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DNA damage-induced replication stress results in PA200-proteasome mediated degradation of acetylated histones

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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 3 January 2018

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, all referees acknowledge that the findings are potentially interesting and novel. However, they also raise a few - most of them overlapping - concerns that should be addressed in order to strengthen the study and enhance its impact. I think that all points raised make a lot of sense and should therefore be addressed.

I 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.

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. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view (EV) figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file. The EMBO reports reference style is numbered and can be found in EndNote.

Regarding data quantification, 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.

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:

This is an excellent study aimed at examining how histone acetylation and histone turnover occurs in response to UV-induced DNA damage. The authors use heavy/light isotope labelling coupled with MS to quantitate this. They report, unexpectedly, that overall histone acetylation levels are reduced following UV exposure on all canonical histones. Interestingly, they note that H2A.Z, which has been linked to DNA repair, is the only variant whose acetylation levels are increased by DNA damage. In a further novel step, they show that the decline in acetylated histones is dependent on the PA-200 proteasome activator. This precise role for PA-200 in histone turnover during UV repair is novel and a strength of the paper. Overall, this paper has the potential to provide new insight into histone turnover during UV-repair, but could be improved by addressing 3 issues: (i) monitoring precise histone acetylation sites which are turned over during UV repair, using site-specific histone acetylation antibodies; (ii) discriminating between chromatin-associated histones and the "free" pool of histones awaiting incorporation, and (iii) more detailed studies of the role of PA-200 and how inhibition impacts e.g. repair, survival, NER function and chromatin re-organization in G1 versus s-phase. This would allow for a more nuanced integration of this data with previous studies showing specific increases in histone acetylation directly at UV damage sites.

1. In the preparation protocol for histone extraction for MS analysis, total nuclear (free + chromatin) associated histones are isolated. Histones are massively transcribed during s-phase due to the need to package newly synthesized DNA. These histones come with a "basal" package of histone modifications, including acetylation. As the authors allude to in the discussion, it is likely that the UV-damage response leads to rapid degradation and/or decreased transcription of this "free" histone pool. The observed loss of acetylation may therefore reflect loss of the "free", unincorporated histone pool. It is therefore important to also analyze the chromatin-associated histones to determine if this pool undergoes deacetylation/degradation by e.g. isolating chromatin and then extracting histones. The observation that the degradation is cell cycle dependent, and that histone acetylation recovers later would be consistent with cell cycle dependent degradation of new, unincorporated histones rather than chromatin-associated histones. Further, this would allow the authors to more precisely align their results with the previously described increases in H3K9Ac/H3K14Ac reported by several groups.
2. The authors need to validate some of their observations using previously validated acetyl-lysine specific antibodies which can be obtained from commercial vendors. For example, H3K9Ac/H3K14Ac and H4Ac. Comparing "free" and chromatin associated histone acetylation marks after UV would provide more precise validation.
3. In many figures (e.g. 4A, 4C, 5A etc) the acetyl-lysine shows as 2 bands, with H4 shown below. Which histones do these 2 acetylated bands represent?
4. In supplementary figure 1B, its not clear why there are multiple bands for e.g. H2A, H2B and H3.

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5. Will the AcLys antibody used to enrich for acetylated peptides be influenced by the presence of other modifications (e.g. methylation/ubiquitination) present on the same histone peptide?

Referee #2:

Chromatin dynamics is known to play a key role in the efficient response and repair of DNA damage. Previous studies by many groups have shown that histone acetylation increases in chromatin adjacent to various types of DNA lesions, including UV-induced DNA damage and DNA double strand breaks. These histone marks are believed to facilitate repair primarily by increasing the recruitment of chromatin remodeling enzymes that harbor bromodomains, such as SWI/SNF. In this report, the authors have used an unbiased, mass spectrometry approach to monitor changes in histone acetylation after UV-induced DNA damage. This method was quite extensive, using SILAC for quantification of changes and multiple proteases to yield excellent coverage of histone peptides. Acetylated peptides were also enriched using anti-acetyl lysine immunoprecipitation. Notably, their strategy monitors total histones - both chromatin bound and the free histone pool. The authors identify many new examples of histone acetylation sites prior to damage, especially on histones H2A and H2B which tend to be under-studied. The authors find that nearly all types of histone acetylation are reduced following DNA damage, with the exception of H2AZ acetylation. These mass spec results were confirmed by extensive western blot analyses with anti-acetyl lysine antibodies. Histone loss does not require transcription or active nucleotide excision repair, though recovery of normal levels of acetylated histones does require repair. Histone loss also occurs if cells are treated with replication stress agents (HU or AraC), and the UV-induced histone loss is not observed in non-cycling cells or in cells blocked prior to S phase. The authors find that inhibition of the proteasome - specifically a form of the proteasome containing PA200 - blocks UV-induced histone loss. Thus, the data strongly indicate that loss of acetylated histones occurs in response to stalled or stressed replication forks and that histones are targeted for proteasome-dependent degradation.

This is a strong manuscript with exceptional data. The conclusions are well-supported by the data and the results should be of wide interest. However, the authors' data does raise a bit of controversy, as many previous studies have shown increased histone acetylation following UV-induced damage. The authors do address this, but there would seem to be a simple experiment that could further enhance the impact of this paper. It seems likely that DNA damage and replication stress are impacting the degradation of the free histone pool and that chromatin bound histones are protected from degradation. Indeed, the authors refer to this possibility on the last section of the Discussion. This possibility can easily be tested by a simple chromatin fractionation experiment where chromatin-associated histones are separated from the soluble pool and histones monitored by western blot. If correct, this would strongly suggest a model in which the cell decides to degrade excess, acetylated histones when forks are stalled (note that the free histone pool is highly acetylated in preparation for assembly).

Minor points:

1. In Supplemental Figure 1B, it appears that the anti-H2A antibody yields a doublet - is this antibody known to react with histone H4? There should be some explanation provided here.

2. Introduction, line 2. "Chromatin is a highly organized structure in which DNA is wound around nucleosomes". This should be changed to "...wound around histone octamers to form nucleosomes". The following sentence could then be modified to state: "The histone octamer is comprised of a histone H3-H4..."

3. Given the results, the title could be changed to be more specific -- "Replication stress results in PA200-proteasome mediated degradatio of acetylation histones"

Referee #3:

The manuscript from Marteijn and colleagues uses an unbiased mass spectrometry based approach to investigate the effects of UV-induced DNA damage on histone acetylation levels. They find that

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there is a global loss of acetylated histones following UV treatment, and that this is likely due to replication stress because it appears to be dependent on movement through the cell cycle. In addition, they demonstrate that the reduction is dependent on proteasomal degradation, but independent of ubiquitylation.

This finding is initially surprising because there are many reports showing that at least some histone acetylation events are increased in response to UV. The authors conclude that the difference in findings is due to the different experimental techniques: here, the authors monitor acetylation in global histone pools, whereas other studies use chromatin IP or immunofluorescence, which would more specifically monitor histones in chromatin near the damage. This is a very reasonable conclusion, and moreover, since the degradation is due to replication stress rather than specifically UV-induced damage, this highlights an important consideration for studies of histone modifications in response to UV (where global changes from replication would swamp any local differences in response to UV).

Overall, this is an interesting and well-performed study. While it is unreasonable to expect the mechanism to be worked out for this manuscript, there are a few experiments that if added, would solidify the conclusions and slightly extend the impact.

1. The use of modification-specific antibodies (for at least a few sites) in the analysis of global histone modifications by Western would be very useful for validating the changes in response to UV. This is particularly important for sites that are reported to increase in response to UV using other experimental approaches to demonstrate the likelihood that the conclusion about global vs local changes is correct. In addition, there were fewer acetylated residues in the mass spec data than expected, so additional data with specific antibodies is informative.

2. The authors should use the pan-acetyl lysine antibody to look at changes in the chromatin bound versus soluble histone fractions to provide some insight into which pool of histones is degraded in response to replication stress.

3. The evidence supporting replication stress as the cause of the reduction in acetylated histones is good, but limited. To support this conclusion, the authors should use other forms of replication stress (for example, aphidicolin or MMC, or use oncogene-induced replication stress).
4. In addition, the authors should test whether the acetylated histone degradation is dependent on the ATR pathway.

1st Revision - authors' response 15 June 2018

Referee #1:
This is an excellent study aimed at examining how histone acetylation and histone turnover occurs in response to UV-induced DNA damage. The authors use heavy/light isotope labelling coupled with MS to quantitate this. They report, unexpectedly, that overall histone acetylation levels are reduced following UV exposure on all canonical histones. Interestingly, they note that H2A.Z, which has been linked to DNA repair, is the only variant whose acetylation levels are increased by DNA damage. In a further novel step, they show that the decline in acetylated histones is dependent on the PA-200 proteasome activator. This precise role for PA-200 in histone turnover during UV repair is novel and a strength of the paper.

We would like to thank this reviewer for the kind words and appreciation of our study and are grateful to the reviewer for the valuable suggestions to further improve the quality and impact of our manuscript. We have carefully addressed all the raised issues, as discussed in detail below in a point-to-point manner and have adapted our manuscript accordingly.

Overall, this paper has the potential to provide new insight into histone turnover during UVrepair, but could be improved by addressing 3 issues: (i) monitoring precise histone acetylation sites which are turned over during UV repair, using site-specific histone acetylation antibodies; (ii) discriminating between chromatin-associated histones and the "free" pool of histones awaiting incorporation, and (iii) more detailed studies of the role of PA-200 and how inhibition impacts e.g.

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repair, survival, NER function and chromatin reorganization in G1 versus s-phase. This would allow for a more nuanced integration of this data with previous studies showing specific increases in histone acetylation directly at UV damage sites.

We thank the reviewer for the suggested experiments to obtain additional insight into histone turnover during the UV-DDR. Issue (i) and (ii) will be discussed at point 1-3 below. Indeed, even though the PA200-mediated histone turnover is NER-independent, we agree that it is interesting to test whether the UV-induced histone degradation is influencing repair and cell survival. To test this, we performed unscheduled DNA synthesis (UDS) experiments to directly asses NER efficiency by directly quantifying the last DNA damage-mediated repair synthesis step of NER. Knock down of PA200, did not affect UDS, indicating that NERmediated repair is not influenced by PA200-mediated histone degradation (revised Figure 7G). Importantly, these results further distinguishes our observations from previously described UV-induced H3K9/14 acetylation near UV-lesions, which stimulates efficient repair (e.g. Teng et al., 2002, Yu et al., 2005, Duan & Smerdon, 2014). Despite the absence of a role of PA200 during NER-mediated repair, we tested whether PA200 depletion is involved in cell survival following UV-induced DNA damage. Survival experiments showed that PA200 depletion results in UV-hypersensitivity (revised Figure 7H). Together, these data strongly suggest that degradation of acetylated by the PA200 proteasome plays an important role in the cellular survival specifically following replication stress.

1. In the preparation protocol for histone extraction for MS analysis, total nuclear (free + chromatin) associated histones are isolated. Histones are massively transcribed during s-phase due to the need to package newly synthesized DNA. These histones come with a "basal" package of histone modifications, including acetylation. As the authors allude to in the discussion, it is likely that the UV-damage response leads to rapid degradation and/or decreased transcription of this "free" histone pool. The observed loss of acetylation may therefore reflect loss of the "free", unincorporated histone pool. It is therefore important to also analyze the chromatin-associated histones to determine if this pool undergoes deacetylation/degradation by e.g. isolating chromatin and then extracting histones. The observation that the degradation is cell cycle dependent, and that histone acetylation recovers later would be consistent with cell cycle dependent degradation of new, unincorporated histones rather than chromatin-associated histones. Further, this would allow the authors to more precisely align their results with the previously described increases in H3K9Ac/H3K14Ac reported by several groups.

We thank the reviewer for this valuable suggestion. Indeed, it is tempting to speculate that the loss of acetylated histones could be caused by the degradation of unincorporated histones. To test this hypothesis, we studied whether chromatin-associated histones or soluble histones are degraded after UV. Therefore, we performed a chromatin fractionation assay and investigated the acetylation levels in both the soluble fraction, containing free unincorporated histones, and the chromatin fraction, mainly consisting of incorporated histones (revised Figure 6F-G).

Acetylated histones could be detected predominantly in the chromatin fraction. Following UV irradiation, we detected a 50% reduction of acetylation signal in the chromatin fraction, which is similar to the reduction of acetylated histones in whole cell lysates. In our initial manuscript we proposed two different models, either the chromatin-bound histones are degraded to stimulate the removal of stalled replication forks or the soluble histones are degraded to prevent an excess of free histones. These results disprove our second model and we have adapted our manuscript accordingly.

2. The authors need to validate some of their observations using previously validated acetyllysine specific antibodies which can be obtained from commercial vendors. For example, H3K9Ac/H3K14Ac and H4Ac. Comparing "free" and chromatin associated histone acetylation marks after UV would provide more precise validation.

As suggested, we have confirmed our MS-data and general acetylation staining using histone modification specific antibodies to study H3K9, H3K14, H3K27 and H4K12 acetylation levels after UV irradiation on both the total histone pool (revised Figure 2 F, G) and on the fractionated samples (revised Figure 6 F, G). While a clear reduction of H3K27 and H4K12 acetylation levels could be observed, a relatively smaller reduction in H3K9 and H3K14 levels was observed following UV-irradiation. This difference might be explained due to the previously described increase of these specific histone marks around UV-induced damaged sites (Teng et al., 2002, Yu et al., 2005, Duan

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& Smerdon, 2014), while in the rest of the chromatin, histones with these marks are being degraded. Of note, in our MS dataset we do not find any peptides that only carry H3K9Ac or only H3K14Ac, but all peptides that contain either the H3K9Ac or H3K14Ac mark carry at least one other acetylation mark. As speculated in our discussion, this might suggest that mainly histones that are poly-acetylated might be degraded, which may explain the small differences observed between histone mark specific staining versus general acetylation staining or MS data.

3. In many figures (e.g. 4A, 4C, 5A etc) the acetyl-lysine shows as 2 bands, with H4 shown below. Which histones do these 2 acetylated bands represent?

As shown in supplementary figure 2B, the two most intense bands of the general acetylation signal, representing small proteins (10-14 kDa) overlaps with signal from different histone stainings. The lower acetylation specific band overlaps with H4 and therefore mainly represents acetylated H4. The upper band overlaps with signal of histone H2A, H2B and H3 antibody and therefore represents the acetylated forms of these histones, see also comment 4. However, we cannot deduce how much every histone contributes to the signal detected in the higher migrating bands of the acetylation signal.

4. In supplementary figure 1B, it is not clear why there are multiple bands for e.g. H2A, H2B and H3.

Thank you for pointing this out to us. We have included a revised supplementary figure 2B, in which we use other histone antibodies in combination with improved western blot procedures, which resulted in a diminished aspecific staining that was observed in the previous figure. This aspecific staining might have been caused by the cross reactivity of the previously used antibodies against H4.

5. Will the AcLys antibody used to enrich for acetylated peptides be influenced by the presence of other modifications (e.g. methylation/ubiquitination) present on the same histone peptide?

We agree with this reviewer that it is indeed important to get a better understanding of the binding characteristics of the used actelyation antibody, i.e. whether it is affected by other PTMs present on the same acetylated peptide. To answer this question, we have re-analyzed the acetylated peptides from the MS experiments depicted in supplemental table 2, for the presence of additional methylation, phosphorylation and ubiquitylation marks. Using this analysis, we identified 457 unique acetylated peptides of which 19.5% carried additional modifications. The results of three representative peptides are depicted in the table below. This indicates that additional phosphorylation, methyl or ubiquitin modifications do not block the binding of the in our study used pan-acetyl antibody to acetylated histone peptides. We have discussed this in the manuscript and added the table below to Supplemental Figure 1.

Histone peptide Modifications
(H2B) KGSKKAVTKAQKKDGKKR 4 Acetyl (K)
4 Acetyl (K);Dimethyl (KR)
(H3) QTARKSTGGKAPRKQLATKAAR Acetyl (K);Methyl (KR)
2 Acetyl (K)
2 Acetyl (K);Phospho (STY)
2 Acetyl (K);Methyl (KR)
2 Acetyl (K);Dimethyl (KR)
3 Acetyl (K)
3 Acetyl (K);Phospho (STY)
3 Acetyl (K);Methyl (KR)
3 Acetyl (K);Dimethyl (KR)
4 Acetyl (K)
(H4) KGLGKGGAKR 3 Acetyl (K)
Acetyl (K);GlyGly (K)
Referee #2:
This is a strong manuscript with exceptional data. The conclusions are well-supported by the data and the results should be of wide interest. However, the a uthors' data does raise a bit of controversy, as many previous studies have shown increased histone acetylation following UV-induced damage.

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The authors do address this, but there would seem to be a simple experiment that could further enhance the impact of this paper. It seems likely that DNA damage and replication stress are impacting the degradation of the free histone pool and that chromatin bound histones are protected from degradation. Indeed, the authors refer to this possibility on the last section of the Discussion. This possibility can easily be tested by a simple chromatin fractionation experiment where chromatin-associated histones are separated from the soluble pool and histones monitored by western blot. If correct, this would strongly suggest a model in which the cell decides to degrade excess, acetylated histones when forks are stalled (note that the free histone pool is highly acetylated in preparation for assembly).

First, we would like to thank the reviewer for the very nice words on our manuscript and the appreciation of our unbiased proteomics screen and western blot analysis that shows that acetylated histones are degraded following replications stress. In our manuscript, we have suggested two different hypotheses on which histones are degraded after replication stress. As suggested, we now performed the fractionation experiments and we observed that most acetylation signal originates from the chromatin-incorporated histones and that the degradation of these chromatin bound histones are the cause of our observed loss of acetylated histones. It is tempting to speculate that the acetylated histones are degraded around stalled replication forks is S-phase cells. These histones could be degraded to promote the processing of stalled replication forks or perhaps repair of ds or ssDNA breaks, which might result from collapsed replication forks. This might explain the differential response of histone acetylation in the vicinity of NER-mediated UV-lesions as detected by chromatin immunoprecipitation experiments compared to UV-induced stalling of replication. Of note, we have provided additional Unscheduled DNA synthesis (UDS) data, showing the PA200 does not affect repair efficiency (Figure 7G). This result further indicates that the in our study uncovered UV-induced loss of acetylated histones is a different process than the previously described histone acetylation events in the vicinity of UV-induced lesion, as these have been described to stimulate NER-mediated repair (Duan & Smerdon, 2014).

Minor points:
1. In Supplemental Figure 1B, it appears that the anti-H2A antibody yields a doublet - is this antibody known to react with histone H4? There should be some explanation provided here.

Thank you for pointing this out to us. We repeated these stainings with other antibodies in combination with improved western blot procedures. This resulted in the detection of one individual band, most likely caused by a-specific cross reactivity with H4. We have included these new western blots in revised supplementary figure 2B.

2. Introduction, line 2. "Chromatin is a highly organized structure in which DNA is wound around nucleosomes". This should be changed to "...wound around histone octamers to form nucleosomes". The following sentence could then be modified to state: "The histone octamer is comprised of a histone H3-H4..."

We thank the reviewer for bringing this mistake to our attention, we adjusted this manuscript accordingly.

3. Given the results, the title could be changed to be more specific -- "Replication stress results in PA200-proteasome mediated degradatio of acetylation histones"

The reviewer has a point that our study shows the effects on histone acetylation caused by replication stress. However, we believe that by including “DNA damage-induced” in the title our manuscript will attract the attention of both scientists working on replication and from the repair-field, thereby extending its impact, of a broader readership. Our findings are important for researchers in the repair field as, thus far, most studies on the interplay of histone acetylation and repair have focused mainly on the effects of histone marks during the repair process. However, our data shows that DNA damage has also important chromatin wide effects, for example caused by DNA damage induced replication blocks. We believe our study provides an important additional message to the DNA repair community, and therefore, we are in favor to keep the title “DNA damage-induced replication stress results in PA200- proteasome mediated degradation of acetylated histones”.

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Referee #3:
The manuscript from Marteijn and colleagues uses an unbiased mass spectrometry based approach to investigate the effects of UV-induced DNA damage on histone acetylation levels. They find that there is a global loss of acetylated histones following UV treatment, and that this is likely due to replication stress because it appears to be dependent on movement through the cell cycle. In addition, they demonstrate that the reduction is dependent on proteasomal degradation, but independent of ubiquitylation.

This finding is initially surprising because there are many reports showing that at least some histone acetylation events are increased in response to UV. The authors conclude that the difference in findings is due to the different experimental techniques: here, the authors monitor acetylation in global histone pools, whereas other studies use chromatin IP or immunofluorescence, which would more specifically monitor histones in chromatin near the damage. This is a very reasonable conclusion, and moreover, since the degradation is due to replication stress rather than specifically UV-induced damage, this highlights an important consideration for studies of histone modifications in response to UV (where global changes from replication would swamp any local differences in response to UV).

Overall, this is an interesting and well-performed study. While it is unreasonable to expect the mechanism to be worked out for this manuscript, there are a few experiments that if added, would solidify the conclusions and slightly extend the impact.

We would like to thank the reviewer for his/hers appreciation of our manuscript and for the helpful suggestions for experiments. We performed the suggested experiments, as discussed below in a point-to-point manner, and believe these have strengthened our data and improved our manuscript.

1. The use of modification-specific antibodies (for at least a few sites) in the analysis of global histone modifications by Western would be very useful for validating the changes in response to UV. This is particularly important for sites that are reported to increase in response to UV using other experimental approaches to demonstrate the likelihood that the conclusion about global vs local changes is correct. In addition, there were fewer acetylated residues in the mass spec data than expected, so additional data with specific antibodies is informative.

As suggested, we confirmed our results obtained by our mass spectrometry approach by studying histone acetylation levels after UV exposure by Western blotting using 4 different acetylation specific antibodies: H3K9, H3K14, H3K27 and H4K12. While a clear reduction of H3K27 and H4K12 acetylation levels could be observed, a more subtle reduction in H3K9 and H3K14 levels was observed following UV-irradiation (revised figure 2F and G). This might be explained due to the previously described increase of these histone marks around damaged sites (Teng et al., 2002, Yu et al., 2005, Duan & Smerdon, 2014), while in the rest of the chromatin histones with these marks are already being degraded, resulting in a less pronounced loss of H3K9 and H3K14 compared to H3K27 and H4K12. Of note, in our MS dataset we do not find any peptides that only carry H3K9Ac or only H3K14Ac, but all peptides that contain either the H3K9Ac or H3K14Ac mark carry at least one other acetylation mark. In our discussion we suggest that mainly histones that are poly-acetylated might be degraded, which may explain the small differences observed using histone mark specific antibodies versus general acetylation staining or MS data.

2. The authors should use the pan-acetyl lysine antibody to look at changes in the chromatin bound versus soluble histone fractions to provide some insight into which pool of histones is degraded in response to replication stress.

We thank the reviewer for this valuable suggestion. In our initial manuscript we proposed two different models, either the chromatin-bound histones are degraded to stimulate the removal of stalled replication forks or the soluble histones are degraded to prevent an excess of free histones. Indeed, it is tempting to speculate that the loss of acetylated histones could be caused by the degradation of unincorporated histones. To test this hypothesis, we studied whether chromatin-associated histones or soluble histones are degraded after UV. Therefore, we performed a chromatin fractionation assay and investigated the acetylation levels in both the soluble fraction, containing free unincorporated histones, and the chromatin fraction, mainly consisting of incorporated histones (revised Figure 6F-G). Acetylated histones could be detected predominantly in the chromatin

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fraction. Following UV irradiation we detected a 50% reduction of acetylation signal in the chromatin fraction, which is similar to the reduction of acetylated histones in whole cell lysates. These results disprove the second model and we have adapted the discussion of our revised manuscript accordingly.

3. The evidence supporting replication stress as the cause of the reduction in acetylated histones is good, but limited. To support this conclusion, the authors should use other forms of replication stress (for example, aphidicolin or MMC, or use oncogene-induced replication stress).

As suggested, we have extended our panel of replication stress inducing agents of UV and HU/AraC with MMC. MMC exposure resulted in a similar decrease in acetylation levels as was observed following UV irradiation and HU/AraC treatment. We have added this data to figure 5 A and B.

4. In addition, the authors should test whether the acetylated histone degradation is dependent on the ATR pathway.

We thank the reviewer for this suggestion, as it is certainly relevant to investigate whether the main DNA damage signaling kinases ATM and ATR are implicated in the decrease in acetylation levels following replication stress. For this purpose, we used a combination of a specific ATM (Ku55933) and ATR (VE-821) inhibitors, thereby blocking the activity of both kinases at the same time, as shown by the loss yH2AX induction after UV (Supplemental figure 3H). In the absence of ATR/ATM signaling a similar decrease of acetylated histones after UV irradiation could be detected, indicating that the histone degradation is independent of ATR and ATM signaling (Supplemental figure 3F-G).

2nd Editorial Decision 26 June 2018

Thank you for the submission of your revised manuscript. We have now received the enclosed comments from all referees.

I am happy to tell you that all referees overall support the publication of your study. However, both referees 1 and 2 have concerns with the newly added data that need to be addressed, and I would like to give you the opportunity to do so. Please also send us a point-by-point response to these last comments along with the newly revised manuscript.

A few other changes are also needed:

Please change the reference format to the numbered EMBO reports format (with up to 10 authors listed) that can be found in EndNote.

Please specify the statistical test used in figure 4.

The microscopy images in figures EV 3 and 4 are missing scale bars.

In figure EV 3B the error bars are not specified.

Figure EV 3G mentions n=2 in which case no statistics can be calculated. The experiment either needs to be repeated one more time, or the error bars and p-values need to be removed. You can show instead all data points from both experiments along with their mean.

The EV figure files are called supplemental figure x or extended view figure 1. Please either correct the names to Expanded View Figure X or remove the names from the figure files.

Fig 2G and 7F are not called out in the manuscript text.

Fig EV1 is called out as "supplemental figure 1". It should be Fig EV1.

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The 4 supplemental tables should be called Dataset EV1, 2, 3, 4. And Dataset EV4 needs to be called out in the manuscript text.

Many of the Western Blots are over-contrasted, please adjust the contrasts.

Fig 3A is spliced and has a '+' symbol between the first and second row of bands, top left. Can you please explain what this is? All spliced out bands need to be clearly indicated as such by leaving some white space between the bands or drawing a black line.

The author checklist is missing a lot of information in the statistics part, please complete.

I also would like to suggest minor changes to the abstract that needs to be written in present tense:

Histone acetylation influences protein interactions and chromatin accessibility and plays an important role in the regulation of transcription, replication and DNA repair. Conversely, DNA damage affects these crucial cellular processes and induces changes in histone acetylation. However, a comprehensive overview of the effects of DNA damage on the histone acetylation landscape is currently lacking. To quantify changes in histone acetylation we developed an unbiased quantitative mass spectrometry analysis on affinity purified acetylated histone peptides, generated by differential parallel proteolysis. We identify a large number of histone acetylation sites and observe an overall reduction of acetylated histone residues in response to DNA damage, indicative of a histone-wide loss of acetyl-modifications. This decrease is mainly caused by DNA damage-induced replication stress coupled to specific proteasome-dependent loss of acetylated histones. Strikingly, this degradation of acetylated histones is independent of ubiquitylation but requires the PA200-proteasome activator, a complex that specifically targets acetylated histones for degradation. The uncovered replication stress-induced degradation of acetylated histones represents an important chromatin-modifying response to cope with replication stress.

Please let me know if you agree with these changes.

I am looking forward to receiving a revised manuscript as soon as possible. Please let me know if you have any questions.

REFEREE REPORTS

Referee #1:

The authors have done an excellent job of responding to the comments of the referees. The new data showing that the loss of histones is linked to cell cycle and that it is the chromatin-associated fraction which undergoes de-acetylation/degradation is good. Further, the role of PA200 has been strengthened by the addition of data showing that PA200 is required for survival following UV exposure. One issue is the lack of histones identified in the soluble (non-chromatin) fraction in figure 7D-G. This suggest that all of the histone loss is due to degradation of chromatin-associated histones. For example, up to 25% (figure 7E) of H2B is lost, which may indicate that 25% of the DNA is now nucleosome free! This could be, as suggested, degradation of histones from stalled replication forks. It would be useful to include at least 1 western showing H2B and histone acetylation levels following UV in (i) Go/G1 arrested cells and (ii) add quantitation of H2B and H4 to the experiments in figure 5A-D.

Referee #2:

This paper nicely shows a decrease in acetylated histone levels in response to various types of replication stress (UV, MMC, AraC). As expected, the decrease in histone acetylation requires that cells be actively proliferating -- transiting S phase. In my previous review, I asked the reviewers to test whether the loss of histone acetylation was occurring on the chromatin-bound histones or within the free histone pool which is highly acetylated as chromatin is assembled in S phase. The authors attempted to do the exact experiment that I requested, but there appears to be a flaw with their

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experimental design. In the methods section, they state that cells at 80% confluence were isolated and fractionated. (1) the authors do not state the exposure time to UV for these experiments. (2) Most importantly, 80% confluence means that most of the cells are in the G1 phase of the cell cycle and therefore not transiting S phase. It is not surprising, therefore, that they do not detect any soluble histone H2B by western blot (Figure 6). There should be a very abundant soluble pool in S phase in mammalian cells. This has been shown numerous times. If the authors are unable to detect a soluble pool, then they really have not addressed my question. Indeed, the decrease in the chromatin fraction seems much less than shown in other figures. Furthermore, I realized that the authors do not state elsewhere in their methods what the proliferation state of their cells are for all other assays. This would seem to be key if for instance some treatments were performed with confluent cells whereas other treatments used cells that were more actively growing.

Referee #3:

The revised manuscript has nicely addressed the points raised by the reviewers, and should be published.

2nd Revision - authors' response 6 July 2018

Referee #1:
The authors have done an excellent job of responding to the comments of the referees. The new data showing that the loss of histones is linked to cell cycle and that it is the chromatinassociated fraction which undergoes de-acetylation/degradation is good. Further, the role of PA200 has been strengthened by the addition of data showing that PA200 is required for survival following UV exposure. One issue is the lack of histones identified in the soluble (non-chromatin) fraction in figure 7D-G. This suggest that all of the histone loss is due to degradation of chromatin-associated histones. For example, up to 25% (figure 7E) of H2B is lost, which may indicate that 25% of the DNA is now nucleosome free! This could be, as suggested, degradation of histones from stalled replication forks. It would be useful to include at least 1 western showing H2B and histone acetylation levels following UV in (i) Go/G1 arrested cells and (ii) add quantitation of H2B and H4 to the experiments in figure 5A-D.

We thank the referee for his/hers appreciation of our response to the reviewers comments and on the newly added data. Indeed, our chromatin fractionation assay (Figure 6D&E) shows that ~25% of chromatin bound H2B is lost 4 hours after induction of replication stress. This is in line with a similar reduction in histone H4 levels due to PA200-proteasome mediated degradation following UV-induced DNA damage as depicted and quantified in both Figure 6A&B and 7C&D. To confirm that this reduction in histone levels is indeed caused by UVinduced replication stress, we have additionally quantified the loss of H4 in cycling vs noncycling cells, which are arrested in G1/early S-phase using the Cdc7/CDK9 inhibitor PHA 767491 (EV figure 3A). As expected, while in cycling cells we observe a 25% reduction of histone H4, the levels of the histone levels remained unchanged in non-cycling cells, indicating that the loss of core histones is caused by replication stress. We have included this figure in the revised manuscript as Figure EV 3I.

Referee #2:
This paper nicely shows a decrease in acetylated histone levels in response to various types of replication stress (UV, MMC, AraC). As expected, the decrease in histone acetylation requires that cells be actively proliferating -- transiting S phase. In my previous review, I asked the reviewers to test whether the loss of histone acetylation was occurring on the chromatin-bound histones or within the free histone pool which is highly acetylated as chromatin is assembled in S phase. The authors attempted to do the exact experiment that I requested, but there appears to be a flaw with their experimental design. In the methods section, they state that cells at 80% confluence were isolated and fractionated. (1) the authors do not state the exposure time to UV for these experiments. (2) Most importantly, 80% confluence means that most of the cells are in the G1 phase of the cell cycle and therefore not transiting S phase. It is not surprising, therefore, that they do not detect any soluble histone H2B by western blot (Figure 6). There should be a very abundant soluble pool in S phase in mammalian cells. This has been shown numerous times. If the authors are unable to detect a soluble

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pool, then they really have not addressed my question. Indeed, the decrease in the chromatin fraction seems much less than shown in other figures. Furthermore, I realized that the authors do not state elsewhere in their methods what the proliferation state of their cells are for all other assays. This would seem to be key if for instance some treatments were performed with confluent cells whereas other treatments used cells that were more actively growing.

All experiments, including the chromatin fractionation assay were executed at a confluency of 70-80%, otherwise it is clearly stated in the figure legends (e.g. confluent cells). We have now emphasized this important information more clearly in the material and methods section. Importantly, as most experiments are executed in HeLa cells, which have lost contact inhibition, these cells are only minimal affected in cell cycle distribution when grown at 70-80% confluency. The presence of a significant fraction of cells in S-phase at the moment of replication stress induction was shown by EdU incorporation after a 2 hr pulse labelling. As shown in Figure EV 3A more than 30% of HeLa cells are in S-phase when grown at our experimental conditions of 70-80% confluency.

We agree with this reviewer that it is for sure possible to detect the acetylated free (nonchromatin incorporated) pool of histones in proliferating cells when relatively large amounts of cell lysate are subjected to western blot analysis. However, for this manuscript the relevant question is, as asked by all three reviewers, whether the degradation of acetylated histones following replication stress observed in total cell lysates, is caused by the degradation of chromatin bound or free histones. To carefully address this question we have performed a cell fractionation assay, in the same cells, grown at the same conditions following the same UVdose to induce replication stress and analysed the reduction in histone acetylation in chromatin bound and free fractions originating from the exact same number of cells. Of note, the times after UV-exposure, when the cells were harvested, are clearly indicated in this figure. As is shown in figure 6D&E, we observed a ~50% reduction of acetylated histones in the chromatin fraction 4 hours after UV irradiation. This reduction is highly similar to the 40-60% reduction of acetylated histones observed in whole cell lysates throughout the manuscript, and are thus not reduced as stated by the reviewer. This clearly suggests that the majority of histone degradation takes place in the chromatin, indicating that incorporated histones are targeted by the PA200-proteasome. This conclusion is further strengthened by the fact that, in soluble lysates from the same amount of cells only a very small fraction of acetylated histones could be observed (Figure 6D, right panel). Therefore degradation of this minor, difficult to detect, non-chromatin bound histone pool cannot be the cause of the strong reduction of acetylated histones observed in total cell lysates. Importantly, this small fraction of histones in the non-chromatin fraction is completely in line with previous studies that showed that in HeLa cells, which are also used in most of our experiments, only a very small pool of free histones are observed, for example a 1% free fraction was detected by cell fractionation assays (Loyola, et al., Mol. Cell, 2006), or <4% free histones were observed by FRAP-based live-cell imaging approaches (Kimura et al., JCB, 2001).

Referee #3:
The revised manuscript has nicely addressed the points raised by the reviewers, and should be published.

We thank this reviewer for the positive comments on our revised manuscript and the recommendation to publish this study.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jurgen Marteijn
Journal Submitted to: EMBO Reports
Manuscript Number:

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Our sample size lies within the range of typical sample sizes used for these types of experiments.
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?	NA
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.	No, the nature of the experiments did not allow randomization or blinding. Of note, in the quantitative Mass Spectrometry analysis, control and DNA damage exposed sample where combined at the earliest possible step, excluding a bias during sample preparation and analyis.
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, statistical significance was routinely calculated by Students T-test. P values were described in each figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, this is the commonly used test for this type of experiments.
Is there an estimate of variation within each group of data?	The variation of the analyzed data using the above described tests are shown by SEM as indicated in the figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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

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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	We have included clone or catalog numbers in methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cells were acquired from ATCC and are mycoplasm tested on a bimonthly basis.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	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 a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We have uploaded our MS data to PeptideAtlas: Identifier PASS01209 and added the data availability section to the Materials and Methods section.
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).	We added all raw datasets to the online depository PeptideAtlas and all analyzed results as Supplemental tables and Expanded view figures.
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. 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

22. 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