components of transcriptional silencing (e.g. ENL, PBAF, etc.).

*It was shown that ENL, PBAF, RNF8/168 and PcG proteins require intact ATM kinase activity to block transcription after DSB induction. Therefore, we tested the effect of ATM inhibition on MS2 expression after DSB induction in mock and NELF-E-depleted cells. Results show that the number of NELF-E depleted cells that show expression of MS2 is comparable to the number of NELF-E depleted cells that were pre-treated with ATM inhibitor. Altogether, these observations suggest that NELF-E and ATM may act in the same pathway to ensure transient transcription silencing after DSB induction (****New Supplementary Figure 2)****. Below we discussed the crosstalk between PARP and ATM activity to ensure transcription silencing after DNA damage (see our response to Figure 4). This important point was also discussed in the revised manuscript.*

Figure 2: Note the title of the manuscript suggests that NELF-E's main role is in transcriptional silencing. This area of the manuscript must be strengthened considerably.

We agree with the reviewer and as indicated in our response to Figure 1, the revised manuscript includes two additional lines of evidence that corroborate the role of NELF-E in DNA damage-induced transcription silencing. ***(i)*** *We showed that NELF-E contributes to global transcription shutdown in response to IR using CLICK-IT methodology* ***(New Figure 1A, B)****.* ***(ii)*** *We showed that the expression level of A20 gene after CRISP-Cas9-induced DSB is significantly higher in NELF-E depleted cells in comparison to control cells* ***(New Figure 3E-G).***

(b) Where are the controls? What does the MS2-YFP locus look like when there is no damage present? What about when there is damage with control siRNA? Is this the same experiment as in Figure 1C? The controls should be performed simultaneously to give an appropriate picture of how NELF and these mutants impact the response.

We thank the reviewer for raising these important points. We would like to confirm that each experiment was done with its own set of controls. We simply didn't include these controls in all the figures because the space limitation of the journal. In the revised manuscript we added all the requested controls ***(New Figure 2B)****.*

With respect to the above point the YFP-MS2 point is much less obvious in this panel than in the earlier ones. The rescue by the mutants is even less evident. It would be useful to perform an intensity analysis of the MS2 spots and quantify the transcripts by qRT-PCR. How does this compare to the previous factors in silencing, especially ATM?

We agree with the reviewer. The clearness and the contrast of the MS2 spots are affected by the intensity and the distribution of the green signal in the entire nucleus. We apologize for the poor quality of the image shown in figure 2B, which was replaced with clearer representative images in the revised manuscript. Measuring the intensity of the MS2 spots is not applicable in this case due to fluctuation in the intensity of the green signal between the different cells as a result of the transient transfection of pYFP-MS2 plasmid. Regarding the quantification of the MS2 transcript by qRT-PCR, we believe that this wouldn't work for the following reasons: (1) The co-transfection efficiency of

U2OS-TRE-I-Sce-19 cells with the three plasmids (pCherry-tTA-ER; pYFP-MS2 and pCMV-NLS-I-SceI) doesn't exceed 30-40% and it varies between the different conditions of the cells. (2) Not all the transfected cells contain DSBs as evidenced by γH2AX staining. (3) Most importantly, there is large variability in the numbers of cells that show migration of the cytoplasmic Cherry-tTA-ER chimera into the nucleus following the addition of tamoxifen to induce transcription of MS2 gene. For the aforementioned reasons, we believe that visualizing MS2 expression should be done in a single cell level as previously described by several research groups [1-3].

The authors suggest that DSIF subunits may be involved in transcriptional silencing. Have they tested this?

We have some preliminary data suggesting that Spt5 protein (member of the DSIF complex) is also involved in DSB-induced transcription silencing, as it is recruited to laser-microirradiated sites in a PARP-dependent manner and alleviates transcription silencing of the MS2 gene after DSB induction [please see Appendix 1, below (data not included in the Peer Review Process File)]. Further characterization of Spt5 role in transcription silencing after DSB induction is an ongoing step in this line of research and will be published elsewhere. In summary, we do believe that our revised manuscript conveys very important information without the preliminary Spt5 data.

As it stands, a single member of the NELF complex is explored in transcriptional silencing. What about the other members that localize to DSBs (i.e. NELF-A)?

Others [4] and we showed that NELF-E knockdown disrupts the integrity of NELF complex as evident by the degradation of NELF-A and NELF-B proteins (Supplementary Fig. 1). Therefore, we disagree with the referee's statement that "a single member of the NELF complex is explored in transcriptional silencing". As indicated in the results of our manuscript the alleviation of transcription repression following DSB is likely due to disruption of the entire NELF complex rather than sole depletion of a single member. Knocking down NELF-E in MCF7 cells leads to dramatic degradation of NELF-A and NELF-B and vice versa (please see Supplementary Figure 1A-C). Therefore and as indicated in the revised manuscript, the alleviation of transcription silencing after DSB induction in NELF-E depleted cells is likely due to degradation of ***all*** *NELF subunits. Nonetheless and following the reviewer's comment, we provided new data showing that NELF-E depletion leads to a severe degradation of NELF-A and NELF-B in U2OS-TRE-I-Sce-19 cell line as well (cells that were used to study the effect of NELF-E on MS2 expression at DSB sites)* ***(New Supplementary Figure 1D)****. On this basis, it is unnecessary to check transcription of MS2 gene in NELF-A depleted cells.*

The introduction suggests that active NELF also has B and C/D members, why are these not recruited? Are they required for silencing?

We are grateful to the reviewer for raising these important questions. We rigorously tested the recruitment of NELF-B and NELF-C/D subunit to laser-microirradiated sites several times and in different cell lines. It remains unclear why NELF-B and NELF-C/D subunits are not recruited to DNA breakage sites.

Is there some form of sub-complex involved?

It has been reported that NELF sub-complexes are found in human cells. For example, it was shown that NELF-A and NELF-C exist in a sub-complex that binds RNA in vitro and in vivo. [5]. In addition, it was shown that NELF-E subunit binds RNA in vivo and mutating its RNA-binding domain impairs transcription repression of the NELF complex without affecting known protein-protein interactions [4, 6]. Similarly, NELF-A is critical for RNAPII binding and for transcriptional pausing. On this basis, it is plausible to assume that NELF-E and NELF-A form a sub-complex at DNA damage sites to ensure transient transcription repression. We have discussed this important point in the revised manuscript.

Why do the del(RRM) mutants only rescue ~60% of the transfected cells? If this is the region required for silencing in other contexts through what mechanism does NELF-E contribute to silencing at DSBs?

Indeed, our data in Figure 2C suggest that both domains (RRM and LZ) are required for intact transcription silencing at DSB sites. It should be noted that del(RRM) mutant is recruited to DNA damage sites (Supplementary Figure 4) and contains the LZ motif that mediates proteins dimerization and facilitates binding to DNA [7, 8]. Hence, we predict that the del(RRM) mutant may confer silencing through the LZ domain that might promote the recruitment of other silencing factors to DNA damage sites. We clarified this point in the revised manuscript.

Figure 3: This is an excellent system for exploring DSBs. The authors would be well served to better characterize the system given its novelty. At the very least the presentation of ChIP data needs to be improved significantly.

We agree with the reviewer and as requested we reanalyzed and changed the presentation of the ChIP data.

-Is NELF required for Cas9 dependent DSB silencing at this locus?

We thank the reviewer for raising this excellent question, which prompted us to test the effect of NELF-E on A20 expression after DSB induction using CRISPR-Cas9 endonuclease. Results show that the expression level of A20 gene after CRISP-Cas9-induced DSB is significantly higher in NELF-E depleted cells in comparison to control cells ***(New Figure 3E-G)***

(F) This is not typically the way ChIP data is presented. They should be normalized (with a standard curve) to the input DNA and fraction of input is presented. Although these data look consistent with what the authors state, one cannot exclude the possibility that there were simply different amounts of DNA present in these samples at the outset. Normalizing to the mock IgG control is insufficient.

We repeated the qPCR of the ChIP experiments and normalized it to the input DNA exactly as requested by the reviewer ***(Revised Figure 3D)****.*

-The authors state several times that to their knowledge, NELF is the first factor that shows differential recruitment to DSBs within transcriptionally active regions. This is an incorrect statement. Several reports have shown increased HR factor recruitment to transcriptionally active regions (Tang et al. NSMB 2013 PMID:23377543; and Aymard et al. NSMB 2014 PMID: 24658350). This should be noted and the claim of NELF as the first removed.

We agree with the reviewer and thank him for drawing our attention to the indicated manuscripts. As requested, we revised the text and cited the indicated reports.

Figure 4: (d) Is this mislabeled as MS2-YFP?

*Yes, and we corrected this typo in the revised version****.***

Does PARPi impact transcriptional silencing? This would add to cohesiveness of the model.

We provided new data in the revised manuscript showing that pharmacological inhibition of PARP disrupts MS2 silencing ***(New Figure 4H)****. It was shown in this regard by Brendan Price group that PARP inhibition leads to a significant reduction in H3K9me3 at DSB sites and thus disrupts ATM activation (as evidenced by absence of Kap1 phosphorylation)[9]. We assume therefore that the alleviation of MS2 repression at DSB sites following PARP inhibition could be mediated via ATM kinase activity. We indicated this important point in the revised manuscript.*

Figure 5: In Figure 3 the authors show that NELF-E recruitment requires active transcription. DRB blocks transcriptional elongation but does not impact NELF-E localization. The authors need to revise the interpretation of the earlier data if they now suggest it isn't active transcription but rather the presence of RNA PolII, active or not.

We revised our conclusions regarding Figure 3.

Referee #2:

Awwad and Ayoub demonstrate that the transcriptional repressor negative elongation factor NELF-E is required for switching off transcription nearby DNA double-strand breaks (DSBs). The authors show that NELF-E is recruited to DNA break sites in a poly(ADP-ribose) (PAR) and PARP1-dependent manner and provide evidence that NELF-E recruitment occurs preferentially at DSBs induced upstream of RNA polymerase II bound, transcriptionally active genes. Both the PAR- and PARP1-dependent recruitment of NELF-E as well as its repressive activity is found to be important for DSB-induced transcriptional repression, suggesting that NELF-E acts locally to switch off genes close to DSB sites. Overall, this is an interesting and timely manuscript, which addresses the important aspect how DNA damage response signals are transmitted from DSBs to the transcription machinery in order to coordinate DSB repair with transcriptional repression. While the authors convincingly demonstrate that NELF-E plays a hitherto unknown role in this process, the relevance of NELF-E-mediated transcriptional repression for maintenance of genome stability is less well developed, and certain claims about the mechanism of DSB-induced transcriptional repression via NELF-E function would benefit from consolidation as outlined below.

Major points:

1) As the authors point out in the introduction, transcription activity is rapidly and transiently paused in response to DNA damage to eliminate the production of abnormal transcripts and to avoid deleterious collisions between transcription and repair machineries. It would be good to test directly whether conditions, in which the NELF-E mediated transcriptional repression is impaired, would indeed lead to such deleterious consequences and undermine genome stability.

*We are grateful to the reviewer for raising this important point. In the revised manuscript we provided two lines of evidence that implicate NELF-E in DNA damage repair. First, colony formation assay revealed that NELF-E depleted cells are hypersensitive to ionizing radiation (IR), suggesting that NELF-E is required for intact IR-induced DNA damage repair (**New Figures 7A-B**). Second, we determined the effect of NELF-E depletion on the integrity of homology-directed repair (HDR) and non-homologous end joining (NHEJ) of DSBs. Results show that NELF-E depletion causes moderate reduction in HDR (**New Figures 7C-D**) and greater reduction in the efficiency of NHEJ (**New Figures 7E-F**). Altogether, these data suggest that NELF-E facilitates HDR and NHEJ of DSBs in cells.*

2) Related to the previous point, would PARP inhibition or PARP1 knockdown also lead to impaired transcriptional repression as expected in light of the abolished NELF-E recruitment?

*As explained above in our reply to Figure 4 of the first reviewer, the revised manuscript includes new data showing that pharmacological inhibition of PARP disrupts MS2 silencing after DSB induction **(New Figure 4H)**. It was shown that PARP inhibition leads to significant reduction in H3K9me3 at DSB sites and thus disrupts ATM activation (as evidenced by absence of Kap1 phosphorylation)[9]. We assume therefore that the alleviation of MS2 repression at DSB sites following PARP inhibition could be mediated via ATM kinase activity.*

And would this in turn result in similar consequences for DSB repair and genome stability?

Several reports implicated PARP1 in regulating the accumulation of DDR proteins that are required for intact repair of DSBs. Here, we cited few examples: (1) Polo et al., showed that CHD4 protein is recruited to DNA damage sites in a PARP1-depenedent manner to promote DSB repair and survival

after DNA damage[10]. (2) A recent paper by Luijsterburg et al, revealed a PARP1-dependent process that regulates histone variant deposition at DSBs to facilitate repair by NHEJ and HDR and preserve genomic stability [11]. (3) PARP1 regulates KDM4D recruitment to DSB sites and promotes repair by HDR and NHEJ [12, 13]. (4) It was shown that PARP1 inhibition inhibits ATM activation after DSB induction as evidenced by the lack of KAP phosphorylation [9]. (5) Bryant et al showed that PARP1 regulates the recruitment of MRN complex to DSB sites at stalled or collapsed replication forks and subsequently PARP1 inhibition disrupts HDR of these DSBs [14]. Altogether, these observations strongly implicate PARP activity in DSB repair and genomic stability. We discussed this important issue in the revised manuscript.

Testing the effect of PARP inhibition or PARP1 knockdown on DSB-induced repression would be particularly important since both perturbations were recently shown to promote (rather than inhibit) NELF-E function on Pol II in the absence of DSBs (Gibson et al. 2016).

*We fully agree with the reviewer and as stated above, our data showed that PARP inhibition alleviates transcription silencing of MS2 gene at DSB sites **(New Figure 4H)**. Gibson et al. claimed that PARP1 PARylates NELF-E and stimulates the elongation activity of RNA Pol II [15]. On the other hand, we demonstrated that (i) the PARylated levels of NELF-E decrease after IR (Figure 4F). (ii) PARP1-NELF-E interaction is reduced after DNA damage (Figure 4E). Altogether, these observations support the notion that NELF-E molecules that accumulate at DNA damage sites are less PARylated and thus can inhibit RNA Pol II activity. We, therefore, do not see an inconsistency between our data and Gibson's observations with regard to the effect of NELF-E PARylation on its silencing activity. This issue is further highlighted in the revised manuscript.*

3) In light of some of the previous publications discussed in the manuscript (e.g. Kruhlak et al.; Shanbhag et al.; Pankotai et al.), the relative contribution of DDR kinase signaling (primarily ATM and DNA-PK) versus PAR signaling should be assessed in the system the authors use to measure DSB-induced transcriptional repression. Experiments to control for the inhibition should be included, and potential differences to other previously used systems should be discussed.

*As stated above in our response to the first reviewer, we provided new data showing the effect of ATM inhibition on MS2 transcription after DSB induction **(New Supplementary Figure 2)**. In addition, we showed that inhibition of both ATM and NELF-E didn't cause synergistic or additive effects on the transcription of MS2 gene indicating that they might function in the same pathway **(New Supplementary Figure 2)**. In support of this, it was recently shown that pharmacological inhibition of PARP1 prevents ATM activation after DNA damage [9].*

4) How is the interaction between the N-terminal region of NELF-E and PAR mediated? Does it contain any known PAR-binding motif? While a complete dissection of the interaction mechanism might be beyond the scope of this manuscript, this aspect should at least be discussed based on the amino acid sequence of the N-terminal region and in the context of know PAR interaction mechanisms.

*We thank the reviewer for raising this important point. Indeed, bioinformatics analysis revealed that the N-terminal region of NELF-E contains a putative consensus motif for binding of poly(ADP-ribose) PAR moieties **(New Figure 4D)**. This result is in agreement with our findings showing that the N-terminal of NELF-E binds PAR moieties.*

5) The authors note that DSIF and NELF co-operate to silence the activity of Pol II. It should be easy to test whether DSIF depletion would also impair transcriptional repression in response to DSBs.

This important point was also raised by the first reviewer. For you convenience, I copied/pasted our response to Reviewer 1: "Regarding DSIF complex, we have some preliminary data suggesting that Spt5 protein (member of the DSIF complex) is also involved in DSB-induced transcription silencing, as it is recruited to laser-microirradiated site in a PARP-dependent manner and alleviates transcription silencing of the MS2 gene after DSB induction [please see Appendix 1, below (data not included in the Peer Review Process File)]. Further characterization of Spt5 in transcription

silencing after DSB induction is an ongoing step in this line of research and will be published elsewhere. In summary, we do believe that our revised manuscript conveys very important information without the preliminary Spt5 data".

6) In Fig. 1 DSBs induction in control and NELF-E-depleted cells was validated by γH2AX immunostaining. However, in the representative cells shown in panel 1C γH2AX levels seem lower in NELF-E siRNA transfected cells. It would be important to clarify that the induced damage is the same in both conditions, e.g. by quantifying the γH2AX intensities in the cherry-TA-ER foci of all analyzed cells.

We quantified γH2AX intensity. Results showed no noticeable difference in γH2AX intensity between control and NELF-E depleted cells ***(New Figure 1F)****.*

Similarly, for laser-microirradiation experiments it would be good to show that the generated damage is comparable between conditions (e.g. by γH2AX co-staining for selected time-points).

As indicated in the material and methods of the revised manuscript all cell lines were subjected to the same number of laser iterations. In addition, we provided new data showing comparable γH2AX intensities in mock and α-amanitin treated cells ***(New Supplementary Figure 11B)****.*

In Supplementary Fig. 6 a γH2AX co-staining would provide the missing control that the ATM inhibitor worked under these conditions.

We would like to draw the reviewer's attention that treating cells with ATM inhibitor was done exactly as previously described in our work [13], where we confirmed the efficiency of ATM inhibitor by looking at γH2AX induction. Nonetheless, we validated again the efficacy of ATM inhibition by visualizing CtIP accumulation at laser-microirradiated sites. Results showed that pretreating cells with ATM inhibitor abolished CtIP accumulation at laser-microirradiated sites ***(New Supplementary Figure 9B),*** *finding that is in agreement with previous report [16].*

7) In Fig. 3 the authors employ a system to monitor the accumulation of endogenous NELF-E upstream the A20 gene in its active and inactive transcriptional states (with and without TNFα respectively). By ChIP the authors show that NELF-E shows a 20-fold increase at sequences surrounding DSB sites of transcriptionally active A20 gene, compared to a 5-fold increase when A20 is silent. It would be important to show that the expression of NELF-E is not affected by TNFα, and the observed increase in NELF-E binding is due to the transcriptional activation of A20 gene and not because of increased levels of NELF-E.

We agree with the reviewer and measured the protein levels of NELF-E in cells before and after TNFα. As shown in ***New Supplementary Figure 8****, TNFα treatment has no detectable effect on the protein levels of NELF-E.*

8) For ChIP analyses, the occupancy of NELF-E and γH2AX was normalized to IgG control. It seems more appropriate to present percentages of input from representative experiments (rather than normalizing to a background control) and include the unrelated IgG antibody separately.

We repeated the qPCR for the ChIP data and reanalyzed it by normalizing to the input ***(Revised Figure 3D).***

9) In Fig. 4b,c it would be nice to include representative images and a quantification of the recruitment kinetics (as was done in Fig. 4a).

We added representative images exactly as requested ***(Revised Figure 4C)***

In Fig 4d the NELF-E panels seem to be missing and the MS2 signal seems to be in conflict with the data shown in Fig. 1.

Figure 4d was mislabeled. The second row should be labeled with NELF-E instead of YFP-MS2. This mistake was corrected in the revised manuscript.

10) Most of the experiments were performed with U2OS cells, while some experiments were performed in MCF7 cells. No explanation is given for this change of cell line. For example, in Supplementary Fig. 1, how NELF-E knockdown affects NELF-A and NELF-B protein expression is shown in MCF7 cells infected with shRNAs different from the siRNAs used throughout the manuscript. The authors suggest that "alleviation of transcription repression following DSB is likely due to disruption of the entire NELF complex rather than sole depletion of NELF-E subunit", however, it would be important to show that NELF-E knockdown disrupts the integrity of NELF complex in U2OS-TRE-I-Sce 19 cells using the same siRNAs applied for the analysis of DSB-induced transcription silencing. In Supplementary Figure 4 is not clear whether U2OS cells or MCF7 cells were used.

We agree with the reviewer and provided therefore new data in the revised manuscript showing that NELF-E depletion in U2OS-TRE-I-Sce-19 triggered degradation of NELF-A and NELF-B, similar to MCF7 cells ***(New Supplementary Figure 1D)****. Regarding Supplementary Figure 4, we used MCF7 cells. The legend was corrected in the revised manuscript. We thank the reviewer again for his careful reading of the manuscript.*

11) The alpha-amanitin experiment should be better controlled; at the very least, DNA damage induction and DDR signaling should be monitored by γH2AX staining after laser microirradiation, and PARP1 levels and PAR induction should also be tested under these conditions.

As requested, we looked at γH2AX, PAR induction and PARP1 recruitment at laser-microirradiated regions in mock and α-amanitin-treated cells. Our results show no discernable changes in the induction of γH2AX and PARP1 recruitment following α-amanitin treatment. We noticed, however, a slight decrease in the intensity of PAR signal at laser-microirradiated sites. This reduction could be due to degradation of ADP-ribosylated proteins following α-amanitin treatment. We have indicated this point in the revised manuscript. ***(New Supplementary Figure 11).***

12) Markers of molecular weight should be included on western blot images.

We added the molecular weight markers to the entire Western blots.

Minor points:

1) In the section "Preferential accumulation of endogenous NELF-E at DSB nearby transcriptionally active genes" the following phrase is twice used: "To the best of our knowledge, NELF-E is the first example of protein that shows differential recruitment to DSB nearby transcriptionally active rather than inactive genes."

We corrected this in the revised manuscript.

2) Panels 3b and 3c could be combined.

As requested, we combined 3b and 3c.

3) Fig. 3e could be moved to the supplementary information. If the data represent a western blot after IP (rather than a ChIP experiment), this should be clarified and described better in the legend.

We revised the legend of Figure 3e and moved it to the supplementary data as requested.

Referee #3:

In this study, Awwad and Ayoub uncover a novel role for the negative elongation factor NELF in blocking transcription of genes nearby DNA double-strand breaks (DSBs) in human cells. They

focus most of the study on NELF-E subunit, showing that it is recruited to DSBs in the promoters of active genes in a poly-ADP-ribose- and RNA polymerase II-dependent manner. They also provide evidence that both NELF-E recruitment to DSBs and its repressive activity are necessary for turning off transcription. This is a very nice piece of work, which brings a series of new and interesting findings. It is not entirely clear though how NELF connects with pathways previously involved in shutting off transcription near DSBs. Furthermore, while the results provided are generally convincing, some important controls are missing, which should be included to improve the manuscript before it can be accepted for publication in EMBO Reports.

Major comments:

1- Fig.1: gammaH2AX spots seem smaller upon siNELF-E. The authors should check whether DSB induction is affected by siNELF-E.

We quantified γH2AX intensity. Results showed no noticeable difference in γH2AX intensity between control and NELF-E depleted cells ***(New Figure 1F)****.*

Does NELF-E siRNA similarly impair transcription inhibition at laser damage?

The revised manuscript contains two additional lines of evidence that substantiate the role of NELF-E in transcription inhibition after DNA damage induced by IR or by CRISPR-Cas9 methodology. First, we showed that control cells exhibit ~40% reduction in transcription activity at 30min after IR. On the other hand, NELF-E deficient cells show milder reduction in transcription activity after IR (~5%) comparing to mock cells ***(New Figure 1A, B)****. Second, we tested the effect of NELF-E on A20 expression after DSB induction using CRISPR-Cas9 endonuclease. Results showed that the expression level of A20 gene after CRISP-Cas9-induced DSB is significantly higher in NELF-E depleted cells in comparison to control cells* ***(New Figure 3E-G).***

2- The expression of the NELF-E mutants used in Fig.2 and S3 should be controlled by WB to verify that they run at the expected size, are expressed at comparable levels and do not show much degradation.

We fully agree with the reviewer and accordingly performed western blot to cells that express the different NELF-E fusions described in Figure 2 and Supplementary Figure 3 ***(New Supplementary Figures 4B and 5)****.*

3- The authors observe the recruitment to DSBs of NELF-A and NELF-E subunits only, suggesting that NELF is not recruited as a whole complex yet still functional for transcription inhibition. The authors should discuss this point.

As requested we discussed this point in the revised manuscript. In this regard, it was reported that NELF sub-complexes are found in human cells. For example, it was shown that NELF-A and NELF-C exist in a sub-complex that binds RNA in vitro and in vivo. [5]. In addition, it was shown that NELF-E subunit binds RNA in vivo and mutating its RNA-binding domain impairs transcription repression of the NELF complex without affecting known protein-protein interactions [4, 6]. Similarly, NELF-A is critical for RNA Pol II binding and for transcriptional pausing. On this basis, it is plausible to assume that NELF-E and NELF-A form a sub-complex at DNA damage sites to ensure transient transcription repression. We discussed this point in the revised manuscript.

4- Fig. 3a-c: MS2-YFP should be included as a control for transcription activation.

We acknowledge the reviewer's comment. However, it is impossible to include a fifth color in this experiment setup. In spite of this, we would like to draw the reviewer's attention that in the absence of DNA damage, over 95% of U2OS-TRE-I-Sce-19 cells that show red spot (reflects binding of the Cherry-tTA-ER to the TRE repeats) show also colocalized green spot (reflect MS2 expression) (Please see Figure 1e) and Figures 2D,4D ,5D of [2].

5- Fig.4: Given that the transcriptional status of the region close to the damage is critical for NELF-E recruitment, the authors should assess the effect of PARP inhibitor and siPARP1 on transcription.

The revised manuscript includes new data showing that PARP inhibition alleviates transcription silencing of MS2 at DSB sites ***(New Figure 4H)****.*

6- Fig. 4e-f: Inputs should be shown in the IP experiments and a control for IR treatment (like gammaH2AX) should be included.

As requested, we added the inputs to the IP experiments ***(Revised Figure 5A, B).*** *Also, we added immunoblot of γH2AX to confirm the induction of DNA damage* ***(Revised Figure 5A, B).***

7- Fig. 4f: the EQ mutant should be examined along side wild-type NELF-E to confirm that this mutant displays impaired PARylation.

We agree with the reviewer and therefore tested the PARylation of NELF-E-EQ mutant. Toward this, we performed GFP-TRAP for EGFP-NELF-E-WT and EGFP-NELF-E-EQ mutant followed by immunoblot using PAR antibody. Our results (based on 3 biological repeat) showed that the EQ mutant exhibits moderate reduction in the PARylation levels ***(New Supplementary Figure 10)****. We have revised the manuscript accordingly. We would like to draw the reviewer's attention that Figure 4D of Gibson et al 2016 that showed reduction in the PARylation levels of EQ mutant in vitro lacked input showing the amount of the total protein level loaded in each lane.*

8- The authors show that NELF-E N-terminal region binds PAR in vitro. Does it contain any of the known PAR binding motifs?

Bioinformatics analysis revealed that the N-terminal of NELF-E contains a putative consensus motif for binding of PAR moieties ***(New Figure 4D)****.*

Different NELF-E mutants are used in Fig. 2 and Fig. 4 to assess NELF-E recruitment to DSBs in vivo and PAR binding in vitro, respectively. To connect the two types of analyses, similar mutants should be used in both assays. The authors should check the recruitment of NELF-E Nt and Ct fragments to laser damage and/or use the LZ delta mutant in the PAR binding assay.

*As requested we tested the recruitment of the N-terminal and the C-terminal regions of NELF-E to laser-microirradiated sites. In agreement with our findings, the N-terminal, but not the C-terminal, region of NELF-E is recruited to DNA breakage sites (****New Supplementary Figure 4A****).*

9- The authors cannot claim that "NELF-E is the first example of protein that shows differential recruitment to DSB nearby transcriptionally active rather than inactive genes" (this sentence appears twice on p.7). See for example work by the Legube group showing that HR factors are preferentially recruited to active chromatin (Aymard et al., NSMB 2014).

We sincerely thank the reviewer for drawing our attention to the aforementioned manuscripts. We cited these works in the revised manuscript.

10- It would be important to examine whether cell treatment with PARP inhibitor or siPARP1 can recapitulate the effect of siNELF-E in the system used in this study for transcription inhibition nearby I-SceI cut.

We agree with the reviewer. The revised manuscript contains new data showing the effect of PARP inhibition on the expression of MS2 gene after DSB induction using I-Sce-I endonuclease ***(New Figure 4H)***

11- Fig. S6: the efficiency of ATM inhibition should be controlled.

We would like to draw the reviewer's attention that treating cells with ATM inhibitor was done exactly as previously described in our work [13], where we confirmed that efficiency of ATM inhibitor by looking at γH2AX induction. Nonetheless, we validated again the efficacy of ATM inhibition by visualizing CtIP accumulation at laser-microirradiated sites, which was abolished in cells treated with the ATM inhibitor ***(New Supplementary Figure 9B)*** *finding that is in agreement with previous report [16].*

12- The authors could discuss more how NELF may connect with previous factors/pathways involved in shutting off transcription near DSBs.

We discussed this point in the revised manuscript.

Minor points:

Introduction p.3: the authors should cite work by the Mailand group on H1 being a ubiquitylation target of RNF8. They should also cite work by the Miller group when mentioning transcription repression via NuRD at DNA damage sites.

We cited the indicated works in the revised manuscript.

p.5 : it should read "shRNA of NELF-E concomitantly disrupts"

We corrected this typo.

Fig. 3: it would help if the authors could indicate + and - I-SceI on panels b and c and also the position of the DSB on panel f –

We modified the Figure as requested.

Fig. 4d: it should read NELF-E instead of YFP-MS2

We corrected the label

Fig. S4: the legend says MCF7 cells and U2OS cells so it is not clear which cells were used for this experiment.

It is MCF7 cells. We corrected the legend.

We express our thanks once again for the careful reviews, and trust that the revisions to our manuscript have addressed the concerns of the referees. We hope that the revised manuscript will now be acceptable for publication in EMBO Reports.

2nd Editorial Decision 19 January 2017

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees. As you will see, while all referees support publication of the revised study, they still have a few remaining concerns that should be addressed before we can proceed with the official acceptance of your manuscript.

Please address all remaining concerns and send us a point-by-point response along with the revised study to facilitate its evaluation.

Several of the figure legends state n=2, or n is not specified (Figs 1B, 2B, 3A,D,G, 4A,C,G,H, 5C, 6A, 7B,D,F, S2B, S3, S4, S7A). Please note that no statistics can be calculated for n<3. The

experiments either need to be performed one more time so that n=3, or the error bars and p-values need to be removed. In the latter case, all data points from the 2 independent experiments can be shown in the graphs, along with their mean. It would be much better though to repeat the experiments one more time and include statistics.

The questions regarding statistics in the author checklist need to be answered for any experiment that involves statistics, not only for animal experiments (for these additional questions need to be answered). Please complete the checklist.

Please also add scale bars to all microscopy images.

Figures 3 and 4 run over two pages, which is not possible. Each figure must fit on a single page. Either rearrange the panels, or split the figures into 2.

The Appendix tables should be part of the materials and methods, please change them into regular tables that are included in the main text file. Alternatively, they can be EV tables that will expand when clicked online. In this case, an extra table file in excel format must be uploaded.

REFEREE REPORTS

Referee #1:

The authors have been very responsive to the prior critiques. The manuscript addresses novel mechanisms of DSB silencing by identifying NELF as a factor in this process and implicates NELF in DSB repair and resistance to IR. It represents a thorough story that should be of broad interest to the genome integrity community and in this Reviewer's opinion, is worthy of publication in EMBO Reports.

Referee #2:

In their revised manuscript "NELF-E is recruited to DNA double-strand break sites to promote transcription repression and repair" Awwad et al. provide additional experiments supporting a role of NELF-E in DNA damage-induced transcriptional repression and DSB repair. While some of the new additions indeed strengthen the overall conclusions and significantly improved the manuscript, there are still several points, which call for a more careful interpretation, or would benefit from additional experimental conditions and controls. For instance, the effect of ATM and PARP inhibition on repression is now included, but the authors do not go all the way to close the circle, although in principle this should be fairly easy with the system they are using and would significantly help to clarify how NELF-E recruitment, PARylation, ATM signaling and repression are connected.

Specific comments:

1) Using the MS2 system the authors now show the effect of NELF-E knockdown and ATM inhibition (Fig. S2) and of PARP inhibition (Fig. 4H) and speculate about how these things are connected. Why did the authors not combine these treatments in one experiment and test for epistasis between PARPi and ATMi and between PARPi and NELF-E knockdown?

2) In their rebuttal the authors use Fig. 3E-G as evidence that further strengthens the role of NELF-E in DNA damage-induced transcription repression. However, these data do not show a transcriptional repression of the A20 gene upon break induction. Rather, Cas9 leads to increased expression and it is therefore unclear how these results relate to the rest of the manuscript.

Additional points:

1) My comment to include gammaH2AX staining controls for the laser recruitment experiments was not properly addressed. This comment referred to Fig. 4A and C, in which a control staining for the damage is missing, and the new Fig. S11B does not provide this internal control.

2) Similarly, the new Fig. S9 cannot be properly interpreted without a cell cycle marker. CtIP does not recruit in G1 phase cells, but how do the authors know that the single cell they show in the lower panel is not in G1?

3) Figure 3D: What do the asterisks indicate? Do the authors want to claim statistically significant differences between the conditions indicated from 2 experiments?

4) The authors show that alpha-amanitin lowers PAR levels at DNA damage sites (S11B) and speculate that this could be due to degradation of PARylated proteins. However, PARP1 itself is known as the main acceptor of PAR, and PARP1 is recruited normally under these conditions (S11A). It is therefore not clear whether the impaired recruitment is due to RNA Pol II degradation, deregulation of some other protein, or due to reduced PAR formation. To be more convincing, the authors could use low levels of PARP inhibitors to reduce PAR levels to a similar degree as with alpha-amanitin and show that this does not influence NELF-E recruitment.

5) The newly identified PAR-binding motif is a nice addition. It would have been even better to generate a mutant lacking this motif and test it for DNA damage recruitment. Without such data, one cannot exclude that additional sequence motifs contribute to the recruitment and the authors should discuss this more carefully. For instance, the PAR binding could well depend on electrostatic interactions mediated by multiple positively charged amino acids at the N-terminus of NELF-E, as already discussed for other PAR binders (e.g. in Nucleic Acids Res. 2016 Feb 18;44(3):993-1006).

6) With the newly added size markers it is now clear that the PAR signal in 5B corresponds to NELF-E. It is surprising, however, that the authors detect PARylation in the absence of DNA damage. Can they rule out that PAR formation is induced during the cell lysis (e.g. during DNA shearing)? Were lysis and pull-down done in the presence of PARP inhibitors? How would the PAR signal in the inputs look (left panel)? Moreover, it would be helpful to include the recovery time after IR for this figure.

7) Several typos and inconsistencies should be corrected, e.g. "depenedent" in the abstract; "GFP-TARP" on page 32; "beta-actin" vs. "b-actin"; was the ATM inhibitor used at 5 or 10 microM?

Referee #3:

The authors have responded satisfactorily to the reviewers' comments and now provide a more complete mechanistic study. In particular, they further exploit the Cas9-based system for analyzing transcription repression at DSBs and strengthen the role of NELF-E in this context. They further analyze the cross-talks between NELF-E, ATM and PAR signaling. They also provide evidence that NELF-E contributes to DSB repair. The revised manuscript is thus much improved and includes a number of previously missing controls.

A few issues still need to be attended to before this manuscript can be accepted for publication:

1- The authors have included additional experiments (Fig. 7) supporting the relevance of NELF-E-mediated transcriptional repression for maintaining genome integrity. However, these results should be interpreted with caution as one cannot exclude the possibility that NELF-E indirectly affects DSB repair by regulating the expression levels of DSB repair factors. This point should be discussed. Noteworthy, IR sensitivity curves (Fig. 7B) are usually plotted on a log scale and considering the small differences between siNELF-E and control cells, it would be important to run a statistical test to assess whether these differences are significant. Furthermore, the DSB repair assays (Fig. 7D, F) should be better controlled by using siRNAs against HR and NHEJ factors instead of an ATM inhibitor in both assays.
The authors should mention in the method section that ISce-I is tagged with mCherry in U2OS-HR-ind cells so that the reader can understand why they score GFP positive cells out of total red cells. Given the modest effect of NELF-E depletion on DSB repair, the following statement should be rephrased: "NELF-E depletion interrupted DSB repair" (p.14)

2- In their discussion, the authors speculate that the alleviation of MS2 repression at DSB sites

following PARP inhibition could be mediated via ATM kinase activity (p.14). They could also discuss the alternative possibility that the recruitment of silencing factors such as NELF-E, NuRD and PcG to DSBs is impaired upon PARP inhibition.

3- Introduction, p. 3: It would be more correct to state that the RNF8/168 ubiquitin ligases catalyze the ubiquitylation of histones H1, H2A and H2AX as some residues are mono- and not poly-ubiquitylated.

2nd Revision - authors' response 25 January 2017

Thank you for your letter dated January 9th 2017, inviting us to resubmit a revised version of our manuscript after addressing the rest of the reviewers' comments. With this letter comes the revised paper and point-to-point response to the reviewers' questions. As you will see, we fully addressed all the issues that were raised by you and by reviewer 2 and 3.

For your convenience, we summarize below the key revisions of the manuscript:

1. We performed new series of experiments to test the effect of PARP and ATM inhibitors on the expression of MS2 gene in mock and NELF-E depleted cells (Figure EV2B and C).
2. We repeated the HDR and NHEJ assays using cells depleted of Rad51 and Ku80 as positive controls (Figure 7).
3. We validated the efficacy of ATM inhibitor by looking at CtIP recruitment in cells at S/G2 using Geminin as a cell cycle marker (Figure S9B).
4. We included one more repeat of the ChIP experiment showing the enrichment of NELF-E at DSBs induced upstream A20 gene (Figure 3D).
5. We did one more repeat of the experiment described in Figure 4A showing the effect of PARPi on NELF-E recruitment to laser microirradiated sites.
6. We included a control showing that the amount of DNA damage induced in mock and PARP deficient cells is comparable (Appendix Figure S10).
7. We added scale bars to all the figures and indicated the number of the biological repeats and completed the checklist.
8. In the revised manuscript figures 3 and 4 run over one page and consequently we generated two new EV figures.
9. We moved the 3 tables to the material and methods section.

We are pleased that you offered us an invitation to revise our work. Also, we truly appreciate you assigning such qualified reviewers to our manuscript. Their efforts and insights were a tremendous help to us during this revision. We hope that these revisions improve the paper such that you and the reviewers now deem it worthy of publication in EMBO Reports. We look forward to hearing from you in due course.

Point by point response:

We are grateful for the three reviewers once again for their thorough evaluation and their constructive criticisms of our manuscript. We thank them also for taking the time and energy to help us improving the paper.

Referee 1:

The authors have been very responsive to the prior critiques. The manuscript addresses novel mechanisms of DSB silencing by identifying NELF as a factor in this process and implicates NELF in DSB repair and resistance to IR. It represents a thorough story that should be of broad interest to the genome integrity community and in this Reviewer's opinion, is worthy of publication in EMBO Reports.

We are quite appreciative of your comments and suggestions, and we are delighted that you found our manuscript suitable for publication in EMBO Reports.

Referee 2:

In their revised manuscript "NELF-E is recruited to DNA double-strand break sites to promote transcription repression and repair" Awwad et al. provide additional experiments supporting a role of NELF-E in DNA damage-induced transcriptional repression and DSB repair. While some of the new additions indeed strengthen the overall conclusions and significantly improved the manuscript, there are still several points, which call for a more careful interpretation, or would benefit from additional experimental conditions and controls. For instance, the effect of ATM and PARP inhibition on repression is now included, but the authors do not go all the way to close the circle, although in principle this should be fairly easy with the system they are using and would significantly help to clarify how NELF-E recruitment, PARylation, ATM signaling and repression are connected.

Specific comments:

1) Using the MS2 system the authors now show the effect of NELF-E knockdown and ATM inhibition (Fig. S2) and of PARP inhibition (Fig. 4H) and speculate about how these things are connected. Why did the authors not combine these treatments in one experiment and test for epistasis between PARPi and ATMi and between PARPi and NELF-E knockdown?

*Thank you for this suggestion. We performed the suggested experiments as requested by the reviewer. PARP inhibition in NELF-E depleted cells increases the percentage of cells showing expression of MS2, suggesting that beside NELF-E, other PARP-regulated factors might be implicated in pausing transcription after DSB induction (**New Fig EV2C).** The percentage of cells that show MS2 in the presence of DSB in cells treated with ATM inhibitor is comparable to the percentage of cells treated with both ATM and PARP inhibitor. Altogether, these observations further support the notion that PARP and ATM may act in the same pathway to ensure transient transcription silencing after DSB induction (**New Fig EV2C**).*

2) In their rebuttal the authors use Fig. 3E-G as evidence that further strengthens the role of NELF-E in DNA damage-induced transcription repression. However, these data do not show a transcriptional repression of the A20 gene upon break induction. Rather, Cas9 leads to increased expression and it is therefore unclear how these results relate to the rest of the manuscript.

*We partially agree with this comment. Indeed, Fig. 3E-G show that the expression of A20 gene increases after DSB induction. But as we clearly pointed out in the revised manuscript, upon DSB induction using CRISPR-Cas9 system, NELF-E depleted cells (**New Fig EV1C)** exhibit **~15 fold increase** in the expression levels of A20 gene compared to **~4 fold increase** in control cells, suggesting that NELF-E negatively regulates A20 expression at DSB sites (**New Fig EV1C)** and this finding is in line with the role of NELF-E in shutting down transcription after DNA damage.*
*We agree with the reviewer that introducing DSBs upstream A20 gene, even in the presence of NELF-E, facilitates A20 gene expression (**New Fig EV1C)**. As indicated in the revised manuscript, this increase is likely because DSBs could trigger DNA unwinding and create a permissive environment for transcription as previously reported in a Cell paper showing that DSB could trigger expression of a subset of early-response genes in neuron [1].*

Finally, we'd like to draw the reviewer's attention that there is a substantial difference in testing the expression of A20 gene and MS2 gene after DSB induction. While in the MS2 system we focus on nascent transcript of the MS2 gene (reflected by green spot), in the A20 we measure the overall A20 transcript in a population of cells at 12 hours interval after introducing Cas9, as explained in the revised manuscript.

Additional points:

1) My comment to include gammaH2AX staining controls for the laser recruitment experiments was not properly addressed. This comment referred to Fig. 4A and C, in which a control staining for the damage is missing, and the new Fig. S11B does not provide this internal control.

*We agree with the reviewer and thus repeated the experiments with the relevant controls. We observed that PARP inhibition and PARP1 knockdown show no noticeable changes in the intensity of γH2AX staining at laser-microirradiated sites (**New appendix Figure S10**).*

2) Similarly, the new Fig. S9 cannot be properly interpreted without a cell cycle marker. CtIP does not recruit in G1 phase cells, but how do the authors know that the single cell they show in the lower panel is not in G1?
*This is another good point. To monitor cell cycle phase we expressed an EGFP fusion of the N-terminal domain of Geminin, which was previously shown to faithfully mark S/G2 and M phases [2]. In line with previous report [3], ATM inhibition abolished CtIP accumulation at laser-microirradiated sites during S and G2 cell-cycle stages (**New appendix Figure S9B**)*

3) Figure 3D: What do the asterisks indicate? Do the authors want to claim statistically significant differences between the conditions indicated from 2 experiments?

We added a third repeat of the ChIP experiment and recalculated the SD and the P-value from three independent biological repeats.

4) The authors show that alpha-amanitin lowers PAR levels at DNA damage sites (S11B) and speculate that this could be due to degradation of PARylated proteins. However, PARP1 itself is known as the main acceptor of PAR, and PARP1 is recruited normally under these conditions (S11A). It is therefore not clear whether the impaired recruitment is due to RNA Pol II degradation, deregulation of some other protein, or due to reduced PAR formation. To be more convincing, the authors could use low levels of PARP inhibitors to reduce PAR levels to a similar degree as with alpha-amanitin and show that this does not influence NELF-E recruitment.

We disagree with this comment. The representative field of cells in appendix figure S11B includes five alpha-amanitin treated cells (bottom row) that show comparable intensity of PAR staining to the DMSO-treated cells (upper row). On the other hand, alpha-amanitin treatment abolished NELF-E recruitment in all tested cells (n=25). We concluded therefore that the defective recruitment of NELF-E following alpha-amanitin is due to RNA Pol II degradation rather than the mild reduction in the intensity of PAR signal in some of the cells.

5) The newly identified PAR-binding motif is a nice addition. It would have been even better to generate a mutant lacking this motif and test it for DNA damage recruitment. Without such data, one cannot exclude that additional sequence motifs contribute to the recruitment and the authors should discuss this more carefully. For instance, the PAR binding could well depend on electrostatic interactions mediated by multiple positively charged amino acids at the N-terminus of NELF-E, as already discussed for other PAR binders (e.g. in Nucleic Acids Res. 2016 Feb 18;44(3):993-1006).

We agree with the reviewer's comment and therefore we will revise the manuscript indicating that we can't rule out a possibility that other sequences in the N-terminal of NELF-E may contribute to its PAR binding. The essence of our main conclusions in this work stands firm and solid despite this. Therefore, we are certain that repeating the whole set of the radioactive experiments using NELF-E mutant lacking the PAR-binding motif, although important, may prove redundant for this current study.

6) With the newly added size markers it is now clear that the PAR signal in 5B corresponds to NELF-E. It is surprising, however, that the authors detect PARylation in the absence of DNA damage. Can they rule out that PAR formation is induced during the cell lysis (e.g. during DNA shearing)? Were lysis and pull-down done in the presence of PARP inhibitors?

Absolutely, we used PARP inhibitors during the lysis of the cells as previously indicated (Khoury-Haddad et al., 2014) and therefore we believe that NELF-E is PARylated in the absence of DNA damage. We added this important information to the material and method of the revised manuscript. Moreover, the recent Science paper showed also that NELF-E is PARylated in undamaged cells using different approaches (Gibson et al. 2016)

How would the PAR signal in the inputs look (left panel)?

We had PAR immunoblot of the inputs using PAR antibody and included it in the revised manuscript (Revised Figure 5B).

Moreover, it would be helpful to include the recovery time after IR for this figure.

We added the recovery time to the figure legend.

7) Several typos and inconsistencies should be corrected, e.g. "depenedent" in the abstract; "GFP-TARP" on page 32; "beta-actin" vs. "b-actin"; was the ATM inhibitor used at 5 or 10 microM?

We used 10μM of ATM inhibitor and we corrected it in the revised manuscript.

Referee 3:

The authors have responded satisfactorily to the reviewers' comments and now provide a more complete mechanistic study. In particular, they further exploit the Cas9-based system for analyzing transcription repression at DSBs and strengthen the role of NELF-E in this context. They further analyze the cross-talks between NELF-E, ATM and PAR signaling. They also provide evidence that NELF-E contributes to DSB repair. The revised manuscript is thus much improved and includes a number of previously missing controls. A few issues still need to be attended to before this manuscript can be accepted for publication:

1- The authors have included additional experiments (Fig. 7) supporting the relevance of NELF-E-mediated transcriptional repression for maintaining genome integrity. However, these results should be interpreted with caution as one cannot exclude the possibility that NELF-E indirectly affects DSB repair by regulating the expression levels of DSB repair factors. This point should be discussed.

We fully agree with the reviewer's comment and accordingly discussed this important point in the revised manuscript.

Noteworthy, IR sensitivity curves (Fig. 7B) are usually plotted on a log scale and considering the small differences between siNELF-E and control cells, it would be important to run a statistical test to assess whether these differences are significant.

*We performed these tests as requested (**see revised Figure 7B**).*

Furthermore, the DSB repair assays (Fig. 7D, F) should be better controlled by using siRNAs against HR and NHEJ factors instead of an ATM inhibitor in both assays.

Thank you for raising this point. We performed a third repeat of the HR and NHEJ assays using siRNA against Ku80 (NHEJ factor) and Rad51 (HR factor) (New Figure 7D and F).

The authors should mention in the method section that ISce-I is tagged with mCherry in U2OS-HR-ind cells so that the reader can understand why they score GFP positive cells out of total red cells.

We added this information to the revised manuscript.

Given the modest effect of NELF-E depletion on DSB repair, the following statement should be rephrased: "NELF-E depletion interrupted DSB repair" (p.14)

We revised this statement and now it appears as "NELF-E is required for fine-tuning of DSB repair."

2- In their discussion, the authors speculate that the alleviation of MS2 repression at DSB sites following PARP inhibition could be mediated via ATM kinase activity (p.14). They could also discuss the alternative possibility that the recruitment of silencing factors such as NELF-E, NuRD and PcG to DSBs is impaired upon PARP inhibition.

Thank you for raising this interesting possibility, which we discussed in the revised manuscript.

3- Introduction, p. 3: It would be more correct to state that the RNF8/168 ubiquitin ligases catalyze the ubiquitylation of histones H1, H2A and H2AX as some residues are mono- and not poly-ubiquitylated.

We corrected this sentence. Again, we appreciate all your insightful comments. We worked very hard to be responsive to them and hope that our revision meets with your approval.

3rd Editorial Decision 31 January 2017

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed report from referee 2 who was asked to assess it.
The referee only has 2 more minor comments that I would like you to address.

I noticed that for Figs 2B, 4C, EV1C, EV2C, and S2B where n=2 only the averages are shown without the single data points. It would be better to include the single data points of both experiments in the graphs together with the average, however, if you disagree, we can go ahead with the figures as they are now.

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

REFEREE REPORT

Referee #2:

The authors have adequately addressed the concerns and provide additional data, previously missing controls and repetitions of experiments, which overall strengthen the story. While it would have been nice to see a gammaH2AX/PAR co-staining in new Figure S10 as an internal control for the PARP inhibitor treatment and the PARP1 knockdown, and an additional EGFP-Geminin-negative cell in new Figure 9b as an internal control for lack of CtIP recruitment in G1, these are not decisive for the main conclusions of the manuscript. Altogether, the work represents a novel and thorough story, which should be of broad interest and which, in this reviewer's opinion, is worthy to be published in EMBO Reports.

The authors may want to consider the following two small modifications:

- As they are depicted, the asterisks indicating statistical significance in Fig. 3d still suggest that the values between P1 and P3 were compared. I am not sure if this is what the authors want to show. By convention, the squared bracket should indicate which two conditions are compared when testing for significance. It would further be good to state which statistical test was performed.

- The sentence on RNF8/RNF168 in the introduction has not been corrected as suggested in one of the previous reviewers' comments as it still does not account for mono-ubiquitylation. It would be more correct to rephrase this sentence accordingly.

3rd Revision - authors' response 07 February 2017

The authors submitted their revised manuscript with the following point by point response:

We thank the reviewer for the excellent revision and we are pleased that he/she found our work novel and suitable for publication in EMBO Reports. We have addressed his/her minor comments as indicated below.

Referee #2:

The authors have adequately addressed the concerns and provide additional data, previously missing controls and repetitions of experiments, which overall strengthen the story. While it would have been nice to see a gammaH2AX/PAR co-staining in new Figure S10 as an internal control for the

PARP inhibitor treatment and the PARP1 knockdown, and an additional EGFP-Geminin-negative cell in new Figure 9b as an internal control for lack of CtIP recruitment in G1, these are not decisive for the main conclusions of the manuscript. Altogether, the work represents a novel and thorough story, which should be of broad interest and which, in this reviewer's opinion, is worthy to be published in EMBO Reports.

The authors may want to consider the following two small modifications:

- As they are depicted, the asterisks indicating statistical significance in Fig. 3d still suggest that the values between P1 and P3 were compared. I am not sure if this is what the authors want to show. By convention, the squared bracket should indicate which two conditions are compared when testing for significance. It would further be good to state which statistical test was performed.

Response: *We agree with the reviewer and modified Figure 3D accordingly. Also, we explained this point in the figure legend as follow: "asterisks depict statistically significant differences in the values of P1, P2 and P3 when compared to the corresponding values of the control samples that were not treated with TNFα and Cas9". In addition, we indicated the statistical test that we used in this case which is a student's t-test.*

- The sentence on RNF8/RNF168 in the introduction has not been corrected as suggested in one of the previous reviewers' comments as it still does not account for monoubiquitylation. It would be more correct to rephrase this sentence accordingly.

We have rephrased the sentence as follows:

Previous version: *"Several targets of ATM kinase such as, the ubiquitin ligases RNF8/168 that catalyze the formation of K63-linked ubiquitination chain in histones H1, H2A and H2AX [13-16], PBAF complex, PcG proteins, and the transcription elongation factor ENL, were recently shown to participate in blocking transcription after DSB induction [11, 17, 18]."*

Rephrased version: *"Several targets of ATM kinase such as, the ubiquitin ligases RNF8/168 that monoubiquitinate H2A-K119 and catalyze the formation of K63-linked polyubiquitination chain of histones H1, H2A and H2AX [13-16], PBAF complex, PcG proteins, and the transcription elongation factor ENL, were recently shown to participate in blocking transcription after DSB induction [11, 17, 18]."*

4th Editorial Decision 08 February 2017

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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Corresponding Author Name:	Nabieh Ayoub
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 justified
- 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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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?	Biological repeats were performed at least twice and the numbers of cells were above 10 in each expirement.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We don't have animal studies.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For flow cytometry analysis presented in Fig 7D and F doublets were eleminated.
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.	Cells were randomely selected for imaging and analysis and blind tests were performed to minimize the effects of subjecteve bias.
For animal studies, include a statement about randomization even if no randomization was used.	We don't have animal studies.
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.	Yes, some tests were blindly perfomred.
4.b. For animal studies, include a statement about blinding even if no blinding was done	We don't have animal studies.
5. For every figure, are statistical tests justified as appropriate?	Yes, we mentioned this information in the figure legends.
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?	No
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 antibodies used in this study are described in Table 1
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The cell lines are from ATCC and are free of mycoplasma .

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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.	We do not have animal studies.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We do not have animal studies.
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.	We do not have animal studies.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	We do not have human subjects.
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.	We do not have human subjects.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	We do not have human subjects.

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	We do not have human subjects.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	We do not have human subjects.
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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.	We do not have human subjects.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	NA
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).	NA
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G- Dual use research of concern

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