EMBO reports

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JMJD5 Cleaves Monomethylated Histone H3 N-tail Under DNA Damaging Stress

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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 13 February 2017

Thank you for your patience while your mannuscript was peer-reviewed at EMBO reports. We have finally received the last referee report, and all comments are pasted below.

As you will see, the referees acknowledge that the findings are potentially novel and interesting. However, they also raise several concerns and point out that significant revisions are required before the study can be considered for publication here. The most important points that need to be addressed are that more direct evidence for the cleavage of H3 by JMJD5 should be provided, that the specificity of JMJD5 for H3 needs to be demonstrated, that the clipping site needs to be identified, and that time-dependent assays for clipping activity should be performed. The other referee concerns also need to be addressed, at the very least in the manuscript text.

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. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS
Referee #1:

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Chin and colleagues have identified a potentially novel role for the histone demethylase JMJD5 in clipping histone H3 at monomethylated K9, particularly during 'stress' conditions. This result is quite intriguing, as this protein has not been implicated in histone tail clipping to date. However, more controls and additional experiments are required to fully convince the reader that this is the case. First, it may be likely that the clipping actually occurs upon G1 arrest (starvation/double thymidine bock), and this has not been carefully considered. The term 'stress' has been used loosely here and the conditions under which the clipping event occurs needs to be defined. Moreover, it is unclear how clipping by JMJD5 is related to its demethylase functions for K36me2 - are these through the same regions of the JmjC domain and residues? Also, it has not been addressed whether other JmJC-containing demethylases might contain such activity. Importantly, the role of the protease activity as well as the clipping event to remove H3K9me1 remains unclear.

Major Comments:

-The fact that this clipping event occurs under 'stress' conditions should be emphasized in both the title and abstract. However, an alternative explanation is a G1 arrest/quiescence situation induces cleavage because both conditions used in this study will arrest cells in G1/S. The authors should utilize other treatments that occur outside of G1/S and look at this clipping event in other conditions that induce senescence or quiescence (see next point). In summary: the cellular event that triggers this clipping needs to be better defined.

-The authors neglect to site two important papers that recently found new biological contexts that display H3 tail cleavage, which may be relevant to their studies:
*Histone H3.3 and its proteolytically processed form drive a cellular senescence programme. Duarte LF, Young AR, Wang Z, Wu HA, Panda T, Kou Y, Kapoor A, Hasson D, Mills NR, Ma'ayan A, Narita M, Bernstein E. Nat Commun. 2014 Nov 14;5:5210. doi: 10.1038/ncomms6210. PMID:25394905
*MMP-9 facilitates selective proteolysis of the histone H3 tail at genes necessary for proficient osteoclastogenesis. Kim K, Punj V, Kim JM, Lee S, Ulmer TS, Lu W, Rice JC, An W. Genes Dev. 2016 Jan 15;30(2):208-19. doi: 10.1101/gad.268714.115. PMID: 26744418

-Do the authors know which H3 variant is being cleaved? H3.1, H3.2 or H3.3? This could be determined from the mass spec analyses if residue 31 (S/A) of H3 was identified.

-Alignment of the secondary structure of JMJD5 and Cathepsin L is insufficient to claim structure-function. Are there crystal structures available that could be super-imposed? It remains mechanistically unclear how JMJD3 possesses peptide protease activity.

-A discussion about the protease vs. demethylase activity is required, especially since the H3 binding activity seems to occur through the JmjC domain. This is only touched upon in the discussion section.

-What is the significance of removing K9me1 from the H3 tail?

Minor comments:

-page 5. line 2. The clipping events should be described as the residues clipped in between or clarify the residue before or after the cleavage. e.g. Cathepsin L cleaves between A21 and T22, creating a new N-terminus starting at residue T22.

-page 6. 1st paragraph. The authors speculate that the 'faster migrating band was most likely the species previously reported.....' citing reference 17 which describes H3 tail clipping in Tetrahymena. The authors have no reason to speculate this without aligning the faster migrating band to other H3 cleaved species. They could only speculate this after the PTM analyses described in this paragraph.

-Figure 4. The raw mass spec data could be moved to the Supplement and plotted/displayed such that non-experts can understand the data.

-The manuscript should be checked for the use of proper English.

Referee #2:

This manuscript entitled 'JMJD5 Proteolytically Processes Monomethylated Histone H3 N-tail' is interesting. Although there has been significant progress recently but still understanding of clipping

process of histone tails is very poor. Following are my concerns:

1. Authors need to show specificity of JMJD5. Does it specifically cleave Histone H3 or it can also act on other core histones.

2. What is the cellular significance of H3 clipping by JMJD5 is not clear. Does it also cleave chromatin bound histone H3?

3. How long recombinant JMJD5 was incubated (minutes or hours) with histone H3 to assess the Clipping in vitro?

4. One thing is not clear to me is that when H3 is cleaved, the amount of intact H3 is not decreasing. Time dependent incubation assay should be performed to examine clipping activity of JMJD5. How many clipping products are generated will also be clear from this experiment.

5. What the clipping site is is also not clear.

6. It will be better to perform starvation experiments to see dynamics of H3 clipping. As it is starvation induced process shown in this study, cells can be allowed to grow further in normal medium to see whether or not clipping stops and expression of JMJD5 comes to normal level.

Referee #3:

In this manuscript, the authors investigated JMJD5 potential role in histone H3 N-tail cleavage. They found that JMJD5 could cleave H3 peptides in vitro and that the cleavage was affected by methylation status of the peptides. In light of this, the authors further explored cleavage activity in cell line and proposed a model for gene expression regulation. Although the proposed histone tail cleavage activity of JMJD5 would in principle be quite novel and interesting, many experiments presented at current stage are too weak to fully support this model.

Main concerns:

1. By using antibodies against H3 C-terminal region or modified H3 tails, the authors observed a faster migrating band and hypothesized it as N-tail clipping fraction of H3 for the following study. However, without isolating the band for mass-spec identification, the proposed "cleavage" of histone tails lacks credibility. Moreover, throughout the manuscript, protein standard is missing from all western blot figures, which makes it hard to interpret the data.

2. To fully characterize JMJD5 in vivo cleavage activity and clearly map cleavage site(s), the authors should adopt either Edman degradation assay or mass-spec coupling with GluC digestion (instead of direct trypsin digestion the authors used) after JMJD5 incubation with purified histone H3. Based on current data with different antibodies targeting H3 modifications, no solid conclusion of JMJD5 cleavage activity should be drawn.

3. The authors tried to link the in vitro histone H3 cleavage activity to gene transcription regulation by over-expression of wild type or N-term JMJD5. However, no direct evidence of JMJD5 binding or histone cleavage at corresponding loci is provided.

Comments on specific figures:

1. In Figure 1B, JMJD5 expression at no starvation condition is higher than that at starvation release 0h, but starvation release 0h has more "cleavage" product exemplified by faster moving K27me2 band. This anti-correlation is in conflict with the statement that JMJD5 is responsible for H3 N-term cleavage. Also, the K27me2 signal doesn't seem to significantly increase upon release (0h, 1h, 3h signals are about the same).

2. Based on Figure 2D, the authors suggested that H3 might switch the protein interaction via S10 and/or T3 phosphorylation. However, S10->A or T3->A mutation can only address the importance of corresponding amino acid. Direct experiments targeting specific phospho-modifications are needed to make this claim.

3. Figure 3E and 3F, coomassie blue staining for purified his-JMJD5 is needed here to show the purity of enzyme.

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4. Figure 5A, the K14me2, K36me1 and K79me2 data are not very convincing (myc-JMJD5 added sample doesn't exhibit significantly more cleaved product).

5. To support the notion that JMJD5 cleaves between K9 and S10 residues, mass-spec data in Table S1 should at least contain "STGGK" peptide which starts with S10. However, neither S1(i) nor S1(ii) contains such peptide, which left the statement unconvincing.

6. Figure 5H, H3K9me1 ChIP signal decreases upon FL-JMJD5 overexpression does not prove the cleavage of N-term H3 (it is possible that H3K9me1 was demethylated/hydroxylated by JMJD5 overexpression). More direct evidence would come from N-term H3 ChIP. Also, an important control experiment is lacking here, which is ChIP of JMJD5 to show overexpression of JMJD5 actually leads to more JMJD5 binding on corresponding promoters.

1st Revision - authors' response 26 June 2017

POINT-BY-POINT RESPONSE

Referee #1:

Chin and colleagues have identified a potentially novel role for the histone demethylase JMJD5 in clipping histone H3 at monomethylated K9, particularly during 'stress' conditions. This result is quite intriguing, as this protein has not been implicated in histone tail clipping to date. However, more controls and additional experiments are required to fully convince the reader that this is the case. First, it may be likely that the clipping actually occurs upon G1 arrest (starvation/double thymidine bock), and this has not been carefully considered. The term 'stress' has been used loosely here and the conditions under which the clipping event occurs needs to be defined.

(We provided new experiments to address this question (Fig EV1E))

Moreover, it is unclear how clipping by JMJD5 is related to its demethylase functions for K36me2 - are these through the same regions of the JmjC domain and residues?

(We discussed this in Discussion section)

Also, it has not been addressed whether other JmjC-containing demethylases might contain such activity.

(We did experiment to show that JMJD4 also contains a much weaker histone H3 N-tail clipping activity (Fig EV2J))

Importantly, the role of the protease activity as well as the clipping event to remove H3K9me1 remains unclear.

(Please see responses below for this question and Result section in page 11, line 4-7)

Major Comments:

-The fact that this clipping event occurs under 'stress' conditions should be emphasized in both the title and abstract. However, an alternative explanation is a G1 arrest/quiescence situation induces cleavage because both conditions used in this study will arrest cells in G1/S. The authors should utilize other treatments that occur outside of G1/S and look at this clipping event in other conditions that induce senescence or quiescence (see next point). In summary: the cellular event that triggers this clipping needs to be better defined.

Answer: We have modified manuscript title to emphasize stressed conditions. Various agents that arrest cells at different phases of cell cycle were used to test histone H3 N-tail cleavage under different stressed conditions (Fig EV1E). Flow cytometry analysis showed that short term treatment of cells by these reagents arrested cells at the S or G2 phase with or without initiation of senescence. Western blot showed concomitantly induced expression of JMJD5 and histone H3 N-tail cleavage between K9 and S10 residue. However, RO-3306, a reagent that arrested cells at the G2 phase, cannot induce H3 N-tail cleavage. Among all reagents employed in the experiment, only etoposide induced senescence (Fig EV1E).

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-The authors neglect to site two important papers that recently found new biological contexts that display H3 tail cleavage, which may be relevant to their studies:
*Histone H3.3 and its proteolytically processed form drive a cellular senescence programme. Duarte LF, Young AR, Wang Z, Wu HA, Panda T, Kou Y, Kapoor A, Hasson D, Mills NR, Ma'ayan A, Narita M, Bernstein E. Nat Commun. 2014 Nov 14;5:5210. doi: 10.1038/ncomms6210. PMID:25394905
*MMP-9 facilitates selective proteolysis of the histone H3 tail at genes necessary for proficient osteoclastogenesis. Kim K, Punj V, Kim JM, Lee S, Ulmer TS, Lu W, Rice JC, An W. Genes Dev. 2016 Jan 15;30(2):208-19. doi: 10.1101/gad.268714.115. PMID: 26744418

Answer: We cited and discussed both papers in the manuscript.

-Do the authors know which H3 variant is being cleaved? H3.1, H3.2 or H3.3? This could be determined from the mass spec analyses if residue 31 (S/A) of H3 was identified.

Answer: We cotransfected Myc tagged JMJD5 with C-terminally HA tagged H3.1, H3.2 or H3.3 expression vectors into cells. Western blot showed that all three H3.1, H3.2 or H3.3 variants can be cleaved by JMJD5, with less efficient cleavage of H3.1 and H3.2 by JMJD5 (Fig EV2H).

-Alignment of the secondary structure of JMJD5 and Cathepsin L is insufficient to claim structure-function. Are there crystal structures available that could be super-imposed? It remains mechanistically unclear how JMJD3 possesses peptide protease activity.

Answer: We compared crystal structure of JMJD5 (PDB: 4gjz) and Cathepsin L1 (PDB: 2xu3) in Fig 3A.

-A discussion about the protease vs. demethylase activity is required, especially since the H3 binding activity seems to occur through the JmjC domain. This is only touched upon in the discussion section.

Answer: We profoundly discussed protease vs. demethylase activity of JMJD5 in the Discussion section.

-What is the significance of removing K9me1 from the H3 tail?

Answer: It has been reported that higher H3K9me1 levels were detected in more active promoters surrounding the transcriptional start sites (TSS), suggesting that this modification may be associated with transcriptional activation. H3K9me1 modifications positively correlated with the levels of gene expression. Furthermore, HK9me1 is enriched at DNase hypersensitive (HS) sites, which is used as a marker of functional enhancer elements. All these studies suggest the positive role of H3K9me1 in transcriptional activation. Therefore, removing K9me1 from H3 N-tail may lead to repression of gene transcription activation (Barski, Cuddapah et al., 2007).

Minor comments:

-page 5. line 2. The clipping events should be described as the residues clipped in between or clarify the residue before or after the cleavage. e.g. Cathepsin L cleaves between A21 and T22, creating a new N-terminus starting at residue T22.

Answer: We revised these sentences in the manuscript.

-page 6. 1st paragraph. The authors speculate that the 'faster migrating band was most likely the species previously reported.....' citing reference 17 which describes H3 tail clipping in Tetrahymena. The authors have no reason to speculate this without aligning the faster migrating band to other H3 cleaved species. They could only speculate this after the PTM analyses described in this paragraph.

Answer: We have deleted this sentence.

-Figure 4. The raw mass spec data could be moved to the Supplement and plotted/displayed such that non-experts can understand the data.

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Answer: Fig 4 was rearranged. Original Fig 4A, C, E, F were simplified as Fig 4A, B, C, D. Original Fig 4D was deleted and original Fig 4B, G and H were moved to Fig EV3A, C, B.

-The manuscript should be checked for the use of proper English.

Answer: We checked English grammar in the manuscript.

Referee #2:

This manuscript entitled 'JMJD5 Proteolytically Processes Monomethylated Histone H3 N-tail' is interesting. Although there has been significant progress recently but still understanding of clipping process of histone tails is very poor. Following are my concerns:

1. Authors need to show specificity of JMJD5. Does it specifically cleave Histone H3 or it can also act on other core histones.

Answer: We transfected JMJD5 expression vector into cells and then checked the cleavage of endogenous H2A, H2B, H3 and H4. Western blot using antibodies targeting histone C-terminal regions showed that only H3 can be cleaved by JMJD5, suggesting specificity of JMJD5 towards H3 (Fig EV2G).

2. What is the cellular significance of H3 clipping by JMJD5 is not clear. Does it also cleave chromatin bound histone H3?

Answer: Histone H3 N-tail is subjected to methylation at K4 and K9. H3K4me1/2/3 and H3K9me1 locate at gene transcription start sites and/or enhancer region, which are associated with gene transcription activation (Barski et al., 2007, Kouzarides, 2007). Therefore, H3 N-tail clipping at site of K9me1 may result in repression of gene transcription activation at some gene promoters. We extracted chromatin from A549 cells transfected with Myc-JMJD5. Western blot was performed to show that chromatin bound histone H3 can also be cleaved by JMJD5 (Fig EV2I).

3. How long recombinant JMJD5 was incubated (minutes or hours) with histone H3 to assess the Clipping in vitro?

Answer: Recombinant JMJD5 was incubated with histone H3 for 2-3 hours in vitro to assess cleavage event.

4. One thing is not clear to me is that when H3 is cleaved, the amount of intact H3 is not decreasing. Time dependent incubation assay should be performed to examine clipping activity of JMJD5. How many clipping products are generated will also be clear from this experiment.

Answer: Decreased level of intact H3 can be seen sometimes, but not always, after incubation with JMJD5. This may due to limited amount of cleavage product compared to uncleaved H3. We extended incubation time to 6 hours and result showed gradually reduced level of intact H3 and increased level of cleaved H3 (Fig 3F).

5. What the clipping site is is also not clear.

Answer: In revised manuscript, we used newly made antibody H3KS (Fig EV5C) targeting histone H3 that lacks N-terminal amino acid 1-9 to further confirm clipping site locates between K9 and S10 (Fig 1B, C; Fig 5A; Fig EV1D, 1E, 2J).

6. It will be better to perform starvation experiments to see dynamics of H3 clipping. As it is starvation induced process shown in this study, cells can be allowed to grow further in normal medium to see whether or not clipping stops and expression of JMJD5 comes to normal level.

Answer: We did time course experiment to test H3 clipping after cells were serum starved and released. Cleavage extent of H3 N-tail gradually reduced after further culture of cells in normal medium for over 5-10 hours. Reduced H3 clipping event correlated with reduced expression of JMJD5 (Fig EV1D).

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Referee #3:

In this manuscript, the authors investigated JMJD5 potential role in histone H3 N-tail cleavage. They found that JMJD5 could cleave H3 peptides in vitro and that the cleavage was affected by methylation status of the peptides. In light of this, the authors further explored cleavage activity in cell line and proposed a model for gene expression regulation. Although the proposed histone tail cleavage activity of JMJD5 would in principle be quite novel and interesting, many experiments presented at current stage are too weak to fully support this model.

Main concerns:

1. By using antibodies against H3 C-terminal region or modified H3 tails, the authors observed a faster migrating band and hypothesized it as N-tail clipping fraction of H3 for the following study. However, without isolating the band for mass-spec identification, the proposed "cleavage" of histone tails lacks credibility. Moreover, throughout the manuscript, protein standard is missing from all western blot figures, which makes it hard to interpret the data.

Answer: We have changed this sentence in revised manuscript. Protein standard was added in revised Figures.

2. To fully characterize JMJD5 in vivo cleavage activity and clearly map cleavage site(s), the authors should adopt either Edman degradation assay or mass-spec coupling with GluC digestion (instead of direct trypsin digestion the authors used) after JMJD5 incubation with purified histone H3. Based on current data with different antibodies targeting H3 modifications, no solid conclusion of JMJD5 cleavage activity should be drawn.

Answer: For Edman degradation assay, we purified cleaved histone H3 and sent samples to commercial biotech company to perform experiment for us; however, we do not receive any positive or negative responses from this company until now. Currently we are seeking another biotech company to perform this experiment for us. In revised manuscript, we used newly made H3KS antibody (Fig EV5C) that targeting N-terminal of histone H3 lacking 1-9 amino acids to clearly show that histone H3 was cleaved by JMJD5 between K9 and S10 (Fig 1B, C; Fig 5A; Fig EV1D, 1E, 2J).

3. The authors tried to link the in vitro histone H3 cleavage activity to gene transcription regulation by over-expression of wild type or N-term JMJD5. However, no direct evidence of JMJD5 binding or histone cleavage at corresponding loci is provided.

Answer: By using H3KS antibody targeting histone H3 lacking 1-9 amino acids and Flag antibody, ChIP assays for specific gene promoters were performed to show linkage of JMJD5 DNA binding activity with H3 N-terminal cleavage at specific chromosomal loci (Fig 5H).

Comments on specific figures:

1. In Figure 1B, JMJD5 expression at no starvation condition is higher than that at starvation release 0h, but starvation release 0h has more "cleavage" product exemplified by faster moving K27me2 band. This anti-correlation is in conflict with the statement that JMJD5 is responsible for H3 N-term cleavage. Also, the K27me2 signal doesn't seem to significantly increase upon release (0h, 1h, 3h signals are about the same).

Answer: This result may probably due to variations between different experiments and/or different cultured cell status. We repeated this experiment several times and the statistical analysis showed positive correlation between expression level of JMJD5 and extent of H3 cleavage (Fig 1B).

2. Based on Figure 2D, the authors suggested that H3 might switch the protein interaction via S10 and/or T3 phosphorylation. However, S10->A or T3->A mutation can only address the importance of corresponding amino acid. Direct experiments targeting specific phospho-modifications are needed to make this claim.

Answer: We have changed this sentence to underline the importance of indicated amino acids, but not their phosphorylation form, in binding with JMJD5.

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3. Figure 3E and 3F, coomassie blue staining for purified his-JMJD5 is needed here to show the purity of enzyme.

Answer: Coomassie blue staining for purified his-JMJD5 was shown in Fig EV2E, F.

4. Figure 5A, the K14me2, K36me1 and K79me2 data are not very convincing (myc-JMJD5 added sample doesn't exhibit significantly more cleaved product).

Answer: We repeated this experiment again by adding newly made antibody H3KS that targeting histone H3 lacking 1-9 amino acids. Results are shown in Fig 5A.

5. To support the notion that JMJD5 cleaves between K9 and S10 residues, mass-spec data in Table S1 should at least contain "STGGK" peptide which starts with S10. However, neither S1(i) nor S1(ii) contains such peptide, which left the statement unconvincing.

Answer: We did this experiment several times, however, for some unknown reasons we cannot recover peptides containing amino acid K9 and S10 by general tryptic digestions and MS analysis. Tan et al. reported that tryptic digestions of histones tend to yield peptides that are relatively small and hydrophilic, which are difficult for subsequent detection by MS, therefore, special method is needed to recover short peptide after trytic digestion and detection by MS (Tan, Luo et al., 2011).

6. Figure 5H, H3K9me1 ChIP signal decreases upon FL-JMJD5 overexpression does not prove the cleavage of N-term H3 (it is possible that H3K9me1 was demethylated/hydroxylated by JMJD5 overexpression). More direct evidence would come from N-term H3 ChIP. Also, an important control experiment is lacking here, which is ChIP of JMJD5 to show overexpression of JMJD5 actually leads to more JMJD5 binding on corresponding promoters.

Answer: We used antibody targeting H3 lacking 1-9 amino acids (H3KS), Flag antibody and H3 antibody to perform ChIP assay after cells were transfected with Flag tagged JMJD5. Result showed that level of N-terminal cleaved H3 was enhanced at specific gene promoters, where exogenous Flag tagged JMJD5 was also enriched, suggesting positive correlation between occupation of JMJD5 and cleavage of H3 at specific gene promoters (Fig 5H).

References:

Barski A, Cuddapah S, Cui KR, Roh TY, Schones DE, Wang ZB, Wei G, Chepelev I, Zhao KJ (2007) High-resolution profiling of histone methylations in the human genome. Cell 129: 823-837
Kouzarides T (2007) Chromatin modifications and their function. Cell 128: 693-705
Tan MJ, Luo H, Lee S, Jin FL, Yang JS, Montellier E, Buchou T, Cheng ZY, Rousseaux S, Rajagopal N, Lu ZK, Ye Z, Zhu Q, Wysocka J, Ye Y, Khochbin S, Ren B, Zhao YM (2011) Identification of 67 Histone Marks and Histone Lysine Crotonylation as a New Type of Histone Modification. Cell 146: 1015-1027

2nd Editorial Decision 01 August 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study (you will find enclosed below). The original referee #3 was not able to look into this again. However, we asked referee #1 to evaluate if the concerns of referee #3 have been adequately addressed. As you will see, the referees now support the publication of your manuscript in EMBO reports. Nevertheless, the referees still have concerns and suggestions that we ask you to address in a final revision. In particular, the cause of stress-dependent JMJD5 cleavage needs to be clarified, and the new antibody needs to be fully characterised and described (points on referee #3 by referee #1).

Further, I have the following editorial requests that also need to be addressed in a final revised version:

Please provide all Western blot panels in high resolution (e.g. 300 px/inch). Some panels seem to contain compression artefacts. Please refer to our guidelines:
http://embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

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Please also provide an ORCID for all the co-corresponding authors (Shao and Huang) and link it to their EMBO reports profile.

I look forward to seeing the final 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 (own points):

The authors have addressed many of the previous concerns, however, further revisions are required.

1. In the sentence: "Histone H3 N-terminal region proteolytic cleavage by Cathepsin L has been shown to be required during early differentiation of mouse embryonic stem cell (mESC) [7]", the authors also need to cite Duarte et al., who show CTSL is also required for cleavage of H3 in cells undergoing senescence.

2. The authors have added a WB panel from treatments to cells in order to show which ones induce cleavage (EV1E). Again this is in line with exogenous stress, but it is still unclear which pathways are driving the cleavage to happen. It is an effect or cell cycle arrest or DNA damage? Serum starvation and TdR should arrest the cell cycle in G1, but CPT and ETO cause DNA damage. The treatment of RO-3306, which is a CDK1 inhibitor and blocks the cells in G2 does not induce cleavage, nor does JMJD5 levels increase. The cause of cleavage needs to be clarified. Stress is a general term. What about UV damage, gamma irradiation, or other cell cycle blocking agents?

3. The authors show preferential cleavage of H3.3, which is consistent with what Duarte et al. reported in senescent cells. This should be discussed.

4. The authors have generated a new antibody called H3KS, which they show right away in Figure 1 and throughout the rest of the figures. However, it is not described in the text. Moreover, there needs to be a complete characterization of this new antibody. The Supp Fig EV5C showing that the antibody recognized H310-28 peptides and not H31-9 is not sufficient. How was the antibody generated? Most of these antibodies cross-react with H3, was this purified? The authors MUST align the bands with the H3 C-term or other PTM antibodies by probing multiple strips of the same membrane to show this is the EXACT same band (again see Duarte et al).

Referee #3's points:

In this revision, Shen et al have largely addressed the main concerns. Previously, it was recommended to use Edman degradation or MS with GluC digest after JMJD5 incubation with purified H3. The authors state they have not gotten this experiment to work, however instead they have generated a new antibody (H3KS) targeting the N-terminus of H3 lacking amino acids 1-9. Because this antibody is a key reagent in the revised manuscript (Western blots and ChIP), it must be fully characterized and described. The methods only state the antibody was generated by a biotech company in China. Is it commercially available? Was it generated with peptides? Or recombinant H3? How can the authors be sure it is the same band as the cleavage product seen with the histone modification antibodies? Once this is sufficiently clarified, the manuscript will be suitable for publication.

Referee #2:

Comments that were asked by the reviewers have almost been incorporated in revised manuscript. My biggest question is about specificity of JMJD5 towards H3 and the nature of the protease; effects of protease inhibitors. Does it specifically cleave H3 or can cleave other core histones and related substrates? Experimental supports in many cases is not sufficient. For example, one query was to test activity of JMJD5 on chromatin and other core histones substrates but has not been addressed sufficiently. Authors did an experiment to detect chromatin associated clipped H3 by western blotting. Pure JMJD5 has been shown to cleave pure H3 through an in vitro biochemical experiment. Same experiment should have also been conducted by taking chromatin as substrate as well other pure core histones to examine the specificity of the enzyme upon increasing concentration of JMJD5 or reaction incubation time (2 - 6 hours). Moreover, authors should have also analyzed presence or

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absence of other core histones in pull down (Fig 2 GST pull down) to examine the interaction between JMJD5 and the core histones.

2nd Revision - authors' response 28 August 2017

We have revised the manuscript by following the constructive suggestions and comments raised by reviewer. We hope our revisions will satisfy the reviewersí concerns. The publication guidelines have been followed including those responsibilities of the corresponding authors. We wish you and your colleagues will enjoy and appreciate our work present in this manuscript. Your consideration of our work for a possible publication in EMBO report is highly appreciated.

POINT-BY-POINT RESPONSE

Referee #1 (own points):

The authors have addressed many of the previous concerns, however, further revisions are required.

1. In the sentence: "Histone H3 N-terminal region proteolytic cleavage by Cathepsin L has been shown to be required during early differentiation of mouse embryonic stem cell (mESC) [7]", the authors also need to cite Duarte et al., who show CTSL is also required for cleavage of H3 in cells undergoing senescence.

Answer: We cited this paper in the manuscript.

2. The authors have added a WB panel from treatments to cells in order to show which ones induce cleavage (EV1E). Again this is in line with exogenous stress, but it is still unclear which pathways are driving the cleavage to happen. It is an effect or cell cycle arrest or DNA damage? Serum starvation and TdR should arrest the cell cycle in G1, but CPT and ETO cause DNA damage. The treatment of RO-3306, which is a CDK1 inhibitor and blocks the cells in G2 does not induce cleavage, nor does JMJD5 levels increase. The cause of cleavage needs to be clarified. Stress is a general term. What about UV damage, gamma irradiation, or other cell cycle blocking agents?

Answer: Serum starvation or TdR, but not RO-3306, has been reported to cause DNA damage response [1-3]. Indeed, we did experiments to show that serum starvation, TdR, CPT and ETO treatment of cells caused increased expression of H2AX, a marker of DNA damage response (Fig EV1G), as well as cleavage of H3 N-tail (Fig 1A, 1B; Fig EV1E) On the contrary, G2 cell cycle inhibitor RO-3306 induced neither H2AX expression nor H3 N-tail cleavage (Fig EV1E, 1G). Altogether, these results indicated that it is stress-induced DNA damage response, but not cell cycle arrest itself, caused cleavage of H3, although DNA damage response can also cause cell cycle arrest. We did experiment to show that UV treat of cells also caused cleavage of H3 (Fig EV1F).

3. The authors show preferential cleavage of H3.3, which is consistent with what Duarte et al. reported in senescent cells. This should be discussed.

Anwer: We discussed this in Discussion section (page 14, line 4-9).

4. The authors have generated a new antibody called H3KS, which they show right away in Figure 1 and throughout the rest of the figures. However, it is not described in the text. Moreover, there needs to be a complete characterization of this new antibody. The Supp Fig EV5C showing that the antibody recognized H310-28 peptides and not H31-9 is not sufficient. How was the antibody generated? Most of these antibodies cross-react with H3, was this purified? The authors MUST align the bands with the H3 C-term or other PTM antibodies by probing multiple strips of the same membrane to show this is the EXACT same band (again see Duarte et al).

Answer: To further validate H3KS antibody, we constructed C-terminal Flag tagged expression vector for H3 1-9(amino acid 1-9 were deleted) and full length H3. After transfection of cells with these vectors, cell lysate was blotted with either Flag or H3KS antibodies (Fig EV5C, lower panel). Results showed that H3KS specifically recognized H3 with deletion of amino acid 1-9. Furthermore, in experiment shown in Fig EV1F, we blotted membrane first with H3 C-term antibody, then the same membrane was stripped and blotted

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with H3KS antibody, result showed that cleaved H3 band detected by H3 C-term antibody was also recognized by H3KS antibody (Fig EV1F). All these results indicate that H3KS antibody is specific for sub-band produced from H3 cleavage between K9 and S10. Generation of H3KS antibody: a 2x branched peptide corresponding to histone H3 amino acid sequence 10-14 was conjugated to KLH and injected into rabbits. Serum was collected, purified and tested for specificity as described in Fig EV5C.

Referee #3's points:

In this revision, Shen et al have largely addressed the main concerns. Previously, it was recommended to use Edman degradation or MS with GluC digest after JMJD5 incubation with purified H3. The authors state they have not gotten this experiment to work, however instead they have generated a new antibody (H3KS) targeting the N-terminus of H3 lacking amino acids 1-9. Because this antibody is a key reagent in the revised manuscript (Western blots and ChIP), it must be fully characterized and described. The methods only state the antibody was generated by a biotech company in China. Is it commercially available? Was it generated with peptides? Or recombinant H3? How can the authors be sure it is the same band as the cleavage product seen with the histone modification antibodies? Once this is sufficiently clarified, the manuscript will be suitable for publication.

Answer: H3KS antibody was generated as follows: a 2x branched peptide corresponding to histone H3 sequence 10-14 was conjugated to KLH and injected into rabbits. Serum was collected, purified and tested for specificity as described in Fig EV5C. This antibody is not commercially available at present. To further validate H3KS antibody, we constructed C-term Flag tagged expression vector for H3 1-9(amino acid 1-9 were deleted) and full length H3. After transfection of cells with these vectors, cell lysate was blotted with either Flag or H3KS antibodies (Fig EV5C, lower panel). Results showed that H3KS specifically recognized H3 with deletion of amino acid 1-9. Furthermore, in experiment shown in Fig EV1F, we blotted membrane first with H3 C-term antibody, then the same membrane was stripped and blotted with H3KS antibody, result showed that cleaved H3 band detected by H3 C-term antibody was also recognized by H3KS antibody (Fig EV1F). All these results indicate that H3KS antibody is specific for sub-band produced from H3 cleavage between K9 and S10.

Referee #2:

Comments that were asked by the reviewers have almost been incorporated in revised manuscript. My biggest question is about specificity of JMJD5 towards H3 and the nature of the protease; effects of protease inhibitors. Does it specifically cleave H3 or can cleave other core histones and related substrates? Experimental supports in many cases is not sufficient. For example, one query was to test activity of JMJD5 on chromatin and other core histones substrates but has not been addressed sufficiently. Authors did an experiment to detect chromatin associated clipped H3 by western blotting. Pure JMJD5 has been shown to cleave pure H3 through an in vitro biochemical experiment. Same experiment should have also been conducted by taking chromatin as substrate as well other pure core histones to examine the specificity of the enzyme upon increasing concentration of JMJD5 or reaction incubation time (2 - 6 hours). Moreover, authors should have also analyzed presence or absence of other core histones in pull down (Fig 2 GST pull down) to examine the interaction between JMJD5 and the core histones.

Answer: We did experiment under the same experimental condition as shown in Fig. 3F to see whether other core histones including H2A, H2B and H4 are cleaved by JMJD5 (Fig EV2G). Results showed that none of those core histones were cleaved by JMJD5 under the experimental condition that resulted in cleavage of H3 N-tail. As H2A, H2B, H3 and H4 forms core histone complex, we predict that JMJD5 may interact with H2A, H2B and H4 indirectly without cleave their N-tails.

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3rd Editorial Decision 07 September 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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The data shown in figures should satisfy the following conditions:

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

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- 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.
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- 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.
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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.

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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).	Antibodies are provided with catalog number,page 15. Supplementary information Fig EV5C.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were originally purchased from ATCC and no mycoplasma contamination. Page 17.

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