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A dual role of H4K16 acetylation in the establishment of yeast silent chromatin

Mariano Oppikofer, Stephanie Kueng, Fabrizio Martino, Szabolcs Soeroes, Wolfgang Fischle, Susan Hancock, Jason Chin, Susan M. Gasser

Corresponding author: Susan M. Gasser, Friedrich Miescher Institut

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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 20 January 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below. As you will see the referees express interest in the study especially the effect of H4K16ac on Sir protein and complex binding to chromatin and require further experimental analysis before the manuscript is suitable for publication in The EMBO Journal. This includes strengthening of the data demonstrating that Sir2-3-4 complex deacetylates H4K16 and deacetylation reaction itself contributes to the observed compaction. Further insight into how the deacetylation of nucleosomes by Sir2 increases the association of Sir2-3-4 complex with nucleosomes and decreases MNase accessibility is also required. However, although always beneficial, given the difficulty of the analysis I do not feel that the experiments in the paper need to be carried out by a second method as suggested by referee #2. Given the interest in the study should you be able to satisfactorily address these issues, we would be happy to consider a revised version of the manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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 initiative, 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 REPORTS:

Referee #1:

Using purified SIR proteins and reconstituted nucleosome arrays, the authors investigated the effects of three histone modifications (H4K16Ac, H3K56Ac, and H3K79me) on the affinity of SIR proteins for the arrays and on the accessibility of the arrays to MNase digestion. Interestingly, H4K16Ac decreased the affinity of the arrays for Sir3, but increased the affinity for Sir2/Sir4. These results help explain seemingly contradictory genetic data suggesting that both unacetylated and acetylated K16 are important for silencing. The other two modifications were found to reduce SIR binding and increase MNase accessibility.

I found this study generally well-executed and clearly described, and it should be of high interest to the field. However, a few issues (including additional experiments) should be addressed prior to publication.

1. The data in the second panel of figure 4A are used to conclude that the Sir2-3-4 complex deacetylates H4K16. This is an important result for interpreting other experiments in the manuscript. However, the controls indicate some technical problems with the experiment. First, the control H3 band is diminished in lanes 3 and 4, suggesting uneven loading or loss of material and precluding a definitive conclusion regarding deacetylation. Second, and perhaps related, H4K16Ac decreases in lane 3, although no deacetylase has been added, again precluding a definitive conclusion regarding deacetylation in lane 4. Please, repeat the experiment so it is interpretable. If possible, it would be nice to quantify the extent of deacetylation, as this information would be useful for understanding other experiments.

2. The impact of this study would be increased if the authors could nail down how the deacetylation of nucleosomes by Sir2 increases the association of the Sir2-3-4 complex with nucleosome arrays (2B) and decreases the accessibility to MNase (3B). There are two possibilities. Either having deacetylated nucleosomes is critical for these properties (inconsistent with 1D but consistent with 3C) or the production of O-AADPR alters the Sir2-3-4 complex such that it behaves differently (consistent with the authors' previous data in Martino et al.). This second (interesting) possibility could be strengthened or refuted by examining the MNase accessibility of unmodified nucleosomes incubated with Sir2-3-4 in the presence or absence of O-AADPR.

3. It would also be helpful to include a control demonstrating that the results observed in the presence of NAD+ (2B and 3B) are due to active deacetylation by Sir2. (This control is especially important given the concerns in point 1.) For example, these experiments could be repeated with a Sir2-3-4 complex in which Sir2 is catalytically inactive due to a point mutation.

4. The x-axes in figures 3B and 3C differ significantly, and this difference causes the curves in 3B to appear dramatically different from one another, whereas the curves in 3C look more similar to one another. This artificial enhancement seems a little unfair. In addition, the EC50 values in 3C are different enough from the values in 3B and 3D that should be the same (4.8 vs 3.7 and 6.6 vs 4.9), that it is not clear how accurate these numbers are. Therefore, I am not persuaded by the authors' conclusion (p 10) that "the deacetylation reaction itself also contributes, because the difference in protection between acetylated and deacetylated chromatin was greater than between acetylated and unmodified chromatin." To resolve these issues, it would be fantastic if the experiments in 3B and 3C could be repeated all at once using consistent MNase concentrations.

5. Please provide more information in the methods section on the statistical methods that were used. How were the n-fold ratios calculated in figure 2? How were data fitted and EC50 values determined in figure 3?

6. Please indicate whether the concentration of 6-mer arrays is represented per array or per nucleosome. Assuming the numbers are per array, it is remarkable that the Sir2-3-4 complex is able to completely shift the nucleosome arrays to a higher mobility when there is less than one complex per array. How do the authors understand this result?

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9. In figure 3, it would be helpful to include the concentrations of array and Sir proteins used.

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The manuscript by Oppikofer et al., entitled "A dual rle of H4K16 Ac in the establishment of silent chromatin" is a description of continuing studies from the Gasser lab on the biochemical characterization of silenced chromatin using a highly defined in vitro system.
In this manuscript the authors have assembled nucleosomes on templates containing tandem array of the 601 sequence that precisely positions nucleosomes and the binding of different Sir protein complexes to these arrays was analyzed. The studies were done using histones modified at specific residues and show that H4K16ac decrease binding of Sir3 while increasing binding of Sir2/4 which is interesting. Deacetylation promotes binding of Sir2/4 in the presence of Sir3. These results provide biochemical in vitro confirmation of in vivo molecular genetic results from the Grunstein and Rine Labs demonstrating that Sir2/4 mediated deacetylation is necessary for Sir3 binding and Sir3 is necessary for enzymatically active Sir2/4 complex to spread.
Additionally the experiments demonstrate that H3K56ac does not affect Sir binding helping clarify some contested results.

Specific points
H4K16Ac histones were generated by native chemical ligation. A Triton acid urea gel of the unacetylated and acetylated histones would be informative regarding the integrity/modification status of the histones used in the reconstitution experiments.
In figure 2B, are the differences statistically significant (compared to figure 2C)?
The authors need to perform competition experiments using increasing amounts of unlabeled modified or unmodified nucleosome arrays to be able to determine preference of Sir2/4 proteins for acetylated nucleosome arrays.
Finally in figure 5, what percentage of histone H3 is methylated on K79?
Figure 6 could be eliminated and the discussion on O-AADPR should be reduced.
The gel shift data are clear but it would be informative if Sir binding were also analyzed by sedimentation analysis to determine chromatin compaction/solubility under various conditions. The rate of digestion of restriction sites in the 601 sequences could have also been used to measure accessibility to provide a second method for the analysis of Sir binding to nucleosomes.

Referee #3:

This manuscript from the group of Susan Gasser analyses the biochemical properties of chromatin bound by the silencing complex Sir2/3/4 under different conditions in vitro. The main finding is that, contrary to expectation, Sir2/4 binds better to chromatin acetylated at H4 K16, whereas Sir3 binding is inhibited by this modification, suggesting that H4 K16Ac has two contrary roles in silencing, 1) to recruit Sir2/4, and 2) to prevent SIR spreading. Importantly, this study reports is a difference in MNAse accessibility between Sir2/3/4-bound chromatin that was concomitantly deacetylated by Sir2 and Sir2/3/4-bound chromatin that was unacetylated to start with. This gives support to the hypothesis that some aspect of the deacetylation reaction itself (which goes beyond mere deacetylation) promotes heterochromatin formation.
It is further shown that H3 K79 methylation by Dot1, in contrast to H4 K14Ac, does not increase

affinity for Sir2/4, but decreases binding of Sir3 and Sir2/3/4. Furthermore, this work shows that H3 K56 Ac decreases Sir2/4 and Sir2/3/4 binding, but that this does not decrease the MNAse accessibility of the chromatin. Contrary to what has been claimed earlier, Gasser et al find that Sir2/3/4 does not deacetylate H3 K56Ac in vitro.

Principally, this study is a continuation of earlier work from the Gasser lab (Martino et al, Mol Cell 2009) that aims at reconstituting yeast heterochromatin in vitro using positioned nucleosomes and Sir proteins expressed in insect cells. While the main problem of the system still is that it is not specific to sequences that are silenced in vivo, Oppikofer et al nonetheless succeed at making important observations that contribute to our mechanistic understanding of heterochromatin formation. The assays used are laborious and technically challenging, not least because the biochemically measurable differences are small in magnitude (in the range of 2-fold). However, with few exceptions (see below), I can follow their interpretation, and this work adds new and significant details to our understanding of heterochromatin formation.

Individual points:
1. In Fig. 2B, the authors claim that Sir2-3-4 binds better to chromatin that is deactylated in vitro (+ NAD). I cannot see this difference in the gel, and according to the graph on the right, it is very minor at most. The data should be interpreted accordingly and the text rephrased to reflect the data.
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6. The figure legend for Suppl. Fig. 3 does not match the figure itself, it seems to be a description of Fig. 4A.

1st Revision - authors' response 19 April 2011

Referee #1:

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decreases in lane 3, although no deacetylase has been added, again precluding a definitive conclusion regarding deacetylation in lane 4. Please, repeat the experiment so it is interpretable. If possible, it would be nice to quantify the extent of deacetylation, as this information would be useful for understanding other experiments.

We agree with the referee and repeated the experiment several times. We have now prepared a new figure which we moved to Figure 2E as a consequence of some figure rearrangement (see also Supplementary Figure S2B). We think that the deacetylation of H4K16ac is now very obvious and in addition there is no question but that it is Sir2 specific, since a catalytic inactive point mutant of Sir2 (Sir2N345A, referred as Sir2cd and assayed in parallel) does not show deacetylase activity (Figure 2E).

2. The impact of this study would be increased if the authors could nail down how the deacetylation of nucleosomes by Sir2 increases the association of the Sir2-3-4 complex with nucleosome arrays (2B) and decreases the accessibility to MNase (3B). There are two possibilities. Either having deacetylated nucleosomes is critical for these properties (inconsistent with 1D but consistent with 3C) or the production of O-AADPR alters the Sir2-3-4 complex such that it behaves differently (consistent with the authors' previous data in Martino et al.). This second (interesting) possibility could be strengthened or refuted by examining the MNase accessibility of unmodified nucleosomes incubated with Sir2-3-4 in the presence or absence of O-AADPR.

This request was the only one that is unfortunately impossible to fulfill. Despite extensive efforts, we were not successful in purifying O-acetyl ADPR (O-AADPR) again. That the presence of O-AADPR is SUFFICIENT for the tighter binding was shown in Martino et al., 2009, yet at that stage MNase experiments were not done. The collaborators who prepared it for Martino et al. are no longer available, and the existing protocol did not work in our hands. Actually we are unable to find anyone who can purify O-AADPR, which is highly labile and hard to purify away from ADPR (which does not affect Sir loading onto chromatin). Nonetheless, we have addressed the question of the importance of O-AADPR as a side product of the Sir2 deacetylation reaction by directly comparing the binding of the Sir2-3-4 complex (and accessibility of the linker DNA) to unmodified nucleosomes as opposed to deacetylated (H4K16ac + NAD) chromatin (Figures 2B and 3E), and by performing similar experiments with the catalytically dead Sir2cd-3-4 complex. We found that in presence of the deacetylation reaction (which produces O-AADPR) chromatin was better protected from MNase attack compared to chromatin that was reconstituted with unmodified histones. More than this cannot be done until someone invents a way to synthesize O-acetyl ADPR.

3. It would also be helpful to include a control demonstrating that the results observed in the presence of NAD+ (2B and 3B) are due to active deacetylation by Sir2. (This control is especially important given the concerns in point 1.) For example, these experiments could be repeated with a Sir2-3-4 complex in which Sir2 is catalytically inactive due to a point mutation.

As mentioned before, we generated a Sir2N345A catalytic dead mutant (Sir2cd) and purified a Sir2cd-3-4 complex by co-expression in insect cells. This mutation has been shown to disrupt the enzymatic activity of Sir2 in vitro and in vivo (Imai et al, 2000; Armstrong et al, 2002). Indeed, our Sir2cd-3-4 failed to deacetylate a chromatin substrate (Figure 2E). We performed several control experiments with this mutant: Figure 2C, 2D and 3C showing that Sir2 catalytic activity is clearly required for the effects we see. We discuss in the paper that this implicates either O-AADPR or a conformational change associated with the activity.

4. The x-axes in figures 3B and 3C differ significantly, and this difference causes the curves in 3B to appear dramatically different from one another, whereas the curves in 3C look more similar to one another. This artificial enhancement seems a little unfair. In addition, the EC50 values in 3C are different enough from the values in 3B and 3D that should be the same (4.8 vs 3.7 and 6.6 vs 4.9), that it is not clear how accurate these numbers are. Therefore, I am not persuaded by the authors' conclusion (p 10) that "the deacetylation reaction itself also contributes, because the difference in protection between acetylated and deacetylated chromatin was greater than between acetylated and unmodified chromatin." To resolve these issues, it would be fantastic if the experiments in 3B and 3C could be repeated all at once using consistent MNase concentrations.

The differences in the X-axis simply reflect experimental conditions and were not used in an

intentional manner to enhance our positive data. MNase assays are difficult to perform in a highly reproducible manner because different preparations of MNase (e.g. different batches but also preparations) show variation in cleavage efficiency. All comparisons from which we draw conclusions are therefore performed strictly in parallel with the same enzyme preparation. To avoid the misleading graphs, we decided to change the representation of our data to a more qualitative vertical bar chart. This renders the differences quite obvious and allows comparison of specific titration points in different experiments. We reinforce our conclusions by including MNase experiments in which we compare (on the same gel with the same titration) SIR-bound deacetylated chromatin (H4K16ac + NAD) and SIR-bound chromatin reconstituted with unmodified histones (Figure 3E).

5. Please provide more information in the methods section on the statistical methods that were used. How were the n-fold ratios calculated in figure 2? How were data fitted and EC50 values determined in figure 3?

We agree that this fitted curve is not the best way to present the MNase data and have replaced these graphs (Figure 3). To simplify the data presentation (as the paper has much more data now) we also dropped the n-fold ratios in Figure 2, which presented the same data shown in Figure 2 in second way. Thanks for this remark, as it certainly makes the data more clear to the reader.

6. Please indicate whether the concentration of 6-mer arrays is represented per array or per nucleosome. Assuming the numbers are per array, it is remarkable that the Sir2-3-4 complex is able to completely shift the nucleosome arrays to a higher mobility when there is less than one complex per array. How do the authors understand this result?

The concentration indicated are per nucleosome as stated in the "Material and methods" section.

7. In figure S3, panel B and the asterisks are missing.

Thank you, we corrected this.

8. I believe the experiment represented in figure 3C had no NAD+, but this is not indicated in the figure or legend. Please clarify.

We clarify this by indicating for every experiment whether NAD was present or not. Thank you for this comment.

9. In figure 3, it would be helpful to include the concentrations of array and Sir proteins used.

We now indicated the amount of chromatin used (constant throughout the entire figure) in the figure legend. We indicated the concentration of Sir proteins directly in the figure.

10. In the figure 4 legend, B and C are reversed.

Sorry - we had changed the figure numbering in the final revision. Thanks for this - we corrected it.

Referee #2:

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In this manuscript the authors have assembled nucleosomes on templates containing tandem array of the 601 sequence that precisely positions nucleosomes and the binding of different Sir protein complexes to these arrays was analyzed. The studies were done using histones modified at specific residues and show that H4K16ac decrease binding of Sir3 while increasing binding of Sir2/4 which is interesting. Deacetylation promotes binding of Sir2/4 in the presence of Sir3. These results provide biochemical in vitro confirmation of in vivo molecular genetic results from the Grunstein and Rine Labs demonstrating that Sir2/4 mediated deacetylation is necessary for Sir3 binding and Sir3 is necessary for enzymatically active Sir2/4 complex to spread.

Additionally the experiments demonstrate that H3K56ac does not affect Sir binding helping clarify some contested results.

Specific points
H4K16Ac histones were generated by native chemical ligation.
A Triton acid urea gel of the unacetylated and acetylated histones would be informative regarding the integrity/modification status of the histones used in the reconstitution experiments.

Supplementary Figure S1 shows now quality control experiments on different steps of the production of H4K16ac histones (H4R21C,K16ac). These make use of ESI-MS and SDS-pages and show that the material produced is homogeneus and clearly forms histone octamers that are indistinguishable from unmodified ones when analysed by SDS-PAGE.

In figure 2B, are the differences statistically significant (compared to figure 2C)?
The authors need to perform competition experiments using increasing amounts of unlabeled modified or unmodified nucleosome arrays to be able to determine preference of Sir2/4 proteins for acetylated nucleosome arrays.

This was a helpful comment - and we agree that the difference observed in the binding of Sir2-3-4 H4K16ac with and without NAD was weak. However it was clearly different than the behavior of Sir2-4 under the same conditions. Nonetheless, we now present the powerful comparison of SIR complex binding H4K16ac nucleosomes with NAD, with SIR complex on unmodified chromatin with NAD (Figure 2B) We also show that this latter binds the same with or without NAD (Supplementary Figure S2A) . We were able to reinforce these observations by performing control experiments with a catalytic inactive Sir2cd-3-4 (Figure 2D; see also comments above). Finally, and importantly, we performed competition experiments as suggested by the referee. In Figure 1D we were able to show that unlabeled H4K16ac competes better than unlabelled unmodified chromatin for the binding of Sir2-4 to a Cy5-labelled unmodified substrate. This, we think, nails the fact that Sir2-4 prefers H4K16ac chromatin.

Finally in figure 5, what percentage of histone H3 is methylated on K79?

As previously published in Frederiks et al, 2008 and Martino et al, 2009, H3K79 is mono-, di- and, to a lesser extent, tri-methylated on at least 50% of the available K79 residues. We confirmed this in our own preparations by western blot, and mention this in Materials and Methods. However, since it was published twice before (and is not the key point of this paper) we do not include the blots here.

Figure 6 could be eliminated and the discussion on O-AADPR should be reduced.

Since the length is not excessive, and since we are refuting a previous model for SIR assembly, we believe that the scheme in Figure 6 is important. As requested, we reduced the argument for the possible involvement of O-AADPR in the "Discussion" section.

The gel shift data are clear but it would be informative if Sir binding were also analyzed by sedimentation analysis to determine chromatin compaction/solubility under various conditions.

We tried to perform experiments using analytical ultracentrifugation on SIR complexes, chromatin and SIR-bound arrays. However, those experiments turned out to be very complex and difficult to analyze, mainly because of the heterogeneousity of the high order assembly formed. A figure demonstrating this is included for the reviewer. We agree with the editor that developing this completely separate type of analysis is not essential for our conclusions, and in addition, its complexity would not only add three or four figures, but would extend the work for several years.

The rate of digestion of restriction sites in the 601 sequences could have also been used to measure accessibility to provide a second method for the analysis of Sir binding to nucleosomes.

We thank the reviewer for this and have now included digestion with AvaI restriction enzyme, which cuts in the linker DNA separating the Widom 601 positioning elements. As with MNase, SIR complex binding greatly protects the linker DNA from AvaI digestion (Supplementary Figure S3B).

Similar results where also obtained using RsaI which also cuts the AvaI consensus.

Referee #3:

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We refer you to our answer to Referee #2 point 2. In brief, we agree that the data as presented were not fully convincing, and we instead performed new experiments and compared directly the binding of the SIR complex to H4K16ac in presence of NAD (which converts it to deacetylated chromatin) with its binding to unmodified nucleosomes (Figure 2B). Here it is clear that the deacetylation reaction per se (and not the simple fact of being unmodified), stimulates the association of Sir2-3-4 to chromatin. We reinforces this conclusion by performing control experiments with a catalytic inactive Sir2cd-3-4 (Figure 2D).

2. I cannot see a difference in MNAse accessibility or Sir2/4-bound chromatin with or without NAD (Fig. 3A).

As mentioned in the manuscript, we agree : we see no significant difference in MNase accessibility for Sir2-4 bound chromatin with or without NAD (Figure 3A) ! Why is this ? One might expect that by removing the H4K16ac mark in presence of NAD chromatin would become less accessible to MNase. However, the Sir2-4 heterodimer clearly binds to H4K16ac chromatin with higher affinity (Figure 1C-E), resulting in higher linker DNA protection in absence of NAD. These two opposite effects cancel each other out, which can explain the lack of difference in MNase sensitivity when Sir2-4 binds chromatin with or without NAD.

Minor points:
3. The introduction describes the interaction between Sir3 and Sir4 and the histone tail, but makes no reference to the observation that the Sir3-BAH domain interacts with nucleosomes (Buchberger et al, MCB 2008; Norris et al, PLoS Genetics 2008; Onishi et al 2007). This information should be included for completeness.
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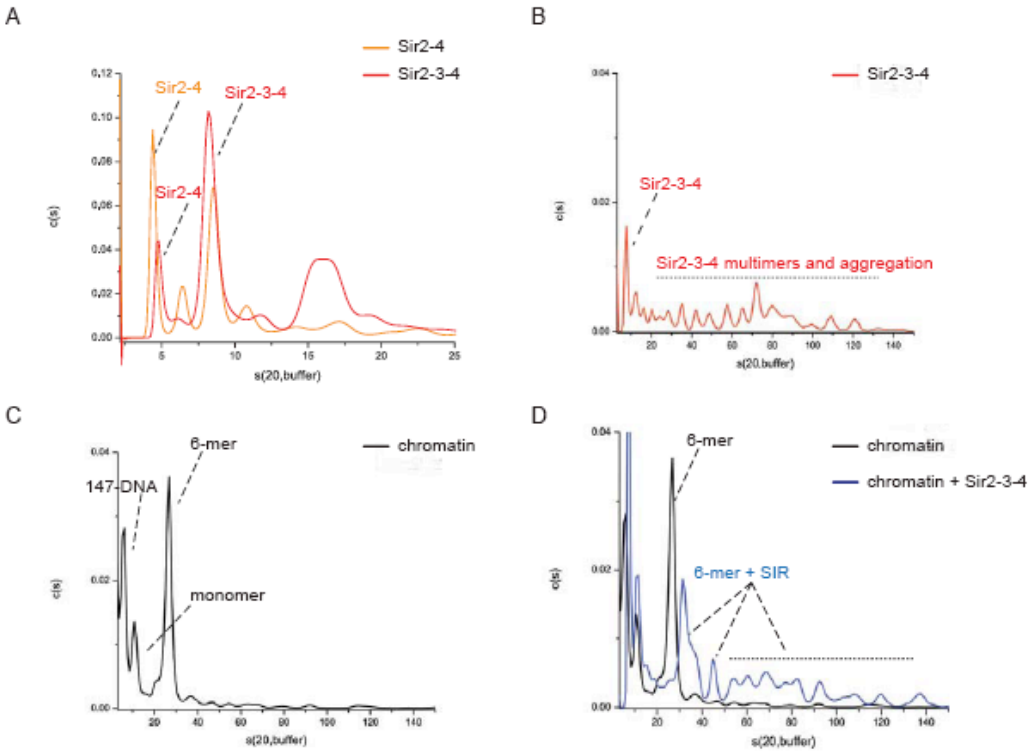
6. The figure legend for Suppl. Fig. 3 does not match the figure itself, it seems to be a description of Fig. 4A.

We thank the referee for these comments. In each case we modified the manuscript accordingly.

Figure for reviewers: analytical ultracentrifugation SIR + chromatin



We have tried to set this up for Sir bound chromatin in collaboration with the Fischle laboratory at the MPI in Goettingen, also hoping to better deterime the size of these complexes formed. However, as these figures illustrate, the sample complexity was too big, so that it was not possible to clearly analyze these results.

A/B: Analysis of Sir2-4 and Sir2-3-4 complexes by aUC. As shown by desity gradients (i.e. Cubizolle et al), the Sir2-3-4 preparations also contain some Sir2-4 complex. Both complexes tend to form higher order assemblies, probably due to the dimerization interfaces of Sir4 and Sir3. A shows only the lower-molecular weight compounds to resolve Sir2-4 and Sir2-3-4 complexes.
C: aUC analysis of 6-mer chromatin preparation. The 6-mer forms a homogenous peak around 30S, free competitor (147) DNA and monomers formed are also clearly visible. This analysis confirms good quality chromatin.

D: Superimposition of free chromatin run and chromatin bound by Sir2-3-4 complex. At this SIR-chromatin ratio, the 6-mer is fully bound by Sirs, and some of the 147 DNA as well, whereas the monomer is SIR-free. This is a state we often observe on our native gels for the complete upshift. There is a major SIR-bound chromatin species around 35S, but also various species of higher molecular weights are detected. The shift of the chromatin peak may be formed by both, addition of molecular weight by Sirs and compaction. Because of sample complexity and the rather large mass added by Sir proteins to

chromatin, it is however impossible to determine the degree of compaction from these experiments.

Acceptance letter 28 April 2011

I have received the comments from the two referees who have evaluated your revised "A dual role of H4K16 acetylation in the establishment of yeast silent chromatin" manuscript, both find that it is significantly improved and recommend publication. I am therefore happy to accept it for publication in The EMBO Journal.

You will receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1

The authors have addressed almost all of the reviewers' comments, and the manuscript is significantly improved.

Referee #2

The manuscript by Oppikofer et al investigates the binding of the Sir complex to nucleosome arrays. The main concern I had was demonstrating a clear preference of this complex for initially binding to acetylated H4K16 followed by deacetylation and stable binding of the holocomplex. The new data in the revised manuscript adequately address this concern. The authors also show using restriction enzymes that accessibility of the chromatin has changed when bound by Sir proteins which is easier to quantitate compared to micrococcal nuclease digested chromatin. The authors have therefore adequately addressed my concerns by performing these new experiments.

