Flexibility in crosstalk between H2B ubiquitination and H3 methylation in vivo

Hanneke Vlaming, Tibor van Welsem, Erik L. de Graaf, David Ontoso, A.F. Maarten Altelaar, Pedro A. San-Segundo, Albert J.R. Heck and Fred van Leeuwen

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Review timeline:

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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.)

Editor: Esther Schnapp

1st Editorial Decision 07 April 2014

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports and referee cross-comments that are copied below.

As you will see, the referees acknowledge that the findings are potentially interesting and the data of high quality. However, they also all point out that functional analyses of the permanently ubiquitinated histones and their effect on gene transcription are required to strengthen the study. All referees mention that the effect of the histone-ubiquitin fusion proteins on gene expression and/or DNA repair in bre1 mutants should be investigated, and referee 3 adds that it should be demonstrated that the fusion proteins are correctly folded and incorporated into chromatin. The referees also indicate where further discussion and more experimental details are required, and overstatements that need to be avoided.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. 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. Please contact us if a 3 months time frame is not sufficient for the revision. Also, the revised manuscript may not exceed 30,000 characters (including spaces, references and figure legends) and 5 main plus 5 supplementary figures, which should directly relate to their corresponding main figure. The current manuscript text exceeds our limits, and the text therefore needs to be shortened. The Results and Discussion sections can be combined, which may help to eliminate some redundancy that is inevitable when discussing the same experiments twice. Commonly used materials and methods can further be moved to the supplementary information, but please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many experiments were performed, and the bars and error bars (e.g. mean +/- SEM, SD) in the respective figure legends. Error bars cannot be shown when less than 3 independent experiments were performed. We strongly recommend that you perform 3 or more experiments and include statistical analyses and error bars. If n<3, please remove the error bars and show all data points in the graphs along with the mean.

We now 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:

Summary:

The manuscript addresses the mechanism of the "histone crosstalk" pathways linking ubiquitination of histone H2B (H2Bub1) to the methylation of histone H3 at lysines 79 and 4 (H3K79me and H3K4me, respectively). Based upon previous in vitro studies documenting plasticity in the required positioning of the ubiquitin moiety on the nucleosome, the authors developed an elegant system for testing the plasticity of the crosstalk pathways in vivo in S. cerevisiae. Various ubiquitin-histone fusion proteins were introduced into a yeast strain deficient in the ubiquitin ligase Bre1. The fusion proteins introduced ubiquitin onto the N- and C-termini of histone H2A, as well as the C-terminus of histone H2B. Rescue of histone crosstalk and consequent histone H3 methylation was assessed by western blotting and mass spectrometry analyses. The findings confirm and extend previous in vitro studies.

General comments:

This is a well-designed and clearly presented study that will be of significant interest to the chromatin field. The data are of high quality and support the stated conclusions, as summarized in the Discussion. I would argue that these conclusions do not constitute a huge conceptual advance in understanding the mechanism of histone crosstalk, as they largely confirm the previous in vitro work. However, this study leads to several novel conclusions that merit its publication in EMBO reports. These include the function of genetically-encoded ubiquitin-histone fusion proteins in the crosstalk pathways in vivo and the independence of crosstalk on other putative targets of the Rad6-Bre1 ubiquitination machinery. Moreover, the system developed by the authors will be of great use in studying functions of H2Bub1 unrelated to downstream methylation which have not been recapitulated in any in vitro system to date. I wonder if the authors have considered adding such a functional assay to this study. For example, the function of the fusion proteins in rescuing the bre1 defect in GAL1 induction could be determined. This defect is known to be independent of H3K4me
and H3K79me. Such a finding may make give the story a broader appeal, given that the crosstalk pathway (at least for H3K4me) may not be universally used in metazoans.

Specific comments:

1. The lethality of HA-S19 in combination with bre1Δ (and its partial rescue by addition of the ub; Figure 2B) is striking and not discussed at all. This is presumably due to some shared function of Bre1 and the H2A N-terminus. Have the authors characterized this any further? Some comment (if only to refer to unpublished results) seems warranted.

2. It is unclear what is to be concluded from the Dot1 protein levels shown in Figure S1C. Are Dot1 levels reduced in the fusion protein strains? Some comment on the significance of this result should be included.

3. There are no legends for the Supplemental Figures (that I could find).

Referee #2:

In this manuscript by Vlaming, et al., the authors utilize various histone replacement yeast mutants to address the flexibility of the placement of ubiquitin on the nucleosome for eliciting cross-talk between histone H2B monoubiquitination (H2Bub) and the methylation of lysine 79 of histone H3 (H3K79me). This question is important since it is currently unknown how H2Bub elicits its effects. Various models have been proposed for the function of ubiquitin, but the data which really address this aspect are very sketchy. The authors have done an excellent job of developing a nice, simple system which clearly addresses this.

The EMBO reports website states the following regarding the suitability for publication. Below are my comments to each of the criteria with relation to this manuscript.

1. Appropriate length and format for the type of article submitted

Yes, the article has an appropriate length for EMBO reports.

2. Physiological/functional relevance demonstrated (detailed insight into the mechanism is not always necessary)

Overall, the paper provides a nice new insight into the flexibility of the placement of a ubiquitin residue within the nucleosome for the effective cross-talk to Dot1-mediated H3K79 methylation. However, a few additional and conclusive physiological studies could significantly increase the interest and novelty of the manuscript (see below).

3. Strong evidence for the conclusions that are drawn

Overall, yes. The data presented are of a very high quality and the results are not over-interpreted, but rather reflect a fair an appropriate conclusion.

4. Novelty (abstracts, meeting reports & online preprints do not compromise novelty)

Overall the point of the paper is a fairly straight-forward and somewhat subtle one (i.e., that ubiquitin simply has to be close to the correct place and in the correct orientation in order to affect H2Bub). Some suggestions to increase the novelty are included below.

5. Broad biological significance

The findings are certainly of importance for the field of epigenetics. They are rather specific for histone H2B ubiquitination, but do provide sufficient and important new information such that it seems appropriate for EMBO reports.

6. Importance to the specific field
In fact, these findings are probably very significant for our understanding of H2Bub and will likely become frequently cited in the field.

Here are a few points that, if addressed, would significantly increase the potential impact of this paper:

1. What happens to H2Bub/SAGA-dependent genes in this system. As an example, the authors could investigate the GAL1 and SUC2 genes as investigated by Henry, et al. The authors discuss briefly about the ubiquitination/deubiquitination cycle. It would be very interesting to know what effect the expression of the fusion proteins have on these genes in Bre1-mutant cells (i.e., can the Henry ubiquitination/deubiquitination model be substantiated in this system?).

2. Another potential physiologically interesting system the authors could investigate (potentially as an alternative to #2) would be to investigate whether expression of the fusion proteins rescues the DNA repair defects observed in the absence of Bre1.

3. Do the ubiquitin-fusion proteins have other lysine residues required for branching (i.e., K48, K63, etc.)? Are the results comparable (i.e., for the H2A N-terminal fusion) with a K0-ubiquitin fusion protein which cannot support ubiquitin branching?

Referee #3:

In the manuscript "Flexibility in crosstalk between H2B ubiquitination and H3 methylation in vivo" Vlaming et al. investigate the plasticity of the positioning of covalently attached ubiquitin to histones with respect to promoting methylation of histone 3 on lysine 4 and 79. Crosstalk between these sites of histone modification has been described several years ago. However, the exact molecular details of this phenomenon are still debated. In particular, the signalling mode of ubiquitin attached to histones is not fully understood. One on hand it has been suggested that ubiquitination affects chromatin dynamics and structure. On the other hand specific binding proteins to this mark have been identified.

The authors engineered several constructs of H2A and H2B trying to mimick constitutive ubiquitination in vivo in budding yeast. They quantified the levels of H3K4 and H3K79 methylation in ubiquitination-deficient background (mutant yeast strains) compared to WT and engineered tagged but not ubiquitinated H2A and H2B expressing strains. The authors deduce from their results a certain plasticity of the positioning of the ubiquitin polypeptide, where ubiquitin attachment sites close to the native H2BK123 show higher levels of H3K4/K79 methylation compared to sites of attachment further from the native position.

The claim made by the authors is intriguing as it potentially provides evidence for plasticity in histone ubiquitination and general modification cross-talk. However, in present form the main conclusions are not fully supported by the data and the study lacks in depth of analysis and controls. The authors tend to overstate/overinterpret their findings.

- The major point of concern is the fact that the ubiquitination is constitutive and uniform across the whole genome and as such is at incomparable levels with wt levels of H2Bub. Huge excess of ubiquitinated histones in the engineered strains should therefore result in significant increase of these methylation levels. The effects on both methylation sites tested (measured by western blot) is comparable, or lower compared to wt strains, and in some cases practically absent for the trimethyl mark. Moreover, monomethylation (and in some cases me2) is already present in bre1delta and histone-tag constructs. These discrepancies need to be addressed conceptionally and experimentally.

- The huge increase in "functional" ubiquitination seems not to have any effect on the viability of the strains. Since ubiquitination is thought to be rather dynamic, this raises the question whether the fusion proteins are functional in first place. Additional experiments and controls are required to demonstrate that the fusion proteins are functional per se (i.e. folded correctly), are incorporated into chromatin normally and do indeed substitute for cellular ubiquitination.

- The authors need to present evidence that the ubiquitination and following methylation events lead to actual transcriptional activation (i.e. are functional) and compare transcription levels with wt strains. Shown for several different loci, this would be a proof that the ubiquitination->methylation
is functional locally and not an artefact (related to point 1).
- The MRM measurements were performed twice ("two biological replicates") and the represented
SEM merely indicates the range of those measurements. The authors need to base their statistical
analysis on three or more replicates, as the differences are only marginal in some cases (e.g. Fig.
4D). The current analysis could overestimate the effects discussed, especially taking into account
that the absolute levels of H3K79me1/2/3 between the constructs are very different (the MRM
analysis here provides only relative abundance within a strain). Additionally, more details on how
the quantification of the MRM data was performed should be provided in the methods section.
- Throughout the text the authors use strong phrasing when discussing the results of the different
constructs examined. However, in many cases the effects were restricted to the lower methylation
states (me1/2 or even only me1 which is present in bre1delta cells already). In this context words
like "dispensable", "remarkable plasticity "and "not critical" clearly overstate the findings
- The lethality of HA-S19-H2A but not the ubiquitinated version of the same truncated construct
needs to be discussed.
- Although, to the best of our knowledge, H3T80ph hasn't yet been detected in yeast, did the authors
check for its presence? The modification could lead to erroneous MRM results if not taken into
account.

Cross-comments from referee #2:

Specifically, concerning Reviewer 3's first comment, this seems like a very valid point. I would
suggest that the authors could perform a few ChIP experiments (e.g. against H3K4me3 and
H3K79me3) at a few different positions to see if the normal placement is maintained or not. Of
course, some "genome-wide" approach would be nice, but I think a few ChIP-PCRs would be
sufficient to make the point (i.e., near the TSS and upstream). My guess is that the placement will
not be changed, but the reviewer's point is very valid and well taken. Concerning his/her second
point, about the functionality, I have little concerns here (since I guess the yeast would not grow
without chromatin...), but it would be easy enough to show that the fusion protein is incorporated
into chromatin (even a simple chromatin extraction, maybe supplemented for example with MNase
data...would also address the "openness" of the chromatin with the constructs). It also seems like a
reasonable suggestion.

Cross-comments from referee #1:

Reviewer 3 is concerned about the functionality of the ubiquitin-histone fusion proteins compared to
the wild-type histones. I think these concerns need to be articulated more clearly. The fusions
maintain the essential functions of the corresponding histones as they support viability on their own.
The fact that they support the histone crosstalk (albeit to varying extents) is further evidence that the
proteins are functional and incorporated into chromatin. To what extent the fusions support other
histone functions that depend on dynamics of the ubiquitin modification is an interesting question
that warrants further investigation but does not need to be addressed immediately. One could argue
that the failure of some of the fusions to rescue crosstalk could be due to defective incorporation into
chromatin. Thus, the authors may have actually underestimated the extent of the plasticity of
crosstalk in vivo. A simple experiment to address this possibility would be to examine the levels of
H3K36me3 (not sensitive to H2Bub1 crosstalk) in the different strains by western blotting. General
defects on nucleosome integrity might be expected to affect levels of this modification. The authors
could also adapt their in vitro methylation system for Set2 (although I would be satisfied with the in
vivo experiment).
As I indicated in my review I think the quality of the data is high and I don't see the need for further
MS experiments.

Referee #1:
Summary: The manuscript addresses the mechanism of the "histone crosstalk" pathways linking
ubiquitination of histone H2B (H2Bub1) to the methylation of histone H3 at lysines 79 and 4
(H3K79me and H3K4me, respectively). Based upon previous in vitro studies documenting plasticity
in the required positioning of the ubiquitin moiety on the nucleosome, the authors developed an
elegant system for testing the plasticity of the crosstalk pathways in vivo in S. cerevisiae. Various
ubiquitin-histone fusion proteins were introduced into a yeast strain deficient in the ubiquitin ligase Bre1. The fusion proteins introduced ubiquitin onto the N- and C-termini of histone H2A, as well as the C-terminus of histone H2B. Rescue of histone crosstalk and consequent histone H3 methylation was assessed by western blotting and mass spectrometry analyses. The findings confirm and extend previous in vitro studies.

General comments: This is a well-designed and clearly presented study that will be of significant interest to the chromatin field. The data are of high quality and support the stated conclusions, as summarized in the Discussion. I would argue that these conclusions do not constitute a huge conceptual advance in understanding the mechanism of histone crosstalk, as they largely confirm the previous in vitro work. However, this study leads to several novel conclusions that merit its publication in EMBO reports. These include the function of genetically-encoded ubiquitin-histone fusion proteins in the crosstalk pathways in vivo and the independence of crosstalk on other putative targets of the Rad6-Bre1 ubiquitination machinery. Moreover, the system developed by the authors will be of great use in studying functions of H2Bub1 unrelated to downstream methylation which have not been recapitulated in any in vitro system to date. I wonder if the authors have considered adding such a functional assay to this study. For example, the function of the fusion proteins in rescuing the bre1 defect in GAL1 induction could be determined. This defect is known to be independent of H3K4me and H3K79me. Such a finding may make give the story a broader appeal, given that the crosstalk pathway (at least for H3K4me) may not be universally used in metazoans.

We thank the reviewer for the supportive comments. We appreciate the suggestion to include additional assays to address the function of the histone-ubiquitin fusion proteins. We examined the kinetics of GAL1 induction since Henry et al (Genes & Dev 2003, 17:2648) reported defects in H2BK123R mutants (as referred to by reviewer 2 and presumably this reviewer). In our hands bre1Δ mutants did not show clear GAL1 induction defects. This may be caused by strain differences. However, in a more recent study the Bhaumik lab also failed to see defects in a bre1Δ strain (Sen et al, 2013, J Biol Chem 288:9619). Therefore, as an alternative approach suggested by reviewer 2, we added a DNA damage response experiment since DNA damage response is also dependent on intact chromatin structure (new Fig. 4B, and see response to point 2 of reviewer 2).

Specific comments:
1. The lethality of HA-S19 in combination with bre1Δ (and its partial rescue by addition of the ub; Figure 2B) is striking and not discussed at all. This is presumably due to some shared function of Bre1 and the H2A N-terminus. Have the authors characterized this any further? Some comment (if only to refer to unpublished results) seems warranted.

We agree with the reviewer that the lethality suggests shared functions. The N-terminus of H2A influences the degree of H2Bub in BRE1+ cells (Zheng et al, MCB 2010 and we verified this in our strains), indeed suggesting interactions between the two sites. This is not surprising given the close proximity. We now discuss this in the legend of Supplementary Fig S1.

2. It is unclear what is to be concluded from the Dot1 protein levels shown in Figure S1C. Are Dot1 levels reduced in the fusion protein strains? Some comment on the significance of this result should be included.

The apparent small changes in Dot1 expression levels correlate with slight loading differences (see the Pgk1 control). Moreover, a decrease in Dot1 expression would lead to less methylation of H3K79. We now explicitly mention in the text that H3K79 methylation was enhanced ‘while Dot1 expression was not increased’.

3. There are no legends for the Supplemental Figures (that I could find).

We apologize for the omission. The legends have now been included in the file with Supplemental Figures.

Referee #2:
In this manuscript by Vlaming, et al., the authors utilize various histone replacement yeast mutants
to address the flexibility of the placement of ubiquitin on the nucleosome for eliciting cross-talk between histone H2B monoubiquitination (H2Bub) and the methylation of lysine 79 of histone H3 (H3K79me). This question is important since it is currently unknown how H2Bub elicits its effects. Various models have been proposed for the function of ubiquitin, but the data which really address this aspect are very sketchy. The authors have done an excellent job of developing a nice, simple system which clearly addresses this. The EMBO reports website states the following regarding the suitability for publication. Below are my comments to each of the criteria with relation to this manuscript.

1. Appropriate length and format for the type of article submitted: Yes, the article has an appropriate length for EMBO reports.
2. Physiological/functional relevance demonstrated (detailed insight into the mechanism is not always necessary): Overall, the paper provides a nice new insight into the flexibility of the placement of a ubiquitin residue within the nucleosome for the effective cross-talk to Dot1-mediated H3K79 methylation. However, a few additional and conclusive physiological studies could significantly increase the interest and novelty of the manuscript (see below).
3. Strong evidence for the conclusions that are drawn: Overall, yes. The data presented are of a very high quality and the results are not over-interpreted, but rather reflect a fair an appropriate conclusion.
4. Novelty (abstracts, meeting reports & online preprints do not compromise novelty): Overall the point of the paper is a fairly straight-forward and somewhat subtle one (i.e., that ubiquitin simply has to be close to the correct place and in the correct orientation in order to affect H2Bub). Some suggestions to increase the novelty are included below.
5. Broad biological significance: The findings are certainly of importance for the field of epigenetics. They are rather specific for histone H2B ubiquitination, but do provide sufficient and important new information such that it seems appropriate for EMBO reports.
6. Importance to the specific field: In fact, these findings are probably very significant for our understanding of H2Bub and will likely become frequently cited in the field.

Here are a few points that, if addressed, would significantly increase the potential impact of this paper:

1. What happens to H2Bub/SAGA-dependent genes in this system. As an example, the authors could investigate the GAL1 and SUC2 genes as investigated by Henry, et al. The authors discuss briefly about the ubiquitination/deubiquitination cycle. It would be very interesting to know what effect the expression of the fusion proteins have on these genes in Bre1-mutant cells (i.e., can the Henry ubiquitination/deubiquitination model be substantiated in this system?).

>>> Please see the response to the first comment of reviewer 1.

2. Another potential physiologically interesting system the authors could investigate (potentially as an alternative to #2) would be to investigate whether expression of the fusion proteins rescues the DNA repair defects observed in the absence of Bre1.

>>> We thank the reviewer for the suggestion to address the functionality of the histone-ubiquitin fusions by physiological studies. To address that point we performed a DNA damage response assay.

The new experiments show that ubiquitin fusions can efficiently substitute for native H2Bub, providing additional evidence for the proper function of the tagged histone proteins (new Fig. 4B). Interestingly, this experiment also shows that dynamic changes in H2Bub are not required for DNA damage checkpoint activation (addressed at the end of the Results section).

3. Do the ubiquitin-fusion proteins have other lysine residues required for branching (i.e., K48, K63, etc.)? Are the results comparable (i.e., for the H2A N-terminal fusion) with a K0-ubiquitin fusion protein which cannot support ubiquitin branching?

>>> To determine whether branched poly-ubiquitin chains are required for the observed crosstalk, we introduced wild-type and K0 versions of human ubiquitin previously used in yeast. K0-ubiquitin
still promoted H3K79 methylation, showing that polyubiquitination is not required (new Supplementary Fig. 1G).

Referee #3:
In the manuscript "Flexibility in crosstalk between H2B ubiquitination and H3 methylation in vivo" Vlaming et al. investigate the plasticity of the positioning of covalently attached ubiquitin to histones with respect to promoting methylation of histone 3 on lysine 4 and 79. Crosstalk between these sites of histone modification has been described several years ago. However, the exact molecular details of this phenomenon are still debated. In particular, the signalling mode of ubiquitin attached to histones is not fully understood. One on hand it has been suggested that ubiquitination affects chromatin dynamics and structure. On the other hand specific binding proteins to this mark have been identified. The authors engineered several constructs of H2A and H2B trying to mimick constitutive ubiquitination in vivo in budding yeast. They quantified the levels of H3K4 and H3K79 methylation in ubiquitination-deficient background (mutant yeast strains) compared to WT and engineered tagged but not ubiquitinated H2A and H2B expressing strains. The authors deduce from their results a certain plasticity of the positioning of the ubiquitin polypeptide, where ubiquitin attachment sites close to the native H2BK123 show higher levels of H3K4/K79 methylation compared to sites of attachment further from the native position. The claim made by the authors is intriguing as it potentially provides evidence for plasticity in histone ubiquitination and general modification cross-talk. However, in present form the main conclusions are not fully supported by the data and the study lacks in depth of analysis and controls. The authors tend to overstate/overinterpret their findings.

1- The major point of concern is the fact that the ubiquitination is constitutive and uniform across the whole genome and as such is at incomparable levels with wt levels of H2Bub. Huge excess of ubiquitinated histones in the engineered strains should therefore result in significant increase of these methylation levels. The effects on both methylation sites tested (measured by western blot) is comparable, or lower compared to wt strains, and in some cases practically absent for the trimethyl mark. Moreover, monomethylation (and in some cases me2) is already comparable, or lower compared to wt strains, and in some cases practically absent for the trimethyl mark. The effects on both methylation sites tested (measured by western blot) is comparable, or lower compared to wt strains, and in some cases practically absent for the trimethyl mark. The effects on both methylation sites tested (measured by western blot) is comparable, or lower compared to wt strains, and in some cases practically absent for the trimethyl mark. The effects on both methylation sites tested (measured by western blot) is comparable, or lower compared to wt strains, and in some cases practically absent for the trimethyl mark.

We agree that the levels of tethered ubiquitin are higher than the steady state levels of native H2Bub. However, native H2Bub is a highly dynamic mark; H2B is subject to cycles of cotranscriptional ubiquitination and deubiquitination. It has been suggested that a transient H2Bub is sufficient to promote (more stable) H3K4/H3K79 methylation throughout the transcribed parts of the yeast genome. This idea is in line with the quantitative discrepancy between H2Bub (low) and H3K79me3 (high) in wild-type yeast cells. Therefore, the relatively low steady state levels of H2Bub do not reflect the transient occurrence of this mark throughout the genome. We now explain this more clearly in the introduction, “Whereas H2Bub is a transient mark, H3K4 and especially H3K79 methylation are more stable and will remain after H2Bub has been removed [2,3].” The high level of H3K79me1 in bre1Δ strains is in agreement with the lower catalytic activity of the non-processive Dot1 enzyme in the absence of H2Bub (as we showed previously in De Vos et al 2011 and see Fig. 1).

Cross-comments from referee #2:
Specifically, concerning Reviewer 3’s first comment, this seems like a very valid point. I would suggest that the authors could perform a few ChIP experiments (e.g. against H3K4me3 and H3K79me3) at a few different positions to see if the normal placement is maintained or not. Of course, some "genome-wide" approach would be nice, but I think a few ChIP-PCRs would be sufficient to make the point (i.e., near the TSS and upstream). My guess is that the placement will not be changed, but the reviewer's point is very valid and well taken.

Following the suggestion of referee 2, we analyzed methylation changes by ChIP-qPCR at three different genomic loci harboring different levels of H3K79me1/2/3 in wild-type cells. At all three loci, the histone ubiquitin fusions promoted Dot1 activity and led to a shift to higher methylation states (as expected based on the distributive mechanism of Dot1; see Fig 1).
Methylation in the strains with tethered ubiquitins is a bit more evenly distributed than in wild-type cells, but the pattern is still partially maintained (i.e. low, intermediate, high methylation at HML,
promoter, coding sequence respectively).

2- The huge increase in "functional" ubiquitination seems not to have any effect on the viability of the strains. Since ubiquitination is thought to be rather dynamic, this raises the question whether the fusion proteins are functional in first place. Additional experiments and controls are required to demonstrate that the fusion proteins are functional per se (i.e. folded correctly), are incorporated into chromatin normally and do indeed substitute for cellular ubiquitination.

Cross-comments from referee #2:
Concerning his/her second point, about the functionality, I have little concerns here (since I guess the yeast would not grow without chromatin...), but it would be easy enough to show that the fusion protein is incorporated into chromatin (even a simple chromatin extraction, maybe supplemented for example with MNase data...would also address the "openness" of the chromatin with the constructs). It also seems like a reasonable suggestion.

Cross-comments from referee #1:
Reviewer 3 is concerned about the functionality of the ubiquitin-histone fusion proteins compared to the wild-type histones. I think these concerns need to be articulated more clearly. The fusions maintain the essential functions of the corresponding histones as they support viability on their own. The fact that they support the histone crosstalk (albeit to varying extents) is further evidence that the proteins are functional and incorporated into chromatin. To what extent the fusions support other histone functions that depend on dynamics of the ubiquitin modification is an interesting question that warrants further investigation but does not need to be addressed immediately. One could argue that the failure of some of the fusions to rescue crosstalk could be due to defective incorporation into chromatin. Thus, the authors may have actually underestimated the extent of the plasticity of crosstalk in vivo. A simple experiment to address this possibility would be to examine the levels of H3K36me3 (not sensitive to H2Bub1 crosstalk) in the different strains by western blotting. General defects on nucleosome integrity might be expected to affect levels of this modification. The authors could also adapt their in vitro methylation system for Set2 (although I would be satisfied with the in vivo experiment).

>>> The fitness of the strains, the positive effects on histone methylation, and the efficient DNA damage response (new Figure 4B) suggest that the strains expressing altered histone proteins show no major defects in chromatin organization. New ChIP-qPCR experiments using the HA-tag present in the fusion proteins (new Supplementary Fig. 1E) provide additional evidence that the histone fusion proteins are incorporated into chromatin. We did not analyze H3K36 methylation because even though at bulk level no changes have been detected in H2Bub mutants, a closer examination revealed complex changes in chromatin (e.g. see Batta et al 2011, Genes & Dev, 25:2254). Therefore, this mark is not a faithful, H2Bub-independent reporter of chromatin structure.

3- The authors need to present evidence that the ubiquitination and following methylation events lead to actual transcriptional activation (i.e. are functional) and compare transcription levels with wt strains. Shown for several different loci, this would be a proof that the ubiquitination-methylation is functional locally and not an artefact (related to point 1).

>>> The causal relationship between histone methylation and gene activation is still unclear and under debate (e.g. see the recent publication from the Gross lab: Zhang et al 2014, PLoS Genet. 10(4):e1004202). Therefore, we did not address downstream transcriptional events.

4- The MRM measurements were performed twice ("two biological replicates") and the represented SEM merely indicates the range of those measurements. The authors need to base their statistical analysis on three or more replicates, as the differences are only marginal in some cases (e.g. Fig. 4D).

The current analysis could overestimate the effects discussed, especially taking into account that the absolute levels of H3K79me1/2/3 between the constructs are very different (the MRM analysis here provides only relative abundance within a strain). Additionally, more details on how the quantification of the MRM data was performed should be provided in the methods section.

Cross-comments from referee #1:
As I indicated in my review I think the quality of the data is high and I don't see the need for further MS experiments.
Because of the distributive nature of Dot1, we do not always observe large changes in every single methyl state for every condition (e.g. see Fig. 1B). Changes in Dot1 activity can be more accurately deduced from the overall pattern of H3K79me0/1/2/3 changes. The MS experiments are very accurate and reproducible. In addition we performed semi-quantitative immunoblot analyses as an independent method (see Supplemental figures). Although we cannot exclude the existence of unknown modifications that could skew the mass spec measurements (see point 7 below) all our results (and those of previous studies) suggest that our mass spec measurements reflect absolute levels. Following the guideline in the decision letter we now show the individual data points along with the mean, rather than error bars. More details on the MS-MRM quantification are provided in the Supplemental Methods section.

5- Throughout the text the authors use strong phrasing when discussing the results of the different constructs examined. However, in many cases the effects were restricted to the lower methylation states (me1/2 or even only me1 which is present in bre1delta cells already). In this context words like “dispensable”, “remarkable plasticity” and “not critical” clearly overstate the findings. Following the reviewer’s suggestion, we have rephrased some of the wording.

6- The lethality of HA-S19-H2A but not the ubiquitinated version of the same truncated construct needs to be discussed.

7- Although, to the best of our knowledge, H3T80ph hasn’t yet been detected in yeast, did the authors check for its presence? The modification could lead to erroneous MRM results if not taken into account.

We did not check for H3T80 phosphorylation ourselves, but the Tyler lab recently showed that they could not identify H3T80ph in yeast (Hammond et al, 2014 Cell Cycle, 13;440-452).

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

REFEREE REPORTS:

Referee #2
I think this is an important story that should be accepted for publication. All my concerns have been addressed.

Referee #3
In the revision the authors have addressed all our concerns in a detailed and adequate manner. With the additional experiments provided, the full overhaul of text and bibliography and especially the much more careful phrasing, the paper is now suitable for publication in EMBOR.