THE EMBO JOURNAL

Manuscript EMBO-2012-81927

Multivalent di-nucleosome recognition enables the Rpd3S histone deacetylase complex to tolerate decreased H3K36 methylation levels

Jae-Wan Huh , Jun Wu, Chul-Hwan Lee, Miyong Yun, Daniel Gilada, Chad A. Brautigam and Bing Li

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Review timeline:

Submission date:	02 May 2012
Editorial Decision:	31 May 2012
Revision received:	20 June 2012
Editorial Decision:	09 July 2012
Accepted:	12 July 2012

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 31 May 2012

Thank you for submitting your research manuscript (EMBOJ-2012-81927) to our editorial office. It has now been seen by three referees and their comments are provided below.

All reviewers appreciate your study and are in general supportive of publication in The EMBO Journal. Nevertheless, they do raise a number of concerns, mainly regarding data presentation and interpretation, although addressing some of them might also require additional experimentation. Importantly, the intelligibility of the manuscript should be improved in a revised version according to the referees' suggestions. Given the comments provided, I would like to invite you to submit a suitably revised manuscript to The EMBO Journal that addresses the raised concerns in full. I should add that it is our policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The manuscript by Huh and colleagues describes their investigations into how the Rpd3S complex binds to nucleosomal templates. In short, they report that Rpd3S binds preferentially to dinucleosomal templates, especially those bearing trimethylated H3K36, in a multivalent mode. Interestingly, this mode of binding is not sensitive to a two-fold dilution of the level of H3K36me3 suggesting that it may have relevance to certain replicating regions of the genome. Overall, the manuscript represents a very solid and well-detailed in vitro analysis of Rpd3S binding to nucleosomal templates. In general, the experiments have been well controlled and the resulting data demonstrate a very high technical competence. The findings will be of interest to workers in the immediate field and will be of general interest to many more. However, I do have a number of comments that the authors need to address.

The manuscript is not written in a style that is easy to follow. The description of experiments is often cryptic and the reader has to really concentrate to understand what has been done. For instance, when first mentioning histones incorporating H3K36me3 (page 6) the reader has to scroll through the materials and methods to find out how this was done. There are annotations in figures that are not described; for instance, in Figure 6A there are letters (a-f) in parentheses to the right of the figure. To what do they refer? Are they in reference to the other panels of this figure that are annotated in uppercase lettering? On the other hand, there are descriptions in figure legends that are not relevant to the figure; in the legend to Figure 2A/B it is written that 'Tailess + H3K36me3' was used. This is not the case.

The authors make repeated use of mutants bearing deletion in CHD and PHD domains of Eaf3 and Rco1 respectively. I wonder whether point mutations rather than deletions might perhaps provide more mechanistic insight, as they are less likely to suffer from severe structural perturbation. It would be particularly interesting to analyse the point mutations in the experiments displayed in Figure 5.

Figure 1: I think more general readers would appreciate the inclusion of a schematic diagram of the Rpd3S complex indicating the various members and the specific domains (e.g. CHD and PHD) which are involved in nucleosome binding etc..

Figure 2: Overall, these data are convincing but the relative apparent binding affinities for the data shown in panels A and B should be quantitated.
The authors should consider placing panel C into the supplementary section. This panel contains lots of information but it is poorly discussed and it is really only displayed to demonstrate the fidelity of the nucleosomal templates. For clarity and simplification these data could be placed into the Supplementary section.

In the text on page 3, it is claimed that 'some PTMs can directly influence chromatin structure'. This is true but the reference of Yun et al is inappropriate. The primary references for these data should be cited.

Referee #2

In this manuscript Huh et al investigate using biochemical approaches the mechanism by which the Rpd3S complex interacts with chromatin substrates. This builds on previous work that demonstrated a requirement for H3K36me, Eaf3/Rco1, and nucleosomes + linker DNA for efficient nucleosome

recognition by this complex. In the current study the authors demonstrate that histone tails inhibit binding, dinucleosomes enhance binding, methylation is only required on one nucleosome within the dinucleosome to achieve enhanced binding, and that methylation of the dinucleosome stimulates the deacetylase activity of Rpd3 on these substrates. The authors posit based on these observations that Rpd3S binding in vivo may be able to tolerate instances where the H3K36me signal on chromatin is diluted and therefore remain associated with target chromatin following fluctuation in modification levels related to transcriptional changes or replication. Overall, I think the quality of the work detailed in this manuscript is exceptional. The chromatin reconstitution systems and purified components used in these experiments are very technically challenging to assemble and they are used precisely and in a quantitative manner to dissect the chromatin binding properties of Rpd3S. Of particular interest is the potential requirement for multivalent interaction not only for Rpd3S binding but also for deacetylase activity. I suppose the inherent weakness of this work is however that it is unclear form the current study whether the observed in vitro activities are sufficient to explain or illuminate further the targeting/function of Rpd3S in vivo. Nevertheless, the study does make some important steps towards understanding in careful molecular detail how a macromolecular chromatin modifying machine interfaces with a physiologically relevant chromatin substrate. This is something that is not routinely undertaken, particularly in this level of detail; therefore this study has a certain degree of novelty in that respect. Below I have detailed specific points that should be considered.

(1) Based on the previously published observation that the Eaf3 chromo and Rco1 PHD domains are required for mononucleosome binding the authors suggest that Rpd3S may achieve binding via multivalent interactions with the nucleosome. Interestingly, removing histone tails appears to increase Rpd3S binding. The authors suggest that removing histone tails may alleviate a steric hindrance that is inhibitory to efficient multivalent interaction of Rpd3S with the mononucleosome template (Figure 1). Therefore the authors examined whether providing a dinucleosome substrate would allow Rpd3S to utilize two less constrained interaction surfaces on independent nucleosomes and thus increase binding compared to a nucleosome. This may be the case based on increased binding of Rpd3S to dinucleosome substrates (Figure 3). This implies that multivalent binding of one Rpd3S molecule may occur between two nucleosomes (ie with Eaf3 and Rco1 binding independent nucleosomes as schematically indicate din Figure 4D). Based on this proposed interaction model I was surprised that one would not observe bridging of two mononucleosomes by Rpd3S when the mononucleosome EMSA is carried out with increasing concentrations of the Rpd3S complex. If the binding is preferentially mediated by bridging interactions between nucleosomes you might expect to see a complex corresponding to one Rpd3S and two mononucleosomes. Also this brings up a second perhaps more important point. Is the increased binding of Rpd3S to the dinucleosome actually mediated by a bridging interaction? What is the evidence in support of this? I think given that this is the main observation in the manuscript (as evidenced by the title) this point should be adequately supported.

(2) In light of the recent work from both the authors lab (Govind et al 2010) and others (Drouin et al 2010) proposing an initial H3K36me independent targeting mechanism for Rpd3S, I was somewhat surprised by the interpretation of the in vitro binding results. In the introduction and discussion it is proposed that multivalent interactions may be important in maintaining binding following relative dilution effects of chromatin modification levels for example following genome replication. However it appears that targeting of Rpd3S to the body of genes first requires a transcription dependent recruitment via elongating Polii. Presumably, as part of this initial targeting process elongating PolII would also reinstate the appropriate level of H3K36me via Set2 co-transcriptionally. Therefore, in this context the Rpd3S complex would not necessarily rely on its capacity to sustain binding in a graded modification environment following replication as retargeting to the gene would first require a productive transcriptional elongation cycle. In light of the enhancement of HDAC activity based on the presence of H3K36me (Figure 6) is it not much more plausible that the H3K36me placement in vivo is important for regulating the capacity of Rpd3S to deacetylate the gene body? Also I think the title which also focusses on signal dilution would benefit from reconsideration.

(3) One point that may be experimentally out of the scope of this current work but I think still remains a key factor that has not been considered in the proposed Rpd3S chromatin binding model is the absolute requirement for non-nucleosomal or linker DNA (Li B 2007 Science). This is particularly interesting as it appears from the current manuscript and the previous work (Li B et al

2007) that Rpd3S does not interact with naked DNA. This implies that the linker DNA requirement is only realized in the context chromatin substrates. Is perhaps a key requirement for Rpd3S chromatin recognition not being overlooked here? If nothing else this point should be discussed in more detail.

(4) I was somewhat confused by the schematic for Rpd3S binding in figure 4D. Here it appears as if there is a core nucleosome dependent binding event and a tail dependent binding event. Based on the work described in this manuscript it seems tail interactions are not required. This schematic needs a rethink.

Referee #3

EMBOJ-2012-81927. Huh et al, Multivalent di-nucleosome recognition enables the Rpd3S histone deacetylase complex to tolerate signal dilution.

This manuscript describes a comprehensive in vitro dissection of the elements that may influence the association of the Rpd3S deacetylase complex with its nucleosomal substrates. Results indicate that the optimal interaction involves multiple contacts, including the Eaf3 chromodomain, trimethylated H3-K36, the Rco1 PHD domain, >= two adjacent nucleosomes, and preferably tail-less histones (although the in vivo significance of the last escapes this reviewer; see below).

This is an extremely well performed study: I was particularly impressed at the range of (thankless but essential) nuclease sensitivity and ATPase remodeling approaches used to confirm that the many different nucleosome arrays were properly assembled, and thus appropriate substrates for further analysis. My primary issues were a lack of consistency in data presentation, interpretation of the tail-less nucleosome analyses, and certain specifics about the deacetylation assays (see below). Assuming that these concerns can be addressed I fully support publication.

Specific comments -

1. The labeling of many panels (throughout) is inconsistent and should be addressed. For example the quantitation panel Fig. 1C refers to Mono-WT, Mono-Me, TL and TL-me, but none of these labels are used in Figs. 1A / 1B or explained in the figure legend or body text. Fig. 6A row 1 refers to ' AcH3* (b) ' where (b) may refer to the use of this preparation in Fig. 1B (?), but this is a guess given the lowercase b and lack of such an indication in the figure legend or body text. Overall many of the figure legends might benefit from a bit more information.

2. Page 7: How do the authors believe that histone N-terminal tails interfere with an interaction between Rpd3S and the histone globular domains? What are the contact points of Rpd3S with DNA that the tails could compete? (referred to in excluded model 1). (See also comment #4)

3. Page 12: ' Recombinant mono-nucleosomes were labeled using either Histone H3 specific acetyltransferases (a mixture of three H3 HATs - SAGA, ADA and SLIK) ... or a histone H4-specific HAT (NuA4) ... in the presence of 3H-acetyl-CoA '. Were the acetyltransferase preparations biochemically purified (such that SAGA, ADA and SLIK could not be distinguished)? If so this should be cited / described. The labeling of Fig. 6a refers to ADA as the H3-HAT: this should be modified (inconsistent with the text). How efficient were the in vitro labeling reactions? (both alone and by comparison across the HATs). The various HATs are stated as highly specific to H3 or H4 although no evidence is presented for such exquisite selectivity on these in vitro substrates. I am not suggesting that the authors revisit > 20 years of substrate specificity papers but we all know about in vitro off-targeting by such preparations. Either present supporting immuno-blotting with a range of Ac-antibodies or tone down the exclusivity statements.

4. Page 12: ' As shown in Figure 6B, Rpd3S deacetylates H4 in a K36me-dependent manner; however its activity on H3 is not as sensitive to K36Me '. Would the authors care to comment further on this intriguing observation? Further it is inconsistent with 6C where H3 and H4 deacetylation are both sensitive to K36Me. Might hyper-acetylated substrates be preferred because they help to stabilize the Rpd3S association? Could this suggest an in vivo importance of the tails to

Rpd3S function, underappreciated because of the tail-less binding data earlier in the manuscript (which are likely in vitro observations of limited functional in vivo relevance?).

5. Fig. 6B-C: Recolor the data columns: it's currently very difficult to distinguish the preparations (also the color order of the first and second columns seems to be flipped between panels?).

Revision - authors' response 20 June 2012

Point-by-point responses to each reviewer's comments

Referee #1

The manuscript is not written in a style that is easy to follow. The description of experiments is often cryptic and the reader has to really concentrate to understand what has been done.

We have now extensively edited the text and figure legends for clarity.

For instance, when first mentioning histones incorporating H3K36me3 (page 6) the reader has to scroll through the materials and methods to find out how this was done.

We applied the methyl-lysine analog method to chemically methylate recombinant histones and this information is now mentioned in the text.

There are annotations in figures that are not described; for instance, in Figure 6A there are letters (a-f) in parentheses to the right of the figure. To what do they refer? Are they in reference to the other panels of this figure that are annotated in uppercase lettering?

We apologize for the missing description in the figure legend. The letters in parentheses represent the panel number of which the substrates prepared from each branch were used in HDAC assays.

On the other hand, there are descriptions in figure legends that are not relevant to the figure; in the legend to Figure 2A/B it is written that 'Tailess + H3K36me3' was used. This is not the case.

This error is now corrected

The authors make repeated use of mutants bearing deletion in CHD and PHD domains of Eaf3 and Rco1 respectively. I wonder whether point mutations rather than deletions might perhaps provide more mechanistic insight, as they are less likely to suffer from severe structural perturbation. It would be particularly interesting to analyse the point mutations in the experiments displayed in Figure 5.

We have performed the experiments requested by this reviewer and showed that even a single mutation of one aromatic residue within the K36me binding pocket disrupts the binding of Rpd3S to methylated di-nucleosomes (Supplemental Figure 5). This is also consistent with our previous data showing that the chromo-domain is not only required for recognizing K36me but also important for overall complex binding (Li et al 2007 Science). Since mutant Rpd3S complexes failed to bind to fully methylated di-nucleosomes, we did not test their binding to hybrid di-nucleosome templates.

Figure 1: I think more general readers would appreciate the inclusion of a schematic diagram of the Rpd3S complex indicating the various members and the specific domains (e.g. CHD and PHD) which are involved in nucleosome binding etc..

We now incorporate requested information into the cartoon shown in Figure 4D

Figure 2: Overall, these data are convincing but the relative apparent binding affinities for the data shown in panels A and B should be quantitated. The authors should consider placing panel C into the supplementary section. This panel contains lots of information but it is poorly discussed and it is really only displayed to demonstrate the fidelity of the nucleosomal templates. For clarity and simplification these data could be placed into the Supplementary section.

We have now included a new figure panel (Figure 3C) in which relative apparent affinity of Rpd3S to each nucleosome was quantified.

As this reviewed suggested, we also moved the sliding assays into the Supplementary Figure 2B.

In the text on page 3, it is claimed that 'some PTMs can directly influence chromatin structure'. This is true but the reference of Yun et al is inappropriate. The primary references for these data should be cited.

The original paper is now cited in the text.

Referee #2

(1) *....Based on this proposed interaction model I was surprised that one would not observe bridging of two mononucleosomes by Rpd3S when the mononucleosome EMSA is carried out with increasing concentrations of the Rpd3S complex. If the binding is preferentially mediated by bridging interactions between nucleosomes you might expect to see a complex corresponding to one Rpd3S and two mononucleosomes. Also this brings up a second perhaps more important point. Is the increased binding of Rpd3S to the dinucleosome actually mediated by a bridging interaction? What is the evidence in support of this? I think given that this is the main observation in the manuscript (as evidenced by the title) this point should be adequately supported.*

Since increasing Rpd3S concentration will eventually lead to two Rpd3S binding to one mono-nucleosome due to the symmetric nature of nucleosomes, we think this reviewer was meant to comment on the results shown in original Supplementary Figure 3C, in which an excess amount of mono-nucleosomes were provided for Rpd3S binding.

We reason that the binding of Rpd3S to any nucleosome requires at least two simultaneous, now we consider fairly weak, interactions. When di-nucleosomes are presented, Rpd3S can contact two nucleosomal binding surfaces from each nucleosome within one molecule, which results in a more favorable binding than that of a mono-nucleosome. However, when only mono-nucleosomes are available, Rpd3S still needs both CHD and PHD to contact different surfaces within a single nucleosome to achieve the first nucleosome binding. Therefore, in theory, we would not expect that one Rpd3S has additional binding surfaces sufficient to contact the second mono-nucleosome.

The bridging model was concluded through a serial of biochemical/biophysics experiments. We first established the binding stoichiometry of Rpd3S-dinucleosome. Next we proposed two formal possible scenarios where di-nucleosome templates are preferred by Rpd3S. An alternative scenario besides our model shown in Fig 4D is that each Rpd3S binds to one of the two nucleosomes within a di-nucleosome template, and it is the dimerization of these two Rpd3S molecules that form a bridge to facilitate the synergistic binding. We subsequently ruled out this possibility by showing that Rpd3S does not dimerize in our solution condition (Supplementary Figure3). More importantly, we performed the competition experiment as shown in Figure 4C where both mono-nucleosomes and di-nucleosomes were presented to Rpd3S at the same time. We showed that at a lower concentration of Rpd3S (Fig4C lane3), only 1Rpd3S-dinucleosome interaction was observed. Since one Rpd3S can only bind to one molecule of either mono- or di-nucleosome, the advantage of di-nucleosomes is to allow Rpd3S simultaneous contacting both nucleosomes, and essentially forming a bridge. We would not expect any favorable binding if a single Rpd3S contacts one of the two nucleosomes within di-nucleosome templates. These results collectively led to our proposed bridging model. Lastly, the HDAC result shown in Figure 6F also supported our model, as within a di-nucleosome, one methylated nucleosome is able to elevate the Rpd3S deacetylation activity on a neighboring unmethylated nucleosome.

(2) *In light of the recent work from both the authors lab (Govind et al 2010) and others (Drouin et al 2010) proposing an initial H3K36me independent targeting mechanism for Rpd3S, I was somewhat surprised by the interpretation of the in vitro binding results. In the introduction and discussion it is proposed that multivalent interactions may be important in maintaining binding following relative dilution effects of chromatin modification levels for example following genome replication. However it appears that targeting of Rpd3S to the body of genes first requires a transcription dependent recruitment via elongating Polii. Presumably, as part of this initial targeting process elongating PolII would also reinstate the appropriate level of H3K36me via Set2 co-transcriptionally. Therefore, in this context the Rpd3S complex would not necessarily rely on its capacity to sustain binding in a graded modification environment following replication as retargeting to the gene would first require a productive transcriptional elongation cycle. In light of the enhancement of HDAC activity based on the presence of H3K36me (Figure 6) is it not much more plausible that the H3K36me placement in vivo is important for regulating the capacity of Rpd3S to deacetylate the gene body? Also I think the title which also focusses on signal dilution would benefit from reconsideration.*

Our previous paper and the results from others suggested that initial targeting of Rpd3S to coding regions is in part mediated by phosphorylated Pol II CTD. However it remains unclear whether the retention of Rpd3S at coding regions during multiple rounds of transcription elongation requires phosphorylated-CTD interaction. Here we showed that via the di-nucleosome recognition, K36me not only enhances the Rpd3S nucleosome engagement (stable binding), but also stimulates Rpd3S HDAC activity. Since newly-synthesized histones are hyper-acetylated, one would expect to observe an elevated acetylation level once replication forks passing by if there is no immediate action to safeguard the genome. The reading tolerance mechanism we proposed here should allow continuing functions of Rpd3S by either quickly re-targeting Rpd3S to methylation diluted chromatin, or by maintaining full HDAC activity of Rpd3S on half-methylated templates when a new round of transcription is reestablished after replication. At this point, the in vivo tools to distinguish these two possibilities are not available, and we hope that our future studies will shed lights on this important issue.

(3) One point that may be experimentally out of the scope of this current work but I think still remains a key factor that has not been considered in the proposed Rpd3S chromatin binding model is the absolute requirement for non-nucleosomal or linker DNA (Li B 2007 Science). This is particularly interesting as it appears from the current manuscript and the previous work (Li B et al 2007) that Rpd3S does not interact with naked DNA. This implies that the linker DNA requirement is only realized in the context chromatin substrates. Is perhaps a key requirement for Rpd3S chromatin recognition not being overlooked here? If nothing else this point should be discussed in more detail.

We agree with this reviewer that linker DNA plays an important role in Rpd3S binding to mono-nucleosome. Here we further demonstrated that increasing the length of linker DNA (>70bp) of mono-nucleosome does not significantly improves Rpd3S binding (Figure 4A). However, due to technical reasons, we could not prepare a di-nucleosome template without any linker DNA in between. Therefore, we can not directly evaluate whether linker DNA is still important for di-nucleosome recognition.

(4) I was somewhat confused by the schematic for Rpd3S binding in figure 4D. Here it appears as if there is a core nucleosome dependent binding event and a tail dependent binding event. Based on the work described in this manuscript it seems tail interactions are not required. This schematic needs a rethink.

The tail interaction we referred in Figure 4D was the binding of Rpd3S to H3K36 adjacent peptide sequence (H3 aa22-42). Since in our tailless histone constructs, we only removed about 20 amino acids at the N-terminus (a few at C-term in case of H2B), the interference effect caused by histone tails were only restricted to these removed regions at extreme ends of tails.

Referee #3

1. *The labeling of many panels (throughout) is inconsistent and should be addressed. For example the quantitation panel Fig. 1C refers to Mono-WT, Mono-Me, TL and TL-me, but none of*

these labels are used in Figs. 1A / 1B or explained in the figure legend or body text. Fig. 6A row 1 refers to ' AcH3 (b) ' where (b) may refer to the use of this preparation in Fig. 1B (?), but this is a guess given the lowercase b and lack of such an indication in the figure legend or body text. Overall many of the figure legends might benefit from a bit more information.*

We apologize for this confusion. All labeling and annotation issues raised by this reviewer have been fixed. In addition, we have added more description to the figure legends and the text for clarity.

2. Page 7: How do the authors believe that histone N-terminal tails interfere with an interaction between Rpd3S and the histone globular domains? What are the contact points of Rpd3S with DNA that the tails could compete? (referred to in excluded model 1). (See also comment #4)

We suggested that histone tails may interfere with Rpd3S binding to globular domains based on the observation that each tail truncation seems to display some independent and additive effects. Given the flexibility of extreme histone tails in relation to the nucleosome core particle structure, the only access point by Rpd3S which has common relationship with all four histone tails (assuming that any projection of each tail is possible) is the globular domains. Considering the minor differences displayed by different tailless nucleosomes (new Figure 2C), we speculate that the top part of globular domains, which is close to the dyad area, is likely to be the primary contact point for Rpd3S. This is because truncation of H2A and H2B alone or in combination showed less increase of Rpd3S binding.

We apologize for the ambiguous description in the original text. Here we meant to refer to Rpd3S contacting nucleosomal DNA where histone tails can potentially exert direct steric hindrance effect. The supporting evidence is that we detected weak binding of Rpd3S to tailless nucleosomes which do not contain any linker DNA (147bp nucleosome in Supplementary Figure 2A).

3. Page 12: ' Recombinant mono-nucleosomes were labeled using either Histone H3 specific acetyltransferases (a mixture of three H3 HATs - SAGA, ADA and SLIK) ... or a histone H4-specific HAT (NuA4) ... in the presence of 3H-acetyl-CoA '. Were the acetyltransferase preparations biochemically purified (such that SAGA, ADA and SLIK could not be distinguished)? If so this should be cited / described. The labeling of Fig. 6a refers to ADA as the H3-HAT: this should be modified (inconsistent with the text). How efficient were the in vitro labeling reactions? (both alone and by comparison across the HATs). The various HATs are stated as highly specific to H3 or H4 although no evidence is presented for such exquisite selectivity on these in vitro substrates. I am not suggesting that the authors revisit > 20 years of substrate specificity papers but we all know about in vitro off-targeting by such preparations. Either present supporting immuno-blotting with a range of Ac-antibodies or tone down the exclusivity statements.

We are sorry for not citing the original reference of our approach to prepare the HAT complexes. The Workman lab has established that Ada2-TAP purification contains SAGA, SLIK and ADA complexes, as confirmed by extensive mass-spec analysis (Lee et al 2005 Biochem Soc Tran). For each preparation, we routinely perform HAT assays to confirm that their primary targetis histone H3. The rationale for our approach is to maximize the yield of H3 HAT activity, instead of discarding the rest of H3 HATs when individual complexes are purified. We agree with this reviewer that our approach may not result in specific lysines being acetylated as characterized previously; therefore we tone down the “specificity claim” of these HATs and define them as “the HATs that primarily acetylate H3”.

4. Page 12: ' As shown in Figure 6B, Rpd3S deacetylates H4 in a K36me-dependent manner; however its activity on H3 is not as sensitive to K36Me '. Would the authors care to comment further on this intriguing observation? Further it is inconsistent with 6C where H3 and H4 deacetylation are both sensitive to K36Me. Might hyper-acetylated substrates be preferred because they help to stabilize the Rpd3S association? Could this suggest an in vivo importance of the tails to Rpd3S function, underappreciated because of the tail-less binding data earlier in the manuscript (which are likely in vitro observations of limited functional in vivo relevance?).

In Figure 6B, these substrates were only acetylated at H3 or H4, whereas in Figure 6C, both H3 and H4 are acetylated but only indicated histones are labeled with ^{3}H. Therefore, in Figure 6C, deacetylation by Rpd3S occurs at both H3 and H4, and our approach allows us to measure them individually and we believe these substrates are more physiologically relevant. Although we do not have any further evidence to explain the phenomenon shown in Figure 6B, we speculate that hyper-acetylated H4 may help orientate Rpd3S on nucleosome in a specific configuration, which restricts Rpd3S HDAC activity so that it becomes dependent on the K36me signal. This model is also consistent with the notion that negative effect caused by extreme ends of histone may help Rpd3S binding to nucleosome in a more specific manner while sacrificing some robustness.

5. Fig. 6B-C: Recolor the data columns: it's currently very difficult to distinguish the preparations (also the color order of the first and second columns seems to be flipped between panels?).

We have adjusted the color coding patterns in Fig 6B-C as this reviewer suggested. Since different nucleosomes were used in Figure 6B and 6C, we now label them with different colors to avoid confusion.

2nd Editorial Decision 09 July 2012

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that it was very well received. Nevertheless, we agree with Referee #2 regarding the detracting effects of overstatements to the value of a study. Therefore, we would like you to reconsider your title as well as to rephrase the main conclusions of your study according to his suggestions. To simplify the processing, you can just sent the amended text file in reply to this e-mail. After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

The revised manuscript from Huh et al addresses most of my original points sufficiently. However I still disagree somewhat with their interpretation of one aspect of the study as I pointed out in my original review point 2

'In light of the recent work from both the authors lab (Govind et al 2010) and others (Drouin et al 2010) proposing an initial H3K36me independent targeting mechanism for Rpd3S, I was somewhat surprised by the interpretation of the in vitro binding results. In the introduction and discussion it is proposed that multivalent interactions may be important in maintaining binding following relative dilution effects of chromatin modification levels for example following genome replication. However it appears that targeting of Rpd3S to the body of genes first requires a transcription dependent recruitment via elongating Polii. Presumably, as part of this initial targeting process elongating PolII would also reinstate the appropriate level of H3K36me via Set2 co-transcriptionally. Therefore, in this context the Rpd3S complex would not necessarily rely on its capacity to sustain binding in a graded modification environment following replication as retargeting to the gene would first require a productive transcriptional elongation cycle. In light of the enhancement of HDAC activity based on the presence of H3K36me (Figure 6) is it not much more plausible that the H3K36me placement in vivo is important for regulating the capacity of

Rpd3S to deacetylate the gene body? Also I think the title which also focusses on signal dilution would benefit from reconsideration.'

In the revised manuscript the title and the abstract still implies that the main new finding of this work is that Rpd3S can tolerate signal dilution, a feature that would be required for Rpd3S targeting following DNA replication. As indicated above the targeting mechanism for Rpd3S in vivo remains contentious and the work in this manuscript does not address this point further (ie does not mechanistically demonstrate that the in vitro binding properties lead functionally to maintain Rpd3S targeting in the face of signal dilution in vivo). Therefore it takes a conceptual leap of faith that I predict few will be willing to take to accept that the in vitro chromatin binding activity described in this body of work is mainly overcoming signal dilution following replication in vivo. As I suggested in my initial review and re-iterate above, an equally likely alternative is that the binding activity described here is required once Rpd3S is targeted to genes as a way of controlling deacetylation. I think the author's observations are important and interesting but they overstate the relevance in their dilution toleration model. This makes the title and the main conclusion of the work somewhat misleading and will likely disappoint the discerning reader ultimately detracting from the value of what I think is a very nice and well executed study. This manuscript would be much more balanced if they were to simply describe what they observe and then propose the dilution tolerance model in the discussion as one of several possible models for how this mechanism may contribute to the function of Rpd3S at genes. Therefore I still think a minor alteration in the title and rebalancing of the conclusions would significantly improve this interesting study.

Referee #3

EMBOJ-2012-81927R. Huh et al, Multivalent di-nucleosome recognition enables the Rpd3S histone deacetylase complex to tolerate signal dilution.

The authors have dealt with my concerns from the first round review. This is a very well performed study, definitely of interest to readers of EMBO J, and I fully support publication.

