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HUWE1 interacts with PCNA to alleviate replication stress

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

1st Editorial Decision 04 December 2016

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

As you will see, the referees agree that the findings are interesting and novel and that the technical quality of the study is high. However, referee 1 also points out that the manuscript reports a list of interesting observations that have not been linked at the mechanistic or functional level. This is an important concern, shared by referee 3, as mentioned in her/his cross-comments, which must be addressed. Referees 2 and 3 further ask for additional and better quality data, and their concerns need to be addressed too.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Please note that this is a borderline revision, and that the revised manuscript will only be sent back to the referees if their concerns have been adequately addressed.

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.

Revised manuscripts should be submitted within three months of a request for revision; they will

otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. Given the 7 main figures, we would publish your manuscript as a full article, with no size limitations. Supplementary data are now called expanded view (EV) figures and tables at EMBO press, which need to be uploaded as individual files. The legends for EV figures need to be added at the end of the main manuscript file.

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

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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

REFEREE REPORTS

Referee #1:

Manuscript by Choe et al. describes a new role for the HUWE1 HECT-type ubiquitin ligase in maintaining genomic stability through the prevention of replication stress. The authors use HUWE1 loss-of-function studies to correlate different functions for HUWE1 in the maintenance of replication fork speed and in preventing spontaneous DNA breaks. HUWEI interacts with the replication factor PCNA at stalled replication forks via its PIP box and that this interaction is required for replication fork restart. Furthermore, they show evidence that HUWE1 promotes H2AX ubiquitination, likely at stalled forks. Collectively, this work identifies HUWE1 as a new regulator of the mammalian replication stress response. The authors use cutting-edge techniques and sound experimental approaches, such as DNA fiber analysis and cell imaging, to support their claims. The experiments, in general, were performed with high quality. However, I have some reservations on the relevance of the overall study due to the somewhat "disjointedness" in the conclusions of their findings. The manuscript feels more like a laundry list of well-executed experiments that describes multiple functions of HUWE1, but doesn't quite fit together how each of these puzzle pieces relate to one another in describing the role of HUWE1 in the replication stress response. For example, the authors show that loss of HUWE1 results in decreased H2AX phosphorylation (pS139) and ubiquitination (Ub-H2AX) events in the presence of high dose UV. If this is true, what then is the functional connection between Ub-H2AX and replication stress? Not all replication stress causes DNA breaks and not all DNA breaks is a result of replication stress. So, is HUWE1 only critical in promoting Ub-H2AX for replication fork-coupled DNA damage events? What happens during a condition when DNA breaks occur in the absence of DNA replication (G1 phase). Does HUWE1 still modulate Ub-H2AX? Is Ub-H2AX cell cycle regulated (only occurring in S-phase)? What about other ubiquitin E3 ligases that have been purported to ubiquitinate H2AX? Is there a way to distinguish between the functions of each of these ubiquitin ligases (cell cycle regulated vs. PCNA-dependent, vs. replication fork-dependent)? After reading the manuscript, I'm left wanting for more to understand what is the mechanistic role of HUWE1 in the replication stress response.

Minor Comments:

1) It is unclear how the RNA seq data in Figure 1B fits into the narrative of the story. The experiment was done in 293 cells, which isn't even a tumor cell from a specific tumor tissue. I don't understand the logic behind checking for genomic instability phenotype just because one sees dysregulation of cancer-related processes by RNA-seq. There is already quite a bit of literature on

HUWE1 in base excision repair and p53 response so the molecular connection with genomic instability and HUWE1 inhibition has already been demonstrated (Parsons et al, EMBO J, 2009).

2) Loss of HUWE1 increases DNA breaks (Comet assay) and reduces replication track length independent of exogenous DNA damage (Figure 1C and F). How do these phenotypes link to the function of HUWE1? Is the DNA break phenotype and the fork speed phenotype mechanistically linked?

3) Figure 2H X-axis labeling is off

4) Is the interaction of HUWE1 with PCNA enhanced with PCNA monoubiquitination? Both HU and UV damage upregulate PCNA monoubiquitination, which is critical for lesion bypass by translesion synthesis polymerases. Since HUWE1-deficient cells are sensitive to both HU and UV, it would be reasonable to check whether HUWE1 is regulated by PCNA monoubiquitination (binding or localization).

5) Is HUWE1 part of the replisome at stalled replication sites? (iPOND study)?

6) The effect on Ub-H2AX is quite small (Figure 7D,E and F) in the absence of endogenous HUWE1. Can the authors test whether other purported Ub E3 ligases that ubiquitinate H2AX can function together with HUWE1? In other words, does depletion of HUWE1 plus another ligase further reduce Ub-H2AX levels?

7) Why is there a need to use 250 J/m2 of UV damage (Figure 7)? This is a really high level. DNA double-strand breaks can be generated more directly in other ways, such as with camptothecin, bleomycin, or ionizing radiation. Using such a high dose will cause the cells to undergo an apoptotic program, if one's looking at survival responses, such as DNA repair, many cellular responses may already be shutting down (down regulation of transcription, translation, etc...).

8) Does controlling H2AX monoubiquitination affect the replication fork speed (track length over time)? This could be one way to link replication fork speed to changes in H2AX monoubiquitination. I think this is important for the authors to figure out.

Referee #2:

Review of Choe et al.

This submission reports an interesting and potentially significant observation pertinent to the cellular response to DNA damage. The paper provides data suggesting a potential involvement of HUWE1, a ubiquitin ligase, in the tolerance of lesions that damage DNA and perturb replication. This observation assigns a novel role for HUWE1 that was previously thought to inhibit DNA repair processes, and suggests that the interaction of HUWE1 with PCNA and possibly modification of histone H2AX promotes recovery from replication fork stalling under some circumstances. The strength of the paper lies in the novelty and potential significance of the results and the well-supported data demonstrating a physical interaction between HUWE1 with PCNA. However, many of the other observations reported in the paper should be better substantiated with higher quality data and carefully interpreted, as listed below.

Critique and suggestions

PCNA travels with replication forks as well as orchestrates DNA repair and bypass synthesis upon replication fork stalling. PCNA foci, therefore, can indicate stalled replication forks and locations of DNA repair as well as progressing forks. This fact should be discussed in the context of localization with HUWE1.

Information about cell viability and cell cycle distribution for HUWE1 depleted cells is shown in Figure 1 and 2, whereas the essential controls demonstrating that the cell cycle phenotype is reversed when HUWE1 is re-expressed is shown in Figure 7. This was done in order to compare the "rescued" phenotype with the phenotype of the mutant HUWE1, but it would be good to consider

including the control upfront. The mutant phenotype could still be discussed separately.

Related to the above: was the DNA breakage phenotype "rescued" by the expression of the full-length HUWE1?

Cell cycle analyses reveal crucial phenotypes essential for understanding the paper. The histograms reporting those changes, however, tend to focus on the level of increase in S-phase arrested cells (e.g. Figure 1E). It is unclear whether there were other changes (for example, if the overall % of cells in S-phase was altered). It would be better to include images of FACS analyses along with the histograms, and show the fraction of cells in particular cell cycle stages instead of the "fold increase". The fraction of cells in each cell cycle should also be informative.

Related to the above: Figure 2C shows a cell cycle flow analysis revealing a small group of cells with mid-to-late S-phase DNA content that do not incorporate BrdU (R2). There do not seem to be cells arrested with early-to-mid S-phase DNA content. Is this reproducible?

Overall, for all the statistical analyses, the paper should report the type of cells used to determine statistical significance, number of samples and p-values.

Fiber experiments: It is curious that UV did not shorten replication tracks in the control. Was this finding significant or was it only observed in the fiber shown in Figure 2J? To control for such instances, it would be good to show examples containing more than a single fiber, ideally with a counterstain evaluating if the shorter label (e.g. IdU in figure 2J) is located at the end of the fiber, indicating a DNA break at the end of the replication track.

PCNA foci are almost invisible in many of the images (e.g. Figure 6C, and to a lesser extent Figure 3). Better quality confocal images should be included. Also, how was "% PCNA colocalized" calculated - overall intensity or number of foci? If the latter, how were cells with no foci (or pan-staining) quantified?

Minor comments

Gene expression analyses were performed in 293T cells. Those cells harbor SV40 T antigen that inactivates many of the cell cycle regulatory pathways that normally prevent tumorigenesis. Referring to those cells as "non-malignant" is somewhat misleading and should be avoided.

Figure 2F: why was a different siRNA used?

Figures 2H and 2I: were the differences between the two siRNA statistically significant?

Figure 4D: It would be good to replace the image of PCNA co-IP with HUWE1 with an image with a lower background. The current image seems to indicate that the siHUWE1 contains some PCNA immunoprecipitated by anti-HUWE1.

Referee #3:

This is an interesting manuscript providing evidence for a novel role for the HUWE1 ubiquitin ligase in the response to DNA replication stress. While the data presented in support of this conclusion are largely convincing, some clarification and additional data are necessary.

Specific comments:

1) There were no page numbers on my copy of the manuscript. They would have been helpful for highlighting specific points in the text. Instead this will have to be done via Figure numbers.

2) Figure 1: C) The authors report a significant increase in the level of strand breaks in HUWE1 -/- cells using the alkaline comet assay. It would have been interesting to see if there was any difference in neutral comet assays as this would have revealed the level of double strand breaks that presumably arise by fork collapse. E) The authors present the fold increase in S-phase arrested cells

in HUWE-/- cultures. However these seem to constitute a very low proportion of cells in the populations. It would have been useful to know the fraction (%) of these cells in the population and the fraction (%) of cells in S-phase given that HUWE1 knockdown by siRNA reduces the fraction of cells in S-phase (see figure 2).

3. Figure 2: A) What is the point of the asterisk in the Western. Is this a non specific band? C) Again the proportion of cells arrested in in S-phase is low and it should be presented as % cells in E. There is also a significant decrease in the fraction of cells in S-phase. Why is this not seen in the knockout cells? H) This is an unusually high level of UV light to reduce cell survival. No details of these experiments is provided. I) Only one of the siRNAs sensitizes the HUWE knockdowns to HU while the other does not. This could be interpreted as an off-target effect. It is notable that the sensitivity of the HUWE1 knockouts is NOT presented. The authors must provide more consistent data in support of their claim that the loss of HUWE1 confers HU sensitivity.

Figure 3. C) Colocalizations for PCNA and HUWE after HU treatment are not presented. It would be useful as colocalization after HU treatment is presented in the graphs in D.

Figure 4. While the immunoprecipitation experiments are largely consistent with an interaction with PCNA the interactions appear weak. Also molecular weight markers should be presented.

Figure 6. C) The colocalizations presented here are not very convincing. There seem to be a lot of red and green foci but not many yellow ones. Also images for HU treated cells are not presented.

Figure 7. As in earlier figures only the fold increase in S-phase arrested cells is presented, it would be useful to see the % of cells arrested in S-phase. Additionally % of cells in S-phase should be presented.

Cross-comments from Referee #3:

I agree with the comments of reviewer 1. The manuscript is a bit frustrating in that there is a novel and interesting finding and that many of the experiments are done to a high standard using "cutting edge" techniques. However, one is left with the feeling that the linkage between the phenotypes reported is not clear. I felt that there were a number of gaps and inconsistencies with the work reported. If these were addressed, it might go a little way to resolve some of the limitations. Nevertheless I think all the reviewers agree that this is a novel function for HUWE1 that will prove of interest to investigators in this field. I think it is likely that the reported findings will stimulate work addressing important question of how this novel function relates to other ubiquitination pathways responding to replication stress or DNA damage. Consequently I feel that the findings are appropriate for publication in EMBO reports, providing that the authors address the gaps and inconsistencies and provide some hypothesis that potentially links some of the phenotypes.

1st Revision - authors' response 07 March 2016

Thank you for allowing us to resubmit the revised version of our manuscript "**HUWE1 interacts with PCNA to alleviate replication stress"** We were very pleased that the three reviewers found our findings potentially very important. We are now providing a significantly revised manuscript, including 12 new figure panels and 9 new supplementary figures, to address the reviewers' concerns. In particular, we would like to highlight the following new features of the revised manuscript:

- To address the major comment that our manuscript is not mechanistic enough for publication in *EMBO Reports*, we now present evidence that HUWE1-knockout cells show reduced engagement of DNA repair enzymes BRCA1 and BRCA2 in response to replication stress. This finding mechanistically connects the DNA damage phenotypes with the γH2AX defects we showed in the original manuscript. Overall, our study shows that HUWE1 promotes H2AX ubiquitination at stalled replication forks, resulting in recruitment of repair proteins including BRCA1 and BRCA2, and thus promoting DNA repair and fork restart.

- We have extended our replication stress studies to include HU and Aphidicolin treatments. Moreover, we show by ChIP that γH2AX binding to the common fragile site FRA3B is reduced in HUWE1-deficient cells. Since common fragile sites are known as sites of breakage under replication stress, which are counteracted by γH2AX-induced signaling, these findings provide a mechanistic example of the impact of HUWE1 on the replication stress response.
- We used the iPOND assay to confirm the localization of HUWE1 to replication forks, thus corroborating the PCNA interaction and co-localization data from the original manuscript.

Moreover, as indicated in your letter, we included the number "n" for the number of experiments performed for each graph (either in the graph or in the figure legend, as appropriate). In each figure legend, we now provide the exact p-value, and describe the bars and error bars. Moreover, we added a separate section under Methods explaining the statistical analyses (since the statistical test used was the same for all experiments). Finally, we included scale bars in all micrographs. Since our manuscript contains 7 large figures, we included all other figures as Supplementary Material, rather than Expanded View. However, we will be happy to add Supplementary figures as Expanded View, if you or the reviewers indicate so.

Referee #1:

Manuscript by Choe et al. describes a new role for the HUWE1 HECT-type ubiquitin ligase in maintaining genomic stability through the prevention of replication stress. The authors use HUWE1 loss-of-function studies to correlate different functions for HUWE1 in the maintenance of replication fork speed and in preventing spontaneous DNA breaks. HUWEI interacts with the replication factor PCNA at stalled replication forks via its PIP box and that this interaction is required for replication fork restart. Furthermore, they show evidence that HUWE1 promotes H2AX ubiquitination, likely at stalled forks. Collectively, this work identifies HUWE1 as a new regulator of the mammalian replication stress response. The authors use cutting-edge techniques and sound experimental approaches, such as DNAfiber analysis and cell imaging, to support their claims. The experiments, in general, were performed with high quality. However, I have some reservations on the relevance of the overall study due to the somewhat "disjointedness" in the conclusions of their findings. The manuscript feels more like a laundry list of well-executed experiments that describes multiple functions of HUWE1, but doesn't quite fit together how each of these puzzle pieces relate to one another in describing the role of HUWE1 in the replication stress response. For example, the authors show that loss of HUWE1 results in decreasedH2AX phosphorylation (pS139) and ubiquitination (Ub-H2AX) events in the presence of high dose UV. If this is true, what then is the functional connection between Ub-H2AX and replication stress? Not all replication stress causes DNA breaks and not all DNA breaks is a result of replication stress. So, is HUWE1 only critical in promoting Ub-H2AX for replication fork-coupled DNA damage events? What happens during a condition when DNA breaks occur in the absence of DNA replication (G1 phase). DoesHUWE1 still modulate Ub-H2AX? Is Ub-H2AX cell cycle regulated (only occurring in S-phase)? What about other ubiquitin E3 ligases that have been purported to ubiquitinate H2AX? Is there a way to distinguish between the functions of each of these ubiquitin ligases (cell cycle regulated vs. PCNA-dependent, vs. replication fork-dependent)? After reading the manuscript, I'm left wanting for more to understand what is the mechanistic role of HUWE1 in the replication stress response.

We are happy that the reviewer found our manuscript of "high quality", using "cutting edge techniques and sound experimental approaches". The reviewer points out that we do not present a mechanistic picture of the role of HUWE1 in H2AX regulation, including its functions outside S-phase and its connections to other E3 ligases for H2AX. We addressed the reviewer's concerns in the revised manuscript, as detailed below.

-We now present clear evidence, in the new Fig. 7A, B, that HUWE1 promotes H2AXubiquitination in response to replication stress induced by treatment with HU, Aphidicolin, or low doses UV. (While loss of HUWE1 does reduce γH2AX ubiquitination also at high doses of UV, we do not have a good understanding of the damage that induces γH2AX ubiquitination under those conditions.) As requested by the reviewer, we investigated H2AX ubiquitination in G1. We found

that, in response to bleomycin treatment (which induces double strand breaks), H2AX ubiquitination is not decreased in HUWE1-knockout cells (if anything, ubiquitination is increased -new Suppl. Fig. S10) –pointing to a replication stress-specific function for HUWE1. However, we cannot exclude a role for HUWE1 inubiquitinating H2AX under other damage conditions than replication stress. Interestingly, a recent paper published during this revision (Atsumi et al, Cell Reports 2015 PMID: 26711340) described a role forHUWE1 in suppressing γH2AX formation in response to Ionizing radiation (however that seemed to be caused by a multi-ubiquitination event inducing H2AX degradation).

-As requested by the reviewer, we have investigated other H2AX ubiquitin ligases (RNF168 and the RNF2/BMI1 complex) attempting to distinguish their functions. We found that knockdown of those ligases also reduced H2AX ubiquitination, both under normal conditions (new Suppl. Fig. S11) and upon induction of replication stress (new Fig. 7B, 7F). Co-depletion of HUWE1 and other ligases resulted in additive reduction in H2AX ubiquitination (new Suppl. Fig. S11). Furthermore, using chromatin immunoprecipitation, we now show (new Fig. 7G) that knockdown of HUWE1 or other H2AX ubiquitin ligases reduces the binding of γH2AX to the common fragile site FRA3B in aphidicolin-treated cells. This binding was originally described by Lu et al, *Mol. Cancer*. 2013, PMID: 23601052. Thus, HUWE1, RNF2/BMI1, and RNF168 are all required for efficient H2AX activation during replication stress. We do not believe that this reduces in any way the importance of our findings. On the contrary, since little is known about the role of H2AX modifications in S-phase (as opposed to the well described roles duringG2 double strand break repair), our results provide important clues on the regulation of H2AX during replication stress.

-To further elaborate on the mechanism of HUWE1 during replication stress and address the reviewer's comment regarding the disjointedness of our original manuscript, we evaluated the recruitment of repair proteins to DNA upon induction of replication stress. We reasoned that reduced γH2AX should result in reduced DNA damage signaling, and thus impaired recruitment of repair proteins. Consistent with this, we found that chromatin recruitment of BRCA1 and BRCA2 was significantly reduced in HUWE1-knockdown cells (new Fig. 7H). This reduced recruitment explains the repair defect of HUWE1-knockout cells (increased DNA breaks, hypersensitivity to replication stress), and thus ties in the seemingly disparate observations in our original manuscript. Indeed, restart of stalled replication fork through recombination or other DNA repair mechanisms has been well documented –see for example citations PMID: 12415303; 23637285; 20188668; and 25907220. In particular, both BRCA1 and BRCA2have been previously shown to promote replication fork stability (measured as fork tract length in DNAfiber assays: Schlacher et al *Cell* 2011, PMID: 21565612; Pathania et al, *Nat. Commun. 2014*, PMID: 25400221). Our results thus indicate that, for replication stress, HUWE1 loss phenocopies BRCA1/2deficiency.

In conclusion, while the regulation of H2AX ubiquitination turns out to be complex, our data clearly shows that in response to replication stress (which is the focus of our current manuscript) HUWE1 monoubiquitinates H2AX and promotes its functional activation, resulting in recruitment of repair proteins and alleviation of replication stress.

Minor Comments:

1) It is unclear how the RNAseq data in Figure 1B fits into the narrative of the story. The experiment was done in 293 cells, which isn't even a tumor cell from a specific tumor tissue. I don't understand the logic behind checking for genomic instability phenotype just because one sees dysregulation of cancer-related processes by RNA-seq. There is already quite a bit of literature on HUWE1 in base excision repair andp53 response so the molecular connection with genomic instability and HUWE1 inhibition has already been demonstrated (Parsons et al, EMBO J, 2009).

We agree with the referee and have now removed the RNA-Seq data from the manuscript, since, as mentioned by the reviewer #1 above, it may not fit very well in the narrative of our story. (We will

be happy to include it as Supplementary material at the reviewers' or the editor's request.)

2) Loss of HUWE1 increases DNA breaks (Comet assay) and reduces replication track length independent of exogenous DNA damage (Figure 1C and F). How do these phenotypes link to the function of HUWE1? Is the DNA break phenotype and the fork speed phenotype mechanistically linked?

In the original manuscript, we showed that the replication tract length is rescued by re-expression of WT, but not PIP-mutant HUWE1. We now show that also the spontaneous breakage phenotype is corrected by WT, but not PIP-mutant HUWE1 (new Fig. 6G). These results strongly suggest that the phenotypes are mechanistically linked: failure to recruit HUWE1 to stalled replication forks results in reduced γH2AX signaling, deficient recruitment of repair proteins, and inability to repair/restart stalled replication forks resulting in accumulation of DNA breaks.

3) Figure 2H X-axis labeling is off

We apologize for this formatting error, and have now fixed it.

4) Is the interaction of HUWE1 with PCNA enhanced with PCNA monoubiquitination? Both HU and UV damage upregulate PCNA monoubiquitination, which is critical for lesion bypass by translesion synthesis polymerases. Since HUWE1-deficient cells are sensitive to both HU and UV, it would be reasonable to check whether HUWE1 is regulated by PCNA monoubiquitination (binding or localization).

We have recently obtained Rad18-knockout cells using CRISPR/Cas9 technology. Since Rad18 is the major ubiquitin ligase for PCNA, these cells show no detectable PCNA ubiquitination. The interaction between PCNA and HUWE1 is normal in these cells (new Fig. 4G), indicating that PCNA ubiquitination does not contribute to the HUWE1 interaction.

5) Is HUWE1 part of the replisome at stalled replication sites? (iPOND study)?

We thank the reviewer for suggesting this experiment. In fact, a recent iPOND coupled with mass-spec study by the Cortez lab identified HUWE1 as a putative replisome component (Dungrawala et al, *Mol. Cell* 2015, PMID: 26365379 -Supplementary Table S6). Indeed, we now show that HUWE1 can be cross linked to nascent DNA by iPOND in cycling cells, and even stronger in UV-treated cells (new Fig. 3A).

6) The effect on Ub-H2AX is quite small (Figure 7D,E and F) in the absence of endogenous HUWE1.Can the authors test whether other purported Ub E3 ligases that ubiquitinate H2AX can function together with HUWE1? In other words, does depletion of HUWE1 plus another ligase further reduce Ub-H2AXlevels?

As mentioned above in the answer to reviewer's major concern, we now show an additive effect when knocking down HUWE1 and the other ubiquitin ligases (new Suppl. Fig. S11).

7) Why is there a need to use 250 J/m2 of UV damage (Figure 7)? This is a really high level. DNA double-strand breaks can be generated more directly in other ways, such as with camptothecin, bleomycin, or ionizing radiation. Using such a high dose will cause the cells to undergo an apoptotic program, if one's looking at survival responses, such as DNA repair, many cellular responses may already be shutting down (downregulation of transcription, translation, etc...).

As mentioned above in the answer to reviewer's major concern, we now show the effect of HUWE1

onH2AX modification in response to replication stress (2mM HU, 40J/m^2 UV, 600nM aphidicolin) –new Fig. 7B, 7F.

8) Does controlling H2AX monoubiquitination affect the replication fork speed (track length over time)? This could be one way to link replication fork speed to changes in H2AX monoubiquitination. I think this is important for the authors to figure out.

We now show that knockdown of H2AX by siRNA (which results in an almost complete depletion of the ubiquitinated form of H2AX –new Suppl. Fig S12B), results in reduced replication fork length (new Suppl. Fig S12A), suggesting that H2AX modifications are essential for replication fork stability. Unfortunately at this time, we have no way to more specifically investigate the role of H2AX ubiquitination, as this would entail obtaining H2AX-knockout cell lines expressing the H2AX ubiquitination-deficient point mutant(s) -a very challenging and time-consuming experiment, which we believe is outside the scope of our current manuscript.

Referee #2:

Review of Choe et al.

This submission reports an interesting and potentially significant observation pertinent to the cellular response to DNA damage. The paper provides data suggesting a potential involvement of HUWE1, a ubiquitin ligase, in the tolerance of lesions that damage DNA and perturb replication. This observation assigns a novel role for HUWE1 that was previously thought to inhibit DNA repair processes, and suggests that the interaction of HUWE1 with PCNA and possibly modification of histone H2AX promotes recovery from replication fork stalling under some circumstances. The strength of the paper lies in the novelty and potential significance of the results and the well-supported data demonstrating a physical interaction between HUWE1 with PCNA. However, many of the other observations reported in the paper should be better substantiated with higher quality data and carefully interpreted, as listed below.
We are glad that the reviewer found our work "interesting and potentially significant", highlighting the "novelty" and "the well-supported data". Below is a point-by-point response to this reviewer's comments.

Critique and suggestions

PCNA travels with replication forks as well as orchestrates DNA repair and bypass synthesis upon replication fork stalling. PCNA foci, therefore, can indicate stalled replication forks and locations of DNA repair as well as progressing forks. This fact should be discussed in the context of localization with HUWE1.

We agree with the reviewer entirely. Our new data using the iPOND assay (new Fig. 3A, 6C) showing that HUWE1 is part of the replisome even in the absence of DNA damage treatment, and the fact that PCNA ubiquitination is not required for the HUWE1-PCNA interaction (new Fig. 4G), suggest thatHUWE1 may be part of the replication fork under normal conditions. In the revised manuscript, we tried to incorporate a more nuanced discussion on this topic (page 16 of the revised manuscript).

Information about cell viability and cell cycle distribution for HUWE1 depleted cells is shown in Figure 1and 2, whereas the essential controls demonstrating that the cell cycle phenotype is reversed when HUWE1 is re-expressed is shown in Figure 7. This was done in order to compare the "rescued" phenotype with the phenotype of the mutant HUWE1, but it would be good to consider including the control upfront. The mutant phenotype could still be discussed separately.

We understand the reviewer's point, but we feel that introducing the correction in figure 1 would break the flow of the manuscript, since important pieces of information (e.g. PCNA interaction) are only presented inlater figures. We think it would be confusing for the reader to be presented with mutants that were not described yet. We sincerely hope that the reviewer agrees with us.

Related to the above: was the DNA breakage phenotype "rescued" by the expression of the full-lengthHUWE1?

Indeed, we now show that the breakage can also be rescued by wild type, but not by the PCNA interaction-deficient variant (new Fig. 6G).

Cell cycle analyses reveal crucial phenotypes essential for understanding the paper. The histograms reporting those changes, however, tend to focus on the level of increase in S-phase arrested cells (e.g.

Figure 1E). It is unclear whether there were other changes (for example, if the overall % of cells in S-phase was altered). It would be better to include images of FACS analyses along with the histograms, and show the fraction of cells in particular cell cycle stages instead of the "fold increase". The fraction of cells in each cell cycle should also be informative.

We did show in the original manuscript that the percentage of S-phase cells is reduced (original Fig. 2D). We are now including FACS plots, and a more detailed quantification of cell cycle distribution, in the Supplementary material (new Suppl. Fig. S3, S4). The only reproducible difference is an altered distribution of mid-late S-phase cells (reduced in HUWE1-deficient cells) vs. S-phase arrested cells(increased in HUWE1-deficient cells).

Related to the above: Figure 2C shows a cell cycle flow analysis revealing a small group of cells with mid-to-late S-phase DNA content that do not incorporate BrdU (R2). There do not seem to be cells arrested with early-to-mid S-phase DNA content. Is this reproducible?

We see also arrest in early-S, but it does seem that most cells arrest in mid-late S. The relevance of this is unclear to us at this time.

Overall, for all the statistical analyses, the paper should report the type of cells used to determine statistical significance, number of samples and p-values.

We now present this information for each graph, in the figure legend and/or on the graph itself.

Fiber experiments: It is curious that UV did not shorten replication tracks in the control. Was this finding significant or was it only observed in the fiber shown in Figure 2J? To control for such instances, it would be good to show examples containing more than a single fiber, ideally with a counterstain evaluating if the shorter label (e.g. IdU in figure 2J) is located at the end of the fiber, indicating a DNA break at the end of the replication track.

In our data analysis, we are not quantifying the CldU fiber, since we cannot pinpoint where that particular replication fork fired (it could derive from a fork that fired during the 30mins of labeling, rather than before, thus would be shorter than expected). We are simply using the CldU staining to identify ongoing forks, and are measuring the IdU tracts that directly follow CldU tracts. The example presented in the original fig 2J (Suppl. Fig. S4D in the revised manuscript) is in fact not representative. When we compare the IdU tracts from cells treated or not with UV between the pulses, we find that indeed UV does shorten replication tracts (see for example new Suppl. Fig.

S12A). Unfortunately, in our experimental setup fibers are not stainable by DAPI or other common dyes, so we cannot identify fiber ends.

PCNA foci are almost invisible in many of the images (e.g. Figure 6C, and to a lesser extent Figure 3).Better quality confocal images should be included. Also, how was "% PCNA colocalized" calculated overall intensity or number of foci? If the latter, how were cells with no foci (or pan-staining) quantified?

For this analysis, we numbered the individual HUWE1 and PCNA foci, and quantified how many of them overlap. Cells that had less than five PCNA or HUWE1 foci were not considered in this calculation, since we wanted to present a measurement of the colocalization. The colocalization experiment is technically challenging, since the conditions needed for co-staining are not ideal for the individual antibodies. In the revised manuscript, we have included new immunofluorescence micrographs (new Fig. 3C, new Supplementary Fig. S5) which we hope the reviewer will find convincing. To further confirm that HUWE1 localizes to replication forks, we present in the revised manuscript new results obtained using the iPOND assay (new Fig. 3A, 6C). We sincerely hope that the reviewer agrees with us that overall, our data (PCNA interaction; iPOND; PCNA colocalization) strongly indicate that HUWE1 is part of the replication machinery in response to replication stress.

Minor comments

Gene expression analyses were performed in 293T cells. Those cells harbor SV40 T antigen that inactivates many of the cell cycle regulatory pathways that normally prevent tumorigenesis. Referring to those cells as "non-malignant" is somewhat misleading and should be avoided.

We agree with the reviewer. Since a similar point was raised by the Referee #1, who found that this data “does not fit in the narrative” of our story (minor comment #1), we decided to remove the RNA-seq data from the revised manuscript. (We will be happy to include it as Supplementary material at the reviewers’ or the editor’s request.)

Figure 2F: why was a different siRNA used?

The availability of the different siRNA oligonucleotides accounted for this. However we show that the5’UTR siRNA used here does knockdown HUWE1 to the same extent, and has similar phenotypes assiRNA#2 (new Suppl. Fig. S4), thus confirming the specificity of the knockdown.

Figures 2H and 2I: were the differences between the two siRNA statistically significant?

The differences between siRNA #1 and #2 are statistically significant, but we believe that this is due to the fact that #2 is more potent than #1 (Fig. 2A, 2B) and thus has a stronger phenotype (Fig 2G, 2H).

Figure 4D: It would be good to replace the image of PCNA co-IP with HUWE1 with an image with a lower background. The current image seems to indicate that the siHUWE1 contains some PCNA immunoprecipitated by anti-HUWE1.

Indeed, there is some PCNA immunoprecipitated by anti-HUWE1 in the siHUWE1 sample, but this is because there is some HUWE1 protein left in those cells (see HUWE1 blot of the IP lane). Unfortunately we do not have a better exposure for this experiment.

Referee #3:

This is an interesting manuscript providing evidence for a novel role for the HUWE1 ubiquitin ligase in the response to DNA replication stress. While the data presented in support of this conclusion are largely convincing, some clarification and additional data are necessary.

We are happy that the referee found our manuscript "interesting", presenting "largely convincing" data. Below is a point-by-point response to the comments of referee #3.

Specific comments:

1) There were no page numbers on my copy of the manuscript. They would have been helpful for highlighting specific points in the text. Instead this will have to be done via Figure numbers.

We apologize for this. We are now including page numbers in the revised manuscript.

2) Figure 1: C) The authors report a significant increase in the level of strand breaks in HUWE1 -/- cells using the alkaline comet assay. It would have been interesting to see if there was any difference in neutral comet assays as this would have revealed the level of double strand breaks that presumably arise by fork collapse.

We performed the requested experiments and found no increase in the neutral comet assay in untreated cells (new Suppl. Fig. S2). This indicates that the majority of breaks observed in HUWE1-knockout cells are in fact not double-strand breaks, but single strand breaks and other types of damages. Since this is a hallmark of replication stress, this data represents additional evidence for a specific role of HUWE1 in suppressing replication stress.

E) The authors present the fold increase in S-phase arrested cells in HUWE-/-cultures. However these seem to constitute a very low proportion of cells in the populations. It would have been useful to know the fraction (%) of these cells in the population and the fraction (%) of cells in S-phase given that HUWE1 knockdown by siRNA reduces the fraction of cells in S-phase (see figure 2).
We now present these results in Supplementary material (new Suppl. Fig. S3). –see also answer to Reviewer #2 above. While they represent indeed a relatively small proportion of cells, they do indicate that replication arrest does occur. We would like to point out that these cells do grow and proliferate, sowe would not expect a strong replication arrest phenotype under standard growth conditions.

3. Figure 2: A) What is the point of the asterisk in the Western. Is this a non specific band?

That was indeed a cross-reactive band; we cropped it out in the respective figure of the revised manuscript, to avoid any confusion.

C) Again the proportion of cells arrested in in S-phase is low and it should be presented as % cells in

We now present these results in Supplementary material (new Suppl. Fig. S4).

E. There is also a significant decrease in the fraction of cells in S-phase. Why is this not seen in the knockout cells?

This is, in fact, seen also in the knockout cells –see new Suppl. Fig. S3.

H) This is an unusually high level of UV light to reduce cell survival. No details of these experiments is provided.

We apologize for this, but the X-axis label was wrong in the original manuscript, because of a formatting error. We have now corrected this. The cell line used for this clonogenic experiment is 8988T. These cells are slightly more resistant to damage compared to HeLa cells for example, but overall the UV sensitivity is in the same general as that previously published for other cell lines in similar clonogenic experiments.

I) Only one of the siRNAs sensitizes the HUWE knockdowns to HU while the other does not. This could be interpreted as an off-target effect. It is notable that the sensitivity of the HUWE1 knockouts is NOT presented. The authors must provide more consistent data in support of their claim that the loss ofHUWE1 confers HU sensitivity.

The HU sensitivity of the HUWE1-knockout cells was in fact presented in Fig. 7A of the original manuscript (Fig. 6D of the revised manuscript). This figure shows that loss of HUWE1 results in HU sensitivity, which can be corrected by expression of wild type, but not mutant HUWE1. Regarding the siRNA experiment in Fig. 2I of the original manuscript (Fig. 2H in the revised manuscript), the different phenotypes of the two siRNAs reflect their different efficacies in knocking down HUWE1: siRNA#2 gives a stronger knockdown (see Fig. 2A, 2B) and thus has a stronger phenotype.

Figure 3. C) Colocalizations for PCNA and HUWE after HU treatment are not presented. It would be useful as colocalization after HU treatment is presented in the graphs in D.

We now include this data in the new Suppl. Fig. S5.

Figure 4. While the immunoprecipitation experiments are largely consistent with an interaction with PCNA the interactions appear weak. Also molecular weight markers should be presented.

While the interaction may be indeed weak, this may also reflect experimental limitations. In general, the stringent buffer conditions required to remove PCNA from chromatin may result in loss of some of the interaction partners. As requested, we have added molecular weight markers to representative figures.

Figure 6. C) The colocalizations presented here are not very convincing. There seem to be a lot of red and green foci but not many yellow ones. Also images for HU treated cells are not presented.

The colocalization experiment is technically challenging (see also our reply to the comment of Referee #2regarding PCNA foci –above, bottom of page 6 of this letter). In the revised manuscript, we have included new immunofluorescence micrographs (new Fig. 3C, new Supplementary Fig. S5) which we hope the reviewer will find convincing. To further confirm that HUWE1 localizes to replication forks, we present in the revised manuscript new results obtained using the iPOND assay (new Fig. 6C). We find that the PIP mutant does not localize correctly to nascent DNA, confirming the immunofluorescence data. We sincerely hope that the reviewer agrees with us that overall, our data (PCNA interaction; iPOND; PCNA colocalization) strongly indicate that HUWE1 is part of the replication machinery in response to replication stress, and this depends on PCNA interaction.

Figure 7. As in earlier figures only the fold increase in S-phase arrested cells is presented, it would be useful to see the % of cells arrested in S-phase. Additionally % of cells in S-phase should be presented.

We present this data now in the new Suppl. Fig. S7.

Cross-comments from Referee #3:

I agree with the comments of reviewer 1. The manuscript is a bit frustrating in that there is a novel and interesting finding and that many of the experiments are done to a high standard using "cutting edge" techniques. However, one is left with the feeling that the linkage between the phenotypes reported is not clear. I felt that there were a number of gaps and inconsistencies with the work reported. If these we readdressed, it might go a little way to resolve some of the limitations. Nevertheless I think all the reviewers agree that this is a novel function for HUWE1 that will prove of interest to investigators in this field. I think it is likely that the reported findings will stimulate work addressing important question of how this novel function relates to other ubiquitination pathways responding to replication stress or DNA damage. Consequently I feel that the findings are appropriate for publication in EMBO reports, providing that the authors address the gaps and inconsistencies and provide some hypothesis that potentially links some of the phenotypes.

We thank the reviewer for the cross-comments. As indicated above in the letter to the editor and in the response to the main comment of reviewer 1, we believe our revised manuscript addresses these concerns by showing that HUWE1 promotes H2AX and γH2AX ubiquitination at stalled replication forks, resulting in recruitment of repair proteins including BRCA1 and BRCA2, and thus promoting DNA repair and fork restart.

2nd Editorial Decision 29 March 2016

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees. Referees 2 and 3 still have a few suggestions that I would like you to address and incorporate before we can proceed with the official acceptance of your manuscript.

At the moment, your manuscript exclusively contains supplementary figures. We offer the publication of 5 supplementary figures as expanded view (EV) figures, that are embedded in the main manuscript text online and expand when clicked. You can therefore change the 5 most important supplementary figures to EV figures, if you want. We can unfortunately only offer 5 EV figures per manuscript at the moment, remaining extra figures need to be part of the so-called Appendix. Please upload each EV figure as individual file and add the legends for EV figures to the end of the main manuscript text. I would also like to suggest to combine some of the SF, e.g. the ones that only contain a single panel, to reduced their number. May be some of the data can also be shown as source data that will be directly linked to the main figure (e.g. "representative flow cytometry profiles"). Please see our guide to authors for more information on EV figures and source data.

Please note that all materials and methods must be included in the main manuscript file, the antibodies and siRNA sequences must be moved.

Regarding statistics, the legends for Fig 1C,D and 2F mention n=2. In this case, no statistics can be calculated. Please either repeat the experiment at least one more time or remove the error bars and p values. It is good to show all data points along with their mean for experiments with n=2. Please also specify the test used to calculate p-values for figures S1, S4A, S7 and S12 in the legends. Figure S9B shows the data of 2 experiments, but the legend says that the experiment was repeated more times. It would be much better to include more data and calculate proper statistics. Otherwise, all data points from both experiments should be shown.

EMBO reports abstracts need to be written in present tense. We will therefore make a change to "Importantly, we find...".

I look forward to seeing a final version of your manuscript as soon as possible. Please let me know

if you have any questions.

REFEREE REPORTS

Referee #1:

The authors have put tremendous effort into addressing most/if not all of the major concerns of the reviewers. The work deserves to be published at EMBO Reports.

Referee #2:

The revision has addressed most of my concerns. Cell cycle and statistical information, data measuring replication track length following UV exposure and the additional iPOND observations are helpful. Overall the data support the conclusions regarding HUWE1-PCNA interactions. Two concerns remain:

1/ The revision includes a critical experiment addressing the potential role of HUWE1, demonstrating that expression of the protein in the knockout background prevented DNA breakage, and that expression of a protein variant that could not bind PCNA had the same effect. These are important observations, however, the paper should discuss what might be the potential role of the interaction between HUWE1 and PCNA if PCNA interaction is not essential for preventing DNA breakage.

2/ The IF images showing PCNA foci include some staining in the cytoplasm (figure 3C). This is not the usual pattern of PCNA staining, which is typically limited to the nucleus. I am wondering whether there was a mis-labeling or a technical issue.

Referee #3:

The authors have addressed most of the issues I raised. Most notably they strengthened their evidence for localisation of HUWE1 at DNA replication forks, improved their images showing co-localization of HUWE1 and PCNA, and provided compelling evidence that HUWE1 is required for the cellular response to DNA replication stress. However there is still one issue that is not clear to me, namely the effect of HUWE1 loss on S-phase. The authors have shown that HUWE1 loss or depletion significantly decreases replication tract length and increases the frequency of DNA breaks (presumably in the form of gaps and single strand breaks, as is found during disruption of DNA replication). DNA replication inhibitors that produce such effects cause accumulation of cells in S-phase because it takes longer to replicate DNA under such conditions. Yet the authors report that there are fewer cells in S-phase and HUWE1 deficiency alone does not appear to be sufficient to trigger the CHK1/pRPA signalling cascade (fig 7H). They report a small subpopulation of cells with an S-phase DNA content that no longer incorporates DNA precursors. These are not likely to account for slowed replication tract length measured in the fibre assay as this assay only measures cells incorporating precursors. It is possible that HUWE1 deficiency affects the entry of cells into S-phase. However I think the authors should make some comment concerning this anomaly.

2nd Revision - authors' response 31 March 2016

We are very happy that the reviewers found that the revised manuscript deserves to be published in *EMBO Reports*.

Editor comments:

At the moment, your manuscript exclusively contains supplementary figures. We offer the publication of 5 supplementary figures as expanded view (EV) figures, that are embedded in the main manuscript text online and expand when clicked. You can therefore change the 5 most important supplementary figures to EV figures, if you want. We can unfortunately only offer 5 EV figures per manuscript at the moment, remaining extra figures need to be part of the so-called

Appendix. Please upload each EV figure as individual file and add the legends for EV figures to the end of the main manuscript text. I would also like to suggest to combine some of the SF, e.g. the ones that only contain a single panel, to reduced their number. May be some of the data can also be shown as source data that will be directly linked to the main figure (e.g. "representative flow cytometry profiles"). Please see our guide to authors for more information on EV figures and source data.

We have now merged all previous supplementary figures into five EV figures.

Please note that all materials and methods must be included in the main manuscript file, the antibodies and siRNA sequences must be moved.

We have moved the antibodies and siRNA lists in the main manuscript file.

Regarding statistics, the legends for Fig 1C,D and 2F mention n=2. In this case, no statistics can be calculated. Please either repeat the experiment at least one more time or remove the error bars and p values. It is good to show all data points along with their mean for experiments with n=2.

We have removed the p-value and error bars from these figures.

Please also specify the test used to calculate p-values for figures S1, S4A, S7 and S12 in the legends.

We have included this information in the legends.

Figure S9B shows the data of 2 experiments, but the legend says that the experiment was repeated more times. It would be much better to include more data and calculate proper statistics. Otherwise, all data points from both experiments should be shown.

We have included the data from a third experiment, and added error bars.

EMBO reports abstracts need to be written in present tense. We will therefore make a change to "Importantly, we find...".

We have made this change in the Abstract.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We have included these items.

Referee #1:

The authors have put tremendous effort into addressing most/if not all of the major concerns of the reviewers. The work deserves to be published at EMBO Reports.

We are happy that the reviewer found our resubmission satisfactory.

Referee #2:

The revision has addressed most of my concerns. Cell cycle and statistical information, data measuring replication track length following UV exposure and the additional iPOND observations are helpful. Overall the data support the conclusions regarding HUWE1-PCNA interactions. Two concerns remain:

We thank the reviewer for his/her comments. Below we are addressing the remaining comments:

1/ The revision includes a critical experiment addressing the potential role of HUWE1, demonstrating that expression of the protein in the knockout background prevented DNA breakage, and that expression of a protein variant that could not bind PCNA had the same effect. These are important observations, however, the paper should discuss what might be the potential role of the interaction between HUWE1 and PCNA if PCNA interaction is not essential for preventing DNA breakage.

We believe that this is a misunderstanding: the experiment mentioned by the reviewer is presented in Fig. 6G. We show that re-expression of WT corrects the breakage phenotype, but re-expression of the FF (PCNA interaction deficient mutant) does not. Thus, PCNA interaction is indeed essential for preventing DNA breakage, as we discussed in our manuscript.

2/ The IF images showing PCNA foci include some staining in the cytoplasm (figure 3C). This is not the usual pattern of PCNA staining, which is typically limited to the nucleus. I am wondering whether there was a mis-labeling or a technical issue.

Indeed, in the PCNA staining there is some cytoplasmic/membrane staining. We believe that this is caused by the pre-extraction (using Triton X-100) and fixation (using PFA) conditions employed for the co-staining. Indeed, when we perform PCNA staining using Methanol fixation (which is more widely used for PCNA staining) we do not observe any cytoplasmic staining. However, that protocol is unfortunately not compatible with HUWE1 staining.

Referee #3:

The authors have addressed most of the issues I raised. Most notably they strengthened their evidence for localisation of HUWE1 at DNA replication forks, improved their images showing co-localization of HUWE1 and PCNA, and provided compelling evidence that HUWE1 is required for the cellular response to DNA replication stress. However there is still one issue that is not clear to me, namely the effect of HUWE1 loss on S-phase. The authors have shown that HUWE1 loss or depletion significantly decreases replication tract length and increases the frequency of DNA breaks (presumably in the form of gaps and single strand breaks, as is found during disruption of DNA replication). DNA replication inhibitors that produce such effects cause accumulation of cells in S-phase because it takes longer to replicate DNA under such conditions. Yet the authors report that there are fewer cells in S-phase and HUWE1 deficiency alone does not appear to be sufficient to trigger the CHK1/pRPA signalling cascade (fig 7H). They report a small subpopulation of cells with an S-phase DNA content that no longer incorporates DNA precursors. These are not likely to account for slowed replication tract length measured in the fibre assay as this assay only measures cells incorporating precursors. It is possible that HUWE1 deficiency affects the entry of cells into S-phase. However I think the authors should make some comment concerning this anomaly.

We thank the reviewer for this comment. To address it, we have incorporated the following paragraph in the Discussion section (pg. 12):

Our results showing reduced fiber tract length in HUWE1-deficient cells, would suggest that these cells spend a longer time in S-phase. However, we observed that the number of cells with S-phase DNA content is similar (but a larger proportion of those are BrdU-negative). These findings may suggest an additional function of HUWE1 in regulating entry into S-phase.

3rd Editorial Decision 05 April 2016

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.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: George-Lucian Moldovan
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2015-41685-T

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).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes are similar to those generally used in the field (no statistical method was used to predetermine them).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	The fiber and comet experiments were analyzed blindly.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	This was not assessed
Is there an estimate of variation within each group of data?	see pg. 20 (statistical methods)
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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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).	see pg. 17 (cell and protein techniques)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	see pg. 17 (cell culture techniques). Cell lines were not recently authenticated.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
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.	NA

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
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA