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Dynamics of Sir3 spreading in budding yeast: secondary recruitment sites and euchromatic localization

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Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>03 September 2010</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>30 September 2010</td>
</tr>
<tr>
<td>New Submission received</td>
<td>16 December 2010</td>
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<tr>
<td>Editorial Decision</td>
<td>11 January 2011</td>
</tr>
<tr>
<td>Revision received</td>
<td>18 January 2011</td>
</tr>
<tr>
<td>Accepted</td>
<td>20 January 2011</td>
</tr>
</tbody>
</table>

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 30 September 2010

Thank you very much for submitting your manuscript to The EMBO Journal. Three scientists assessed merits and potential suitability of your study for publication here. All of them conclude that the work does contain interesting information on Sir3-genomic localization, mode of its spreading and potential unanticipated role(s) in gene regulation. However, all three also notice that significant further experimental work would be needed to raise conclusiveness and actual definitive insight by your study to the level that would enable their support for publication here. Specifically, we would have to insist on the major points raised by refs#1 and #2:

- addressing whether endogenous Sir3 spreading would actually follow the mode described for Sir3-HA.
- evaluate whether the whole Sir-complex spreads by performing Sir3-HACHIP in sir4 or Sir2 strains including the GAL genes to address the point whether shifting to nuclear pores might influence occupancy
- most importantly, some functional data would be necessary to investigate functionality of Sir3 at the novel binding sites.

Given these rather demanding and time consuming tasks requested by the crucial referees, we are not convinced that offering a straightforward and timely very limited round of revision would be justified. Also in light of the currently uncertain outcome of these experiments, we are simply unable to essentially commit to publication. Therefore, I do have no other choice than to formerly reject the paper.

Still appreciating the potential of your work, and conditioned on your ability and willingness to solve the specific points outlined above, we would be willing to assess a significantly developed study as NEW SUBMISSION of the manuscript.
Please do understand that this decision is entirely based on the currently rather premature state of analysis. Thus, I am really sorry that we are unable to reach a more positive conclusion. I still hope that clearly communicating our demands and expectations together with the comments of our referees might guide your decision on either attempting thorough (and timely-unlimited) development of the study for future consideration here OR to seek rapid publication elsewhere.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REVIEW

Referee #1 (Remarks to the Author):

This manuscript addresses the question of how silent chromatin spreads in budding yeast on a genome wide scale. Briefly, they perform ChIP/CHIP and ChIP/Seq time course experiments during an induction of Sir3-HA expression using a galactose inducible promoter. The data shows not only the genome wide distribution of Sir3, but also how the protein binds to recruitment sites and then spreads further, a model that has been widely established in the field. They find that the spreading model is only true at a limited number of telomeric loci that are often used as model loci to study silencing (like TelVIR), whereas at other places, especially at telomeres with subtelomeric X elements, a uniform binding rate can be observed over a larger area indicating "cooperative" binding or very fast spreading. A similar spreading mode is found for the HMR locus, which has been suggested also in a recent study by the Rusche laboratory. This uniform binding pattern is mainly observed when there are several recruitment sites, indicating that they may influence each other (possibly by looping) or the local chromatin structure.

In addition to the temporal binding of Sir3, the authors also looked for new binding sites of Sir3 throughout the genome. They observe very early binding to PAU genes and Ume6 elements. The PAU genes contain sequences similar to the Ume6 motif and the authors hypothesize that such an element may act as a Sir recruiting element. Surprisingly, they also observe delayed binding of Sir3 at highly expressed genes like the GAL genes that are activated under these conditions. They make the very unlikely hypothesis that depletion of H4K16ac at these loci favors binding of the Sir complex. However, the expression of GAL genes is unchanged in Sir mutants, and the low level Sir association at late time points probably correlates more with GAL gene positioning at pores than anything else.

Finally, in order to find changes in chromatin structure modulated by Sir binding they also map nucleosome occupancy in a wild type and a sir3Δ strain. Overall, deletion of Sir3 does not induce major changes, but subtelomeric histone occupancy is decreased and genes in this area also tend to have a nucleosome dip at the TSS-1 site indicative of their activation. This is rather unrelated to the question of promiscuous Sir3 binding, and probably rather reflects characteristics of unusual promoter structures.

The paper has a number of important points, names how Sir3 spreads on a genome-wide level and how different telomeric contexts influence that event. On this it contributes some new conclusions to the yeast silencing and transcription field. However, the paper is extremely sloppily written (superficially smooth and easy to read, but details are not respected or reported, making it very frustrating for a critical, informed reader). It is overinterpreted throughout since the authors conclude that Sir3 spreading represents SIR complex spreading which was shown years ago not to be the case in strains overexpressing Sir3. Gross errors like this have to be corrected, since it misrepresents the data. This and a number of other points listed below have to be addressed before acceptance for publication.

1. The key problem is that the authors use over expressed Sir3 to do their experiments although it is well known that under these conditions Sir3 spreads WITHOUT Sir2/4 (as shown in Hecht et al., 1996, which is cited by the authors in the introduction). Throughout the text they use Sir Complex, as synonymous for Sir3 which is clearly not the case. It raises two major problems:
   a. Is the binding of overexpressed Sir3 the same as endogenous? (most likely not)
   b. Does the binding of overexpressed Sir3 always coincide and depend on Sir2-4 like normal Sir3? (again most likely not)
The one experiment that addresses this is their control ChIP with anti-Sir3 in sir3Δ and wt strains (galactose and dextrose growth media). The figure is however presented in a way that it is very difficult to compare the data. In fact, it looks as if there is considerable amount of difference between the anti-Sir3 result and the mapping of overexpressed Sir3-HA. The Sir3-HA (i.e. 80mins/240mins) data should be combined with the Sir3 wt in one figure allowing a direct comparison. Also, a zoom in, especially for the new loci (region on 15L or other serinapaurin genes, GAL genes), should be presented. The observation that Sir3 distribution changes considerably in wildtype cells on two different carbon sources may also be worth mentioning.

How similar is the data when examined closely? It is not obvious that overexpressed Sir3-HA resembles that of endogenous Sir3, and a careful comparison needs to be made. The spread of Sir3-HA should be normalized to WT on galactose as well, and not to Sir3-HA on glucose.

To answer the question whether the whole Sir complex ever spreads like overexpressed Sir3-HA, the same experiments (Sir3-HA GAL induced) should be repeated doing Sir2 or Sir4 ChIP. This does not necessarily need to be genome wide; but should include the GAL genes. An alternative experiment that might show that the Sir complex binds active GAL genes would be to repeat the Sir3-HA ChIP in a sir4 or sir2 strain. Finally it should be done in a strain in which GAL genes are not shifted to nuclear pores (mex 67 deletion?).

3. The authors make interesting observations based on their whole genome data concerning novel binding sites of Sirs. Unfortunately, they don't test any of the hypotheses and so the data stays correlative, which often is a major drawback of whole genome studies. Nonetheless the correlative data could be easily tested, for instance by seeing whether the Ume6 sites or PAU genes are Sir recruiting signals.

- Are UME6 sites really SIR recruiting signals? One could just deplete one of them and then check whether this abolishes SIR binding at this place - or alternatively insert the sequence into TELVIR that now shows linear spreading and test whether the newly inserted site does induce SIR binding. There have been other promoter sequences proposed to recruit SIRs which correlated with Rpd3 recruitment (Taddei et al., 2009). The relationship needs to be explored.

- The hypothesis that the UME6 sites are favored binding sites for Sir proteins because they recruit Rpd3, is unsubstantiated. Rpd3 has been shown to be a boundary element for SIR spreading with the idea that the acetyl marks resp. the active de-acetylation by Sir2 are important to promote silent chromatin formation. This discrepancy is not discussed by the authors. Several other publications (Zhou et al NAR 2009; Ehrentraut et al PNAS 2010) show that the SIR complex spreads further in the absence of Rpd3 which is the opposite of what the authors suggest. There is not much known about how Rpd3 regulates this This need to be included in the discussion - a Ume6 deletion experiment would also shed light on that issue.

4. PAU genes as recruiting signal: the authors show in Fig. 1C/D/S3 that there is often a "dip" in Sir3 levels in XCR telomeres. It seems like this "dip" coincides with the PAU genes (compare Fig.3C-F and S4). Does the "dip" match to the conserved possible binding element - or is that after the "dip"? Is this observation meaningful? The PAU genes are not regulated by the Sirs per se (i.e. they are not up regulated in a sir3Δ) and this needs to be explicitly mentioned in the discussion. It is still possible that, like the authors hypothesize, Sir3 also needs to be removed to activate them under stress conditions.

5. Fig4: Nucleosome occupancy in sir3Δ: Fig4A/B concentrate on genes that are up regulated under these conditions: this has no direct correlation with Sir binding, but is just based on the fact that these genes become transcribed like other genes when their transcription factors become expressed - this is mentioned in the text but could be stressed more explicitly. This data may also be moved to
supplemental material.

6. Fig4C/D: Why do the authors look at all genes up to 20kb from the telomere? It is well established (Wyrick 1999) that only genes 6-8kb away from the telomeres are silenced by the Sir complex. The authors also find Sir3 spreading only up to 5-10kb consistent with this previous data. "To identify more direct effects... we focused on subtelomeric genes shown to bind Sir3 above". Did they now select only genes bound or all telomeres up to 20kb? The first may give better results as only these genes are Sir regulated.

7. Fig.6 GAL-gene binding: Why is there no increase in the Sir3-HA ChiP during the time course whereas for wild type Sir3 the authors can only see binding 5hrs after GAL induction? Is the binding in Fig6B significant? To test whether Sir proteins influence GAL gene expression they test expression levels of GAL genes 1-2hrs after induction yet endogenous Sir3 can only be detected after 5hrs - could that explain why they don't see a difference? This experiments are not convincing to show a) that Sir3 really binds to GAL genes (see point 1) and b) that GAL expression is not regulated by Sirs. To test whether the detection of Sir3 at the GAL locus is an "artifact" of recruiting these genes to the periphery the same experiments could be repeated in a mlp1, sac3 or mex67 mutant (Dieppois et al 2006; Abruzzi et al 2006) that disrupts this recruitment yet some still allows the activation.

Minor points:
8. Fig1: data for the histone modifications 1 lacks the indication what the colors mean (low, high levels for red/green), it should best be indicated in the figure like for the yellow/purple.
9. Fig.3 : explain more explicitly what the red count means: location of PAU genes plus indicating their Sir3 counts in one? Maybe just one color for the PAU genes would be better - the sir3 count information is given in 3B? The table in 3B may be better suited for the supplement, for this figure the information about PAU genes name, and distance from ACS/which one is sufficient.
10. Figure 4B should be sir3Δ instead of sir2Δ

Referee #2 (Remarks to the Author):

Using ChIP-Seq technology, the authors mapped the distribution of the silencing protein Sir3 throughout the S. cerevisiae genome at various times after induction of Sir3. Association of Sir3 with chromatin was found to occur in two ways. Assembly was cooperative at HMR and some telomeres, whereas other telomeres displayed slower, more linear spreading of Sir3. These findings are consistent with a recent report (Lynch and Rusche, 2009). New observations in this study are that Sir3 also associates with PAU genes located within 10 kb of telomeres and with highly transcribed genes, such as ribosomal protein genes. However, no evidence was obtained linking Sir3 to the transcriptional regulation of these genes.

This paper is clear and well-organized and provides a genome-wide picture of Sir3 association with chromatin. However, a few additional experiments, outlined below, would significantly increase its impact.

1. I couldn't tell from the description of the methods whether the input DNA was also sequenced to identify genomic loci that have higher than expected sequence reads for technical reasons. The authors should comment on whether this control was conducted, as it would be nice to exclude the possibility that the newly detected sites of Sir3 association are not simply technical artifacts. If the authors have not conducted this control, they should confirm by ChIP-PCR that Sir3 does, indeed, associate with these previously undescribed sites.

2. The authors ChIPed Sir3 but not Sir2 or Sir4. Given that Sir3 is thought to associate with chromatin independently of Sir2 and Sir4 (Hecht et al., 1996; McBryant et al., 2008), it is important that the authors ascertain whether Sir2 or Sir4 associates with the PAU genes and highly transcribed genes. A chromatin structure containing Sir3 alone may have very different properties from a structure containing Sir2,3,4, and it is important to know which type of structure has been detected.

3. The authors detected a sequence motif in subtelomeric silencers and secondary recruitment sites that resembles a Ume6 binding site. If Ume6 does, indeed, contribute to the recruitment of Sir3 to
chromatin, this would be an important discovery and the impact of this work would be increased. Two experiments to test this notion are first, to repeat the Sir3 ChIP experiments in a ume6Δ strain, and second, to ChIP Ume6 and determine whether it co-localizes with Sir3 near the detected motifs.

4. I was not convinced by the data presented in figure 6 that Sir3 is recruited to GAL genes in galactose.
4a. Panel A is supposed to show the galactose-dependent association of Sir3 at the GAL7,10,1 locus, but lacks the critical zero time point control. These data should be included. In addition, the longest time point shown in panel A is 4 hours, but the conclusion drawn from panel B is that there isn't an increase until 5 hours. Please explain.
4b. Panel B is confusing and seems to contradict panel A. First, the scale of the y-axis is baffling and should be expressed in terms of relative enrichment, as is normally done for ChIP data. Second, if I understand the scale correctly, it appears that Sir3 is actually depleted at the GAL locus, rather than enriched as was concluded in panel A. How is this discrepancy explained? Finally, even at five hours the "enrichment" of Sir3 is so minimal compared to what is observed at HMR (inset), that I am skeptical that Sir3 is actually present.

5. In figure 1B, the authors conclude that the loss of H3K56Ac and H3K4me3 follow the same dynamics as Sir3 binding. However, to my eye it looks like the loss of H3K56ac is delayed, and the distribution of H3K4me3 is not uniform across HMR. If these data are going to be presented (they don't seem important for the rest of the paper) they should be accurately described.

6. The authors should tone down their conclusion at the end of the introduction that "heterochromatin complexes may play a more widespread role in gene regulation than previously anticipated," given that their data generally indicate that transcription of euchromatic loci is unaffected by the deletion of Sir3.

7. It would be helpful in figure 5 if the four clusters could be more easily linked with the descriptions provided in the text (no Sir3 enrichment, Sir3 associated with promoter, etc.). Also in figure 5C, it is not clear what the y-axis represents.

8. In figure 3B, I'm guessing that the data are aligned relative to the atg of the PAU genes? Is the +5kb indicated at the top relative to the left-most data point or relative to the atg? Please clarify in the figure legend.

Referee #3 (Remarks to the Author):

The SIR complex is a well-studied system of silencing at telomeres and mating-type loci in Saccharomyces analogous to H3K9-mediated heterochromatic silencing in other eukaryotes. Silencing is known to be able to 'spread' along the chromosome. Past evidence has suggested both a linear polymerization-like model of spreading of silencing involving histone deacetylation and methylation, as well as looping interactions at telomeres, and more recently two rates of spreading that differ between the mating type locus and a telomere. The manuscript by Radman-Livaja et al. describes the first global look at binding of the key SIR complex component Sir3, using an inducible tagged Sir3-HA to track Sir3 location throughout the genome at the single nucleosome level and over a time course of 4.5 hours. They show that their Sir3-HA tag gives similar results to anti-Sir3 antibody. The authors find well-defined regions that show rapid high-level Sir3 binding, and adjacent weaker and more variable regions that increase binding over time and decrease binding over distance from high-level binding regions, similar to recent observations of others. They observe Sir3-dependent alterations in nucleosome positions around transcription start sites for telomeric and mating-type-related genes. They also find that the seripauperin genes, located predominantly near telomeres, act as secondary recruitment sites for Sir3, and share a DNA sequence motif similar to a binding site for Ume6, a regulator of meiotic genes that recruits histone deacetylases and represses transcription. Somewhat paradoxically, they also find Sir3 over highly expressed genes, although they could find no evidence that Sir3 affected the transcription levels of these genes. The authors argue that traditional polymerization models do not account for their observations of two modes of spreading kinetics, "cooperative-like" and "linear".

This global ChIP-Seq view of Sir3 binding provides new insights into SIR complex biology,
identifies several new binding sites of the SIR complex, and provides more complete evidence than
previously available challenging the prevailing view of how spreading of silencing occurs. The data
here are likely to be of importance in formulating new models of spreading that better account for
the observed bimodal kinetics and the roles of different types of silencers. The authors also draw
attention to the possible role of the SIR complex in regulating the seripauperin genes, and a possible
evolutionary link to Ume6 silencing. The paper seems likely to have a significant impact on the field
of chromatin silencing. My comments are largely restricted to minor issues of presentation, clarity
and interpretation.

On p. 4, end of the 1st paragraph the authors say "H4K16 and H3K56 deacetylation as well as
H3K4me3 demethylation appear to follow the dynamics of Sir3 binding". Is this a fair assessment?
The extent of H3K56ac deacetylation seems limited, slow, and inconsistent compared with the other
changes in 1B, although the difference is less dramatic in 1C. Also, the colors used in 1B are not
explained.

In the next paragraph, the authors refer to the promoter region of OCA4 and GIT1 and a region "2kb
upstream to 4kb downstream of HMR-E". Unfortunately, the orientations of genes or positions of
promoters, as well as the location of HMR-E, are not indicated in the cartoon in Figure 1B, and it is
not clear what "upstream" and "downstream" refer to. In the Figure Legend 1C, it is stated that the
"curves are centered at the HMR-E silencer", which seems to be at the left side of HMR at the
vertical axis, but the horizontal coordinates do not suggest that this is the center of the curves. This
figure could be clarified.

On p. 5, refers to anti-Sir3 ChIP in wildtype cells in Figure S2, which is titled "Comparison of wt
Sir3 and pGAL-induced Sir3-HA binding to yeast telomeres". Figure Legend S2B merely says "As
in Figure 2A". Is Figure S2B anti-S3 in wildtype cells, which would be the simplest guess from the
cryptic Legend, or is it Sir3-HA, as implied by the figure title? My guess is the former, but both the
Figure title and Legend are very unclear.

On p. 6, first full paragraph, the authors identify a "weaker additional site of Sir3 binding" on 2R,
and later in the paragraph they mention binding to PAU24 on 2R. I cannot see any binding on 2R in
Figure 2 that seems significant, and in Figure 3A, PAU24 seems to be black, meaning no Sir3
counts. Can the authors clarify, please? The black in Figure 3A is hard to see against the purplish-
brown background - would white be a better choice?

In Figure S5, it would seem more natural to me if the labels of B and C were switched to proceed
left to right then top to bottom.

Figure 4 and the accompanying text on p.7 are somewhat unclear. Instead of referring to "pseudo-
diploid gene expression patterns" it would be helpful to readers to specify what the expected
expression patterns of STE2 and other genes are in SIR3 and sir3Δelta strains. Figure 4B refers to
'sir2delta', while all other references are to 'sir3Delta'. The Legend to 4B is confusing, in part
because of the dual meaning of "close" (verb or adjective?) and I am unsure what it intends to
convey. The Legends to 4C and 4D say "As in A" and "As in B", but they do not specify what is "as
in A" - symbols and colors, presumably?

In Figure Legend S6 it would be simpler and more helpful to restate what the colors represent than
to send the reader to another figure to find out. 'WT' (purple) and 'sir3Δelta' (green) are already
mentioned in the legend, so it actually takes more space to refer the reader to Figure 4.

On p. 9, at the end of the 2nd paragraph, the authors say that 'Sir3 "spreads" from GAL promoters
into the body of the gene 5hrs after the dextrose to galactose shift'. For the GAL7 coding sequence,
Figure 6C is consistent with this interpretation, but there is already substantial Sir3 binding on the
GAL10 and GAL1 coding sequences in dextrose, and it is unclear that the increase at both
promoters and gene bodies after 5 hrs in galactose is indicative of spreading. The same figure might
be used to argue that Sir3 spreads from the FUR4 coding sequence to the FUR4 promoter.

In the Figure Legends, "Figure S87" should be "Figure S8".
Referee #1 (Remarks to the Author):

This manuscript addresses the question of how silent chromatin spreads in budding yeast on a genome wide scale. Briefly, they perform ChIP/CHIP and ChIP/Seq time course experiments during an induction of Sir3-HA expression using a galactose inducible promoter. The data shows not only the genome wide distribution of Sir3, but also how the protein binds to recruitment sites and then spreads further, a model that has been widely established in the field. They find that the spreading model is only true at a limited number of telomeric loci that are often used as model loci to study silencing (like TelVIR), whereas at other places, especially at telomeres with subtelomeric X elements, a uniform binding rate can be observed over a larger area indicating "cooperative" binding or very fast spreading. A similar spreading mode is found for the HMR locus, which has been suggested also in a recent study by the Rusche laboratory. This uniform binding pattern is mainly observed when there are several recruitment sites, indicating that they may influence each other (possibly by looping) or the local chromatin structure.

In addition to the temporal binding of Sir3, the authors also looked for new binding sites of Sir3 throughout the genome. They observe very early binding to PAU genes and Ume6 elements. The PAU genes contain sequences similar to the Ume6 motif and the authors hypothesize that such an element may act as a Sir recruiting element. Surprisingly, they also observe delayed binding of Sir3 at highly expressed genes like the GAL genes that are activated under these conditions. They make the very unlikely hypothesis that depletion of H4K16ac at these loci favors binding of the Sir complex. However, the expression of GAL genes is unchanged in Sir mutants, and the low level Sir association at late time points probably correlates more with GAL gene positioning at pores than anything else.

Finally, in order to find changes in chromatin structure modulated by Sir binding they also map nucleosome occupancy in a wild type and a sir3Δ strain. Overall, deletion of Sir3 does not induce major changes, but subtelomeric histone occupancy is decreased and genes in this area also tend to have a nucleosome dip at the TSS-1 site indicative of their activation. This is rather unrelated to the question of promiscuous Sir3 binding, and probably rather reflects characteristics of unusual promoter structures.

The paper has a number of important points, names how Sir3 spreads on a genome-wide level and how different telomeric contexts influence that event. On this it contributes some new conclusions to the yeast silencing and transcription field. However, the paper is extremely sloppily written (superficially smooth and easy to read, but details are not respected or reported, making it very frustrating for a critical, informed reader). It is overinterpreted throughout since the authors conclude that Sir3 spreading represents SIR complex spreading which was shown years ago not to be the case in strains overexpressing Sir3. Gross errors like this have to be corrected, since it misrepresents the data. This and a number of other points listed below have to be addressed before acceptance for publication.

We thank the reviewer for her/his detailed comments. We address each specific comment below.

1. The key problem is that the authors use over expressed Sir3 to do their experiments although it is well known that under these conditions Sir3 spreads WITHOUT Sir2/4 (as shown in Hecht et al., 1996, which is cited by the authors in the introduction). Throughout the text they use Sir Complex, as synonymous for Sir3 which is clearly not the case.

Throughout the text we have rectified this issue, being more specific about exactly what is observed at a given locus. We have presented better comparisons between the Sir3-HA data and the Sir3 ChIP-Seq carried out in an untagged wild-type strain (Figures S2, S3, S5, S6). Finally, we have carried out ChIP for HA-tagged Sir2 at a handful of interesting loci, and confirm that Sir2 binds along with Sir3 (Figure S6).

It raises two major problems:

a. Is the binding of overexpressed Sir3 the same as endogenous? (most likely not)

b. Does the binding of overexpressed Sir3 always coincide and depend on Sir2-4 like normal Sir3? (again most likely not)
The one experiment that addresses this is their control ChIP with anti-Sir3 in sir3Δ and wt strains (galactose and dextrose growth media). The figure is however presented in a way that it is very difficult to compare the data. In fact, it looks as if there is considerable amount of difference between the anti-Sir3 result and the mapping of overexpressed Sir3-HA. The Sir3-HA (i.e. 80mins/240mins) data should be combined with the Sir3 wt in one figure allowing a direct comparison. Also, a zoom in, especially for the new loci (region on 15L or other serinapar in genes, GAL genes), should be presented. The observation that Sir3 distribution changes considerably in wildtype cells on two different carbon sources may also be worth mentioning.

In answer to point a, Figures S3, S5 and S6 now provide the requested zoom ins for telomeres and for the PAU genes. While overexpressed Sir3HA certainly binds more strongly to the PAU genes than does wild-type Sir3 (particularly at 15L), we note that Sir3 does significantly associate with these loci in wild-type cells (significant relative to Sir3 ChIP in a sir3Δ background). As the reviewer insightfully observes, dex and gal conditions induce differential Sir binding to PAU genes, which we now point out more specifically in the text.

In answer to point b, we note above that Sir2-HA does bind to these loci, and Sir3 binding is dependent on Sir2 (Figure S6).

How similar is the data when examined closely? It is not obvious that overexpressed Sir3-HA resembles that of endogenous Sir3, and a careful comparison needs to be made. The spread of Sir3-HA should be normalized to WT on galactose as well, and not to Sir3-HA on glucose.

We have compared these datasets as requested, showing, as described above. As for normalization, all normalizations are done within one sequencing run (ie sequencing reads are reported as parts per million, essentially), as is commonly done for genome-wide datasets from microarrays to deep sequencing datasets – this is necessary to control here for sequencing depth, for example. We are not certain what would be gained by normalizing these data to Sir3 association on galactose.

To answer the question whether the whole Sir complex ever spreads like overexpressed Sir3-HA, the same experiments (Sir3-HA GAL induced) should be repeated doing Sir2 or Sir4 ChIP. This does not necessarily need to be genome wide; but should include the GAL genes. An alternative experiment that might show that the SIR complex binds active GAL genes would be to repeat the Sir3-HA ChIP in a sir4 or sir2 strain. Finally it should be done in a strain in which GAL genes are not shifted to nuclear pores (mex 67 deletion ?).

As requested, we have carried out Sir2 ChIP in a Sir2-HA strain, now shown in Figure S6. We have also shown that Sir3 localization to all tested PAU genes is Sir2-dependent. Interestingly, Sir3 binding to GAL genes is Sir2-independent, consistent with our model for turnover rather than Sir2-mediated deacetylation as the mechanism for H4K16 deacetyl generation at these genes. As for nuclear pore association, we view a thorough investigation of nuclear pore association as beyond the scope of this study, and we focused most of our efforts in revision on the PAU genes and the role of Ume6 in Sir recruitment.

2. The results section must be written more carefully, and the figures need to be labeled and explained better. As it is, the reader is left very often with guessing how the figure relates to the text and the figure legends to not clarify that either. The authors should especially make very clear whether they ChIP Sir3-HA (and not HA-Sir3 which is a non-functional fusion !) or wild type Sir3 (in overexpression or non-overexpression conditions?, i.e. fig. 6B/C?). The legends and text are so vague that the reader has to guess which protein is being precipitated when and whether the protein is fully functional or not.

We apologize for the difficulties this reviewer had with the text – we have attempted to improve the clarity of the writing throughout the text.

3. The authors make interesting observations based on their whole genome data concerning novel binding sites of Sirs. Unfortunately, they don’t test any of the hypotheses and so the data stays correlative, which often is a major drawback of whole genome studies. Nonetheless the correlative data could be easily tested, for instance by seeing whether the Ume6 sites or PAU genes are Sir recruiting signals.
Actually, we spent over a year working on potential transcriptional consequences of Sir binding to the GAL genes – this is why we initially presented the negative results in Figure 6, as we felt it was important to save others the effort of fruitless interrogation of Sir function at GAL genes. We have now moved this information to the Supplement (Figure S13).

As for ume6Δ, this is an excellent suggestion, and this experiment was also requested by other reviewers below. We now present data for Sir3 ChIP in ume6Δ in Figures 4 and S9. We find that Sir3 association with several subtelomeric loci decreases in ume6Δ, consistent with our computational predictions. This connection between Ume6 and the Sir complex is novel in S. cerevisiae (although one interesting aspect of it is the recent identification of a similar connection in K. lactis), and will be of interest to a range of investigators interested in chromatin and heterochromatin.

- Are UME6 sites really SIR recruiting signals? One could just depletion one of them and then check whether this abolishes SIR binding at this place - or alternatively insert the sequence into TELVIR that now shows linear spreading and test whether the newly inserted site does induce SIR binding. There have been other promoter sequences proposed to recruit Sirs which correlated with Rpd3 recruitment (Taddei et al., 2009). The relationship needs to be explored.

We thank the reviewer for this excellent suggestion, and as noted above found that Sir3 binding to several loci is diminished in ume6Δ mutants.

- The hypothesis that the UME6 sites are favored binding sites for Sir proteins because they recruit Rpd3, is unsubstantiated. Rpd3 has been shown to be a boundary element for SIR spreading with the idea that the acetyl marks resp. the active de-acetylation by Sir2 are important to promote silent chromatin formation. This discrepancy is not discussed by the authors. Several other publications (Zhou et al NAR 2009; Ehrentraut et al PNAS 2010) show that the SIR complex spreads further in the absence of Rpd3 which is the opposite of what the authors suggest. There is not much known about how Rpd3 regulates this. This need to be included in the discussion - a Ume6 deletion experiment would also shed light on that issue.

See above. As requested, we have added a discussion comparing our results with the published literature in this area.

4. PAU genes as recruiting signal: the authors show in Fig. 1C/D/S3 that there is often a "dip" in Sir3 levels in XCR telomeres. It seems like this "dip" coincides with the PAU genes (compare Fig.3C-F and S4). Does the "dip" match to the conserved possible binding element - or is that after the "dip"? Is this observation meaningful? The PAU genes are not regulated by the Sirs per se (i.e. they are not up regulated in a sir3Δ and this needs to be explicitly mentioned in the discussion. It is still possible that, like the authors hypothesize, Sir3 also needs to be removed to activate them under stress conditions.

The dip seen actually largely results from an inability to map sequencing reads over repetitive sequences like the PAU genes, so the PAU genes themselves typically have no reads, but we infer their role in Sir recruitment by the behavior of Sir3 in the regions immediately surrounding the PAU genes. As for regulation of the PAU genes by Sir3, we actually do find that the majority of PAU genes are upregulated at the RNA level in a sir3Δ mutant – see Figure S8.

5. Fig4: Nucleosome occupancy in sir3Δ: Fig4A/B concentrate on genes that are up regulated under these conditions: this has no direct correlation with Sir binding, but is just based on the fact that these genes become transcribed like other genes when their transcription factors become expressed - this is mentioned in the text but could be stressed more explicitly. This data may also be moved to supplemental material.

While the nucleosome mapping results for mating genes is not central to the Sir3 spreading story, it is a relatively rare case where in vivo nucleosome positions change (nucleosomes do not shift positions appreciably in a wide range of changing growth conditions, or in a large number of mutants analyzed to date) and as such is of great interest to the nucleosome mapping field. We would therefore prefer to maintain this result in the main text if the reviewer does not object. But we are happy to move this to the supplement if it proves a sticking point.
6. Fig4C/D: Why do the authors look at all genes up to 20kb from the telomere? It is well established (Wyrick 1999) that only genes 6-8kb away from the telomeres are silenced by the Sir complex. The authors also find Sir3 spreading only up to 5-10kb consistent with this previous data. "To identify more direct effects... we focused on subtelomeric genes shown to bind Sir3 above". Did they now select only genes bound or all telomeres up to 20kb? The first may give better results as only these genes are Sir regulated.

We have modified this figure to only include genes within 15 kb of telomeres. Going any closer results in too few genes to obtain a reasonable average, given the extensive homology between many subtelomeric gene families.

7. Fig.6 GAL-gene binding: Why is there no increase in the Sir3-HA ChiP during the time course whereas for wild type Sir3 the authors can only see binding 5hrs after GAL induction? Is the binding in Fig6B significant? To test whether Sir proteins influence GAL gene expression they test expression levels of GAL genes 1-2hrs after induction yet endogenous Sir3 can only be detected after 5hrs - could that explain why they don’t see a difference? This experiments are not convincing to show a) that Sir3 really binds to GAL genes (see point 1) and b) that GAL expression is not regulated by Sirs. To test whether the detection of Sir3 at the GAL locus is an “artifact” of recruiting these genes to the periphery the same experiments could be repeated in a mlp1, sac3 or mex67 mutant (Dieppois et al 2006; Abruzzi et al 2006) that disrupts this recruitment yet some still allows the activation.

As for the kinetics, several reviewers had this issue – the Sir3-HA binding was done in a raf->gal shift, whereas the binding shown in 6B was done in dex->gal conditions, which induce GAL genes significantly more slowly. We have clarified this issue in the text – the reason for this admittedly irritating change in experiment is to compare native Sir3 binding to changes in GAL gene induction. Since we originally found changes in GAL gene induction in dex->gal but not raf->gal shifts (which subsequently were lost in the hmlD strains), we carried out these Sir3 binding studies under those conditions.

In keeping with the tighter focus of this manuscript, we have moved much of the data concerning euchromatic Sir binding regions to the supplement, and as a result have chosen not to focus on the peripheral recruitment questions raised here.

Minor points:

8. Fig1: data for the histone modifications 1 lacks the indication what the colors mean (low, high levels for red/green), it should best be indicated in the figure like for the yellow/purple.

Thank you, we have rectified this.

9. Fig.3 : explain more explicitly what the red count means: location of PAU genes plus indicating their Sir3 counts in one? Maybe just one color for the PAU genes would be better - the sir3 count information is given in 3B? The table in 3B may be better suited for the supplement, for this figure the information about PAU genes name, and distance from ACS/which one is sufficient.

We have trimmed the PAU table as requested. As for the read counts, we have attempted to clarify this issue in the figure legend, and we have made the requested change.

10. Figure 4B should be sir3Δ instead of sir2Δ.

Actually, the data are from sir2Δ – this was the gene expression data we had available.
that Sir3 also associates with PAU genes located within 10 kb of telomeres and with highly transcribed genes, such as ribosomal protein genes. However, no evidence was obtained linking Sir3 to the transcriptional regulation of these genes.

This paper is clear and well-organized and provides a genome-wide picture of Sir3 association with chromatin. However, a few additional experiments, outlined below, would significantly increase its impact.

1. I couldn't tell from the description of the methods whether the input DNA was also sequenced to identify genomic loci that have higher than expected sequence reads for technical reasons. The authors should comment on whether this control was conducted, as it would be nice to exclude the possibility that the newly detected sites of Sir3 association are not simply technical artifacts. If the authors have not conducted this control, they should confirm by ChIP-PCR that Sir3 does, indeed, associate with these previously undescribed sites.

We have now generated a figure for deep sequencing of sheared genomic DNA (from Teytelman et al), presented in Figure S12. We don’t provide an equivalent for nucleosomal DNA, but nucleosomal DNA shows the opposite behavior from Sir binding – highly-expressed genes tend to have slightly lower nucleosome occupancy than the genomic average, which is in contrast to the higher Sir binding. If necessary we can make a figure for this, but we feel it unnecessary. We have also confirmed by q-PCR that native Sir3 binds to GAL and PAU genes in wild-type cells (Figures 7, S2, S3, S6).

2. The authors ChIPed Sir3 but not Sir2 or Sir4. Given that Sir3 is thought to associate with chromatin independently of Sir2 and Sir4 (Hecht et al., 1996; McBryant et al., 2008), it is important that the authors ascertain whether Sir2 or Sir4 associates with the PAU genes and highly transcribed genes. A chromatin structure containing Sir3 alone may have very different properties from a structure containing Sir2,3,4, and it is important to know which type of structure has been detected.

Thank you – as detailed above in response to Reviewer 1, we have now carried out this important control for several PAU genes, showing that Sir2 binds to these loci and Sir3 binding is Sir2-dependent.

3. The authors detected a sequence motif in subtelomeric silencers and secondary recruitment sites that resembles a Ume6 binding site. If Ume6 does, indeed, contribute to the recruitment of Sir3 to chromatin, this would be an important discovery and the impact of this work would be increased. Two experiments to test this notion are first, to repeat the Sir3 ChIP experiments in a ume6Δ strain, and second, to ChIP Ume6 and determine whether it co-localizes with Sir3 near the detected motifs.

As described in more detail above, we have now carried out Sir3 ChIP in ume6Δ strains, finding that Sir3 binding is decreased around several tested PAU genes in this mutant. We have been unsuccessful in our attempts at Ume6 ChIP to date for unclear technical reasons, but we feel the genetic result is strong enough to include on its own.

4. I was not convinced by the data presented in figure 6 that Sir3 is recruited to GAL genes in galactose.

4a. Panel A is supposed to show the galactose-dependent association of Sir3 at the GAL7,10,1 locus, but lacks the critical zero time point control. These data should be included. In addition, the longest time point shown in panel A is 4 hours, but the conclusion drawn from panel B is that there isn't an increase until 5 hours. Please explain.

Thank you – as detailed above in response to Reviewer 1, this kinetic issue is the difference between galactose induction starting from raffinose (Figure 7A) or dextrose (Figure 7B) – GAL gene induction is rapid (~30 min) from raffinose media, but slow (~4 hours) from dextrose. As noted above, we apologize for this change, which was motivated by our mRNA experiments (Figure S13). As for the zero time point, we do not have t=0 for the Sir3-HA induction system, and the relevant control we do have (Sir3 ChIP in a sir3D) does not strike us as being an ideal comparison. But if requested we can include it.
4b. Panel B is confusing and seems to contradict panel A. First, the scale of the y-axis is baffling and should be expressed in terms of relative enrichment, as is normally done for ChIP data. Second, if I understand the scale correctly, it appears that Sir3 is actually depleted at the GAL locus, rather than enriched as was concluded in panel A. How is this discrepancy explained? Finally, even at five hours the "enrichment" of Sir3 is so minimal compared to what is observed at HMR (inset), that I am skeptical that Sir3 is actually present.

We agree that the Sir3 enrichment at the GAL genes, and at all euchromatic loci, is modest relative to binding to classic heterochromatic loci, and we have attempted to emphasize this in the text. But it has been reproduced not only in 5 ChIP-Seq datasets, but in several q-PCR validations, and is consistent with results regarding HP1 in Drosophila. As far as the scale is concerned, the scale is indeed relative enrichment (corrected for nonspecific binding in a sir3D background).

5. In figure 1B, the authors conclude that the loss of H3K56Ac and H3K4me3 follow the same dynamics as Sir3 binding. However, to my eye it looks like the loss of H3K56ac is delayed, and the distribution of H3K4me3 is not uniform across HMR. If these data are going to be presented (they don’t seem important for the rest of the paper) they should be accurately described.

Actually, your read of the figure is affected by the baseline for each modification – you see that the color changes through black at different times. However, when these data are graphed one sees that the slopes for the modification losses are nearly-identical mirror images to those for Sir3 binding. H3K56 decaetylation is less complete (as we now note in the text), but the kinetics are similar. If required we can present these analyses as a Supplemental Figure.

6. The authors should tone down their conclusion at the end of the introduction that "heterochromatin complexes may play a more widespread role in gene regulation than previously anticipated," given that their data generally indicate that transcription of euchromatic loci is unaffected by the deletion of Sir3.

Thank you, we have made this change.

7. It would be helpful in figure 5 if the four clusters could be more easily linked with the descriptions provided in the text (no Sir3 enrichment, Sir3 associated with promoter, etc.). Also in figure 5C, it is not clear what the y-axis represents.

We have clarified this in the text and in the figure. Y axis is gene # histogram, which we note in the figure legend.

8. In figure 3B, I'm guessing that the data are aligned relative to the atg of the PAU genes? Is the +5kb indicated at the top relative to the left-most data point or relative to the atg? Please clarify in the figure legend.

We have now clarified this issue.

Referee #3 (Remarks to the Author):

The SIR complex is a well-studied system of silencing at telomeres and mating-type loci in Saccharomyces analogous to H3K9-mediated heterochromatic silencing in other eukaryotes. Silencing is known to be able to 'spread' along the chromosome. Past evidence has suggested both a linear polymerization-like model of spreading of silencing involving histone deacetylation and methylation, as well as looping interactions at telomeres, and more recently two rates of spreading that differ between the mating type locus and a telomere. The manuscript by Radman-Livaja et al. describes the first global look at binding of the key SIR complex component Sir3, using an inducible tagged Sir3-HA to track Sir3 location throughout the genome at the single nucleosome level and over a time course of 4.5 hours. They show that their Sir3-HA tag gives similar results to anti-Sir3 antibody. The authors find well-defined regions that show rapid high-level Sir3 binding, and adjacent weaker and more variable regions that increase binding over time and decrease binding over distance from high-level binding regions, similar to recent observations of others. They observe Sir3-dependent alterations in nucleosome positions around transcription start sites for telomeric
and mating-type-related genes. They also find that the seripauperin genes, located predominantly near telomeres, act as secondary recruitment sites for Sir3, and share a DNA sequence motif similar to a binding site for Ume6, a regulator of meiotic genes that recruits histone deacetylases and represses transcription. Somewhat paradoxically, they also find Sir3 over highly expressed genes, although they could find no evidence that Sir3 affected the transcription levels of these genes. The authors argue that traditional polymerization models do not account for their observations of two modes of spreading kinetics, "cooperative-like" and "linear".

This global ChIP-Seq view of Sir3 binding provides new insights into SIR complex biology, identifies several new binding sites of the SIR complex, and provides more complete evidence than previously available challenging the prevailing view of how spreading of silencing occurs. The data here are likely to be of importance in formulating new models of spreading that better account for the observed bimodal kinetics and the roles of different types of silencers. The authors also draw attention to the possible role of the SIR complex in regulating the seripauperin genes, and a possible evolutionary link to Ume6 silencing. The paper seems likely to have a significant impact on the field of chromatin silencing. My comments are largely restricted to minor issues of presentation, clarity and interpretation.

We thank the reviewer for the supportive comments.

On p. 4, end of the 1st paragraph the authors say "H4K16 and H3K56 deacetylation as well as H3K4me3 demethylation appear to follow the dynamics of Sir3 binding". Is this a fair assessment? The extent of H3K36ac deacetylation seems limited, slow, and inconsistent compared with the other changes in 1B, although the difference is less dramatic in 1C. Also, the colors used in 1B are not explained.

As the reviewer states, the extent of H3K56 deacetylation is less than the extent of H4K16 deacetylation of H3K4 demethylation. However, the kinetics are very similar. If necessary we'd be happy to include a supplemental figure like S1 to demonstrate this. We have added colorscale panels to Figure 1 to explain the color scheme.

In the next paragraph, the authors refer to the promoter region of OCA4 and GIT1 and a region "2kb upstream to 4kb downstream of HMR-E". Unfortunately, the orientations of genes or positions of promoters, as well as the location of HMR-E, are not indicated in the cartoon in Figure 1B, and it is not clear what "upstream" and "downstream" refer to. In the Figure Legend 1C, it is stated that the "curves are centered at the HMR-E silencer", which seems to be at the left side of HMR at the vertical axis, but the horizontal coordinates do not suggest that this is the center of the curves. This figure could be clarified.

We have made the requested revisions to Figure 1 – promoters for OCA4 and GIT1 are indicated with arrows, and HMRE is labeled in the annotations area. Upstream and downstream have been changed in the text to centromere and telomere-proximal (?). I do not understand the problem with the x axis labeling of part C – the x axis label explicitly states “distance from HMR-E (bp)”, which I think indicates what the reviewer is asking for?

On p. 5, refers to anti-Sir3 ChIP in wildtype cells in Figure S2, which is titled "Comparison of wt Sir3 and pGAL-induced Sir3-HA binding to yeast telomeres". Figure Legend S2B merely says "As in Figure 2A". Is Figure S2B anti-S3 in wildtype cells, which would be the simplest guess from the cryptic Legend, or is it Sir3-HA, as implied by the figure title? My guess is the former, but both the Figure title and Legend are very unclear.

We have clarified Figure S2 with explicit titles above the heatmaps.

On p. 6, first full paragraph, the authors identify a "weaker additional site of Sir3 binding" on 2R, and later in the paragraph they mention binding to PAU24 on 2R. I cannot see any binding on 2R in Figure 2 that seems significant, and in Figure 3A, PAU24 seems to be black, meaning no Sir3 counts. Can the authors clarify, please? The black in Figure 3A is hard to see against the purplish-brown background - would white be a better choice?
Figure S5 shows a closeup of Sir3 binding around PAU24. We have changed the background in 3A to white as requested.

In Figure S5, it would seem more natural to me if the labels of B and C were switched to proceed left to right then top to bottom.

We have made the requested change.

Figure 4 and the accompanying text on p.7 are somewhat unclear. Instead of referring to "pseudo-diploid gene expression patterns" it would be helpful to readers to specify what the expected expression patterns of STE2 and other genes are in SIR3 and sir3delta strains. Figure 4B refers to 'sir2delta', while all other references are to 'sir3delta'. The Legend to 4B is confusing, in part because of the dual meaning of "close" (verb or adjective?) and I am unsure what it intends to convey. The Legends to 4C and 4D say "As in A" and "As in B", but they do not specify what is "as in A" - symbols and colors, presumably?

We have added discussion of the fact that sir mutants downregulate mating-related genes such as STE2 due to the derepression of the silent mating loci. Regarding the sir2D, the reason for this is that the gene expression data we have comes from sir2D, while our nucleosome mapping was done in the sir3D. We have attempted to clarify the 4B-D legends as requested.

In Figure Legend S6 it would be simpler and more helpful to restate what the colors represent than to send the reader to another figure to find out. 'WT' (purple) and 'sir3delta' (green) are already mentioned in the legend, so it actually takes more space to refer the reader to Figure 4.

We have clarified this as requested.

On p. 9, at the end of the 2nd paragraph, the authors say that 'Sir3 "spreads" from GAL promoters into the body of the gene 5hrs after the dextrose to galactose shift'. For the GAL7 coding sequence, Figure 6C is consistent with this interpretation, but there is already substantial Sir3 binding on the GAL10 and GAL1 coding sequences in dextrose, and it is unclear that the increase at both promoters and gene bodies after 5 hrs in galactose is indicative of spreading. The same figure might be used to argue that Sir3 spreads from the FUR4 coding sequence to the FUR4 promoter.

We have clarified this as requested.

In the Figure Legends, "Figure S87" should be "Figure S8".

We have fixed this.

2nd Editorial Decision 11 January 2011

Thank you very much for submitting a new version of your earlier reviewed manuscript (EMBOJ-2010-75902) for consideration to our editorial office. Appreciating the amendments it was sent for peer-review and the reports are enclosed for your information. As you can see, all scientists are essentially in favour of publication here and raise a couple of technical issues as well as on the appropriateness of the title.

I kindly ask you to attend to these points and provide us with the ultimate version of your study as soon as possible for eventual acceptance.

Yours sincerely,

Editor
The EMBO Journal
The authors addressed most of the previous concerns and the article is substantially improved.

However, I'm still having trouble with figure 7. First, it was not until I read the response to reviewers that I appreciated that two different experimental protocols (glucose or raffinose) were used. This difference should be made clear in the body of the text, not in the figure legend. Second, figures B and D (the experiments that started in glucose) just don't have much signal, yet they are described in the text as providing evidence of Sir3 association. Perhaps it would be better to emphasize that the recruitment of Sir3 to the GAL locus is slow when shifting from glucose.

There are still a few minor issues that can be clarified or improved. The title "Dynamics of Sir complex spreading in budding yeast: secondary recruitment sites and euchromatic localization" slightly overstates the conclusions. The authors found Sir2-dependence at the secondary recruitment sites, but not at GAL4 euchromatic sites, so it would be more accurate to replace "Sir complex spreading" with "Sir3 spreading".

In the abstract the authors state that "association of heterochromatin complexes with highly-expressed euchromatic genes is evolutionarily-conserved." Although this might be true between closely related yeasts, they seem to mean that similar association with euchromatic genes is seen for both Sir3 in yeast and HP1 in flies. However, if this is the intended meaning, this is not evolutionary.
conservation (since the two silencing systems are not homologous) but parallel evolution of two silencing systems that also bind to euchromatic genes. This mischaracterization of evolutionary trajectories should be corrected, particularly since the authors fail to demonstrate any function (conserved or otherwise) of Sir3 binding to highly expressed genes, despite considerable efforts to find one. Instead they suggest that binding is a (possibly fortuitous) consequence of underacetylated nucleosomes present in highly expressed genes independent of Sir2 deacetylation activity. Binding of HP1 to euchromatic fly genes might be similarly fortuitous, but it is not conserved in budding yeast.

Figure Legend S2B says "As in Figure 2A". Do these two figures represent the exact same data set, or do they differ in some way?

On p.6, first full paragraph the authors list "weaker additional sites" of Sir3 binding "at 3R, 2R, 8L, 9R, 15R, 7L, 11L, and 14R." Since one cannot easily see the site on 2R in Figure 2A and I can find no reference to Supplementary Figure S4 in the text, this would be a good place to refer readers to it: "(Figure 2A and Supplementary Figure S4)", conveniently located before the reference to Supplementary Figure S5 in the next sentence.

Supplementary Figure S6C shows Sir2-PA binding relative to input at subtelomeric Sir3 targets. Can the authors include a non-target as a negative control?

In Figure 3A, the PAU 18 label for 12L is misplaced over 9L. In 3B, I presume that replottting the data in Figure 2A to align at the PAU gene ATG reverses the left-right orientation. Is this correct?

I can find no text reference to Supplementary Figure S9, and it seems at least partially redundant with Figure 4. Perhaps it is unnecessary?

Changes in nucleosome positions between wild type and sir3 deletion are shown for mating genes in Figure 5A and Supplementary Figure S11, but the average behavior in 5B seems to show no changes in nucleosome positions and a modest increase in nucleosome occupancy upstream of the transcription start site in the sir3 deletion. Is this meaningful? Is this different than, say, housekeeping genes? I do not see how 5B supports the assertion in the legend that "Mating-related genes generally close in sir3 deletion pseudo-diploid yeast", unless this statement is merely intended to summarize previously established gene expression data, not current nucleosome position data. Perhaps upstream nucleosome occupancy increases in a sir3 deletion because the genes are not being remodeled for transcription? Please clarify the interpretation. Also, the label in Figure 5B describes genes with >4-fold repression in a sir2 deletion strain, whereas the corresponding Figure Legend describes genes with >4-fold repression in a sir3 deletion strain.

On p. 13 and in the bibliography, the citation for Barsoum et al. is incomplete.

Referee #3 (Remarks to the Author):

Dynamics of Sir complex spreading in budding yeast: secondary recruitment sites and euchromatic localization
Marta Radman-Livaja, Assaf Weiner, Giulia Ruben, Nir Friedman, Rohinton Kamakaka, and Oliver J. Rando

This study provides a genome wide overview of Sir3 binding sites and the kinetics with which they are established. It clearly shows that the polymerization model only holds true for some Sir sites like the telomere 6R, but that most sites show rapid Sir binding over few kbs. This rapid assembly correlates with the strength of Sir recruitment sites. In addition, they describe novel binding sites of Sir3, the PAU genes close to telomeres and strongly transcribed genes like the GAL genes in galactose containing media. Whereas the first one is Sir2 dependent and therefore probably reflects classical "Sir complex" binding sites, Sir3 seems to be bound independent of the other Sirs to the GAL loci. The authors provide evidence that the PAU genes are indeed Sir recruitment sites and that Ume6, the regulator of PAU genes, contributes to this. This is a novel finding which seems to shed light on evolution of silencing in yeasts. For the GAL genes it is less clear whether the observed association is meaningful.

We feel that this study now is clear and more careful written, and that it does contribute significantly to our understanding of Sir protein biology. We do have some minor comments that should be corrected, otherwise it is suitable for publication in EMBO Journal.

- Fig4/FigS9: Why is there a drop at HMR (and to a lesser extent 1L) in the pau13 deletion (it may not be significant but is still striking)? It seems rather surprising that deletion of this gene affects
Sir3 binding to other silent loci and the authors do not comment on that in the text. The data may also be removed as it does rather confuse then contribute at this point.
- Fig4 may be structured clearer: the scheme in Fig4A and Fig4E (2nd part) is almost the same and could be done in one which would leave more space for the rest of Fig4E. For Fig4C, the qPCR rectangles may be colored in red like throughout the rest of the figure. Chromosome 6 should be removed, or it should be presented consistently (with red rectangles for qPCR primers and maybe like chromosomes in Fig4A), otherwise it is rather confusing.
- Fig4S is never referenced in the text - if it stays in it should be, but it may also be removed as it shows additional close-ups of PAU genes. Also, for clarity the same color code should be used for the time course as in Fig3 (the same colors could also be used also in Fig7A).
- FigS2: As stated, overexpressed Sir3-HA and wt Sir3 have overall quite similar binding sites. However, at telomere 7L there seems to be a big stretch not bound by Sir3-HA that is bound by wt Sir3 - is there any explanation?
- The reference "Barsoum et al" lacks the year (2010).

Referee #1 (Remarks to the Author):

The authors addressed most of the previous concerns and the article is substantially improved.

However, I'm still having trouble with figure 7. First, it was not until I read the response to reviewers that I appreciated that two different experimental protocols (glucose or raffinose) were used. This difference should be made clear in the body of the text, not in the figure legend. Second, figures B and D (the experiments that started in glucose) just don't have much signal, yet they are described in the text as providing evidence of Sir3 association. Perhaps it would be better to emphasize that the recruitment of Sir3 to the GAL locus is slow when shifting from glucose.

- We have clarified this issue in the text as requested.

Referee #2 (Remarks to the Author):

The SIR complex is responsible for silencing at telomeres and silent mating type loci in budding yeast, and can spread silencing along the chromosome, similar to the spreading of position-effect variegation in fruit flies. This manuscript provides a genome-wide view of the binding pattern and temporal spreading of the key SIR complex component Sir3. Using a tagged Sir3 protein (Sir3-HA) under a galactose-inducible promoter in a sir3 deletion strain and ChIP-Chip and ChIP-Seq technology, Radman-Livaja et al report a bimodal kinetics of Sir3-HA binding that is inconsistent with a simple nucleation and linear spreading model. They find rapid cooperative-like high-level binding at the silent mating type loci, ARS consensus sequences (ACS) within the subtelomeric XCS elements, and some other loci, flanked by regions of weaker binding and linear spreading, in which binding decreases nucleosome by nucleosome along the chromosome, and increases over time. They find novel binding sites for Sir3-HA at subtelomeric seripauperin (PAU) genes. Using an anti-Sir3 antibody, they show that wildtype Sir3 has a similar binding pattern to overexpressed Sir3-HA, including at PAU genes, although Sir3 binding is not always as extensive as the overexpressed Sir3-HA. They show that a SIR3 deletion results in increased PAU gene expression, find that Sir3-HA binding at PAU genes is dependent on Sir2 suggesting the presence of the entire SIR complex, and confirm that Sir2-HA binds to the same loci. They provide evidence that some PAU genes can act to recruit Sir3, that these genes share an Ume6-like binding sequence, and that binding is reduced by the deletion of UME6, a regulator of meiotic genes. They find an alteration of nucleosome spacing around the transcription start sites of subtelomeric genes in a SIR3 deletion, suggesting a possible role of Sir3 in stabilizing nucleosomes. Surprisingly, they also find that Sir3-HA and wild-type Sir3 bind to highly expressed active genes, including dynamic rec localization to the induced GAL genes. Despite this binding, no effect on GAL expression levels could be found in a Sir3 deletion, and the Sir3 binding of GAL genes is not dependent on Sir2.

This re-submitted manuscript clarifies several presentation issues in the earlier version and provides more detailed qPCR comparison of Sir3-HA and wild-type Sir3 binding patterns, together
with new genetic data showing that PAU genes can act to recruit Sir3, that Sir3 recruitment is at least partially dependent on UME6, and that Sir3 binding to PAU genes is Sir2-dependent while Sir3 binding to GAL genes is Sir2-independent. These additional genetic data increase the strength of the paper by going beyond correlational data to demonstrate functional relations. The result is a global view of Sir3 binding that reveals two modes of Sir3 spreading (cooperative-like and linear), a new class of secondary Sir3 recruitment sites, regulation of seripauperin genes by Sir3, and modest binding of Sir3 to highly expressed genes independently of Sir2. The data here provide a better understanding of Sir3 and are likely to be of importance for formulating better models of SIR complex spreading and functions. They also suggest an evolutionary link between Sir complex silencing and Ume6 silencing of meiotic genes and of the silent mating type loci of Kluveromyces.

- We thank the reviewer for the detailed comments.

There are still a few minor issues that can be clarified or improved. The title "Dynamics of Sir complex spreading in budding yeast: secondary recruitment sites and euchromatic localization" slightly overstates the conclusions. The authors found Sir2-dependence at the secondary recruitment sites, but not at GAL4 euchromatic sites, so it would be more accurate to replace "Sir complex spreading" with "Sir3 spreading".

- Changed as requested.

In the abstract the authors state that "association of heterochromatin complexes with highly-expressed euchromatic genes is evolutionarily-conserved." Although this might be true between closely related yeasts, they seem to mean that similar association with euchromatic genes is seen for both Sir3 in yeast and HP1 in flies. However, if this is the intended meaning, this is not evolutionary conservation (since the two silencing systems are not homologous) but parallel evolution of two silencing systems that also bind to euchromatic genes. This mischaracterization of evolutionary trajectories should be corrected, particularly since the authors fail to demonstrate any function (conserved or otherwise) of Sir3 binding to highly expressed genes, despite considerable efforts to find one. Instead they suggest that binding is a (possibly fortuitous) consequence of underacetylated nucleosomes present in highly expressed genes independent of Sir2 deacetylation activity. Binding of HP1 to euchromatic fly genes might be similarly fortuitous, but it is not conserved in budding yeast.

- This is reasonable, and we have changed the sentence

Figure Legend S2B says "As in Figure 2A". Do these two figures represent the exact same data set, or do they differ in some way?

- They are the same data, as we now state.

On p.6, first full paragraph the authors list "weaker additional sites" of Sir3 binding "at 3R, 2R, 8L, 9R, 15R, 7L, 11L, and 14R." Since one cannot easily see the site on 2R in Figure 2A and I can find no reference to Supplementary Figure S4 in the text, this would be a good place to refer readers to it: "(Figure 2A and Supplementary Figure S4)", conveniently located before the reference to Supplementary Figure S5 in the next sentence.

- As requested.

Supplementary Figure S6C shows Sir2-HA binding relative to input at subtelomeric Sir3 targets. Can the authors include a non-target as a negative control?

- These data are normalized to a non-target – we have now added the non-target and the targets as IP/input to Figure S6C.

In Figure 3A, the PAU 18 label for 12L is misplaced over 9L. In 3B, I presume that replotting the data in Figure 2A to align at the PAU gene ATG reverses the left-right orientation. Is this correct?

- Thank you, we have corrected the label. As for 3B, that is correct.
I can find no text reference to Supplementary Figure S9, and it seems at least partially redundant with Figure 4. Perhaps it is unnecessary?

- Figure S9 also includes the galactose version of the ume6D experiment. We have referenced the figure at the appropriate place in the text, and given the locations of the call-outs have also changed the relative numbering of S9 and S10.

Changes in nucleosome positions between wild type and sir3 deletion are shown for mating genes in Figure 5A and Supplementary Figure S11, but the average behavior in 5B seems to show no changes in nucleosome positions and a modest increase in nucleosome occupancy upstream of the transcription start site in the sir3 deletion. Is this meaningful? Is this different than, say, housekeeping genes? I do not see how 5B supports the assertion in the legend that "Mating-related genes generally close in sir3 deletion pseudo-diploid yeast", unless this statement is merely intended to summarize previously established gene expression data, not current nucleosome position data. Perhaps upstream nucleosome occupancy increases in a sir3 deletion because the genes are not being remodeled for transcription? Please clarify the interpretation. Also, the label in Figure 5B describes genes with >4-fold repression in a sir2 deletion strain, whereas the corresponding Figure Legend describes genes with >4-fold repression in a sir3 deletion strain.

- The increase in nucleosome occupancy seen upstream of mating genes is indeed significant – it is similar to the differences between "stress" and "growth" genes, the canonical comparison for differently-packaged genes. The "closing" to which we refer is the increase in occupancy noted by the reviewer. We attempt to make this clearer in the text now. As for the sir2Δ vs. sir3Δ, we have fixed the legend as requested.

On p. 13 and in the bibliography, the citation for Barsoum et al. is incomplete.

- Thank you

Referee #3 (Remarks to the Author):

Dynamics of Sir complex spreading in budding yeast: secondary recruitment sites and euchromatic localization Marta Radman-Livaja, Assaf Weiner, Giulia Ruben, Nir Friedman, Rohinton Kamakaka, and Oliver J. Rando

This study provides a genome wide overview of Sir3 binding sites and the kinetics with which they are established. It clearly shows that the polymerization model only holds true for some Sir sites like the telomere 6R, but that most sites show rapid Sir binding over few kbs. This rapid assembly correlates with the strength of Sir recruitment sites. In addition, they describe novel binding sites of Sir3, the PAU genes close to telomeres and strongly transcribed genes like the GAL genes in galactose containing media. Whereas the first one is Sir2 dependent and therefore probably reflects classical "Sir complex" binding sites, Sir3 seems to be bound independent of the other Sirs to the GAL loci. The authors provide evidence that the PAU genes are indeed Sir recruitment sites and that Ume6, the regulator of PAU genes, contributes to this. This is a novel finding which seems to shed light on evolution of silencing in yeasts. For the GAL genes it is less clear whether the observed association is meaningful.

We feel that this study now is clear and more careful written, and that it does contribute significantly to our understanding of Sir protein biology. We do have some minor comments that should be corrected, otherwise it is suitable for publication in EMBO Journal.

- Thank you for the supportive comments.

- Fig4/FigS9: Why is there a drop at HMR (and to a lesser extent 1L) in the pau13 deletion (it may not be significant but is still striking)? It seems rather surprising that deletion of this gene affects Sir3 binding to other silent loci and the authors do not comment on that in the text. The data may also be removed as it does rather confuse then contribute at this point.
We agree, HMR has been removed.

- Fig4 may be structured clearer: the scheme in Fig4A and Fig4E (2nd part) is almost the same and could be done in one which would leave more space for the rest of Fig4E. For Fig4C, the qPCR rectangles may be colored in red like throughout the rest of the figure. Chromosome 6 should be removed, or it should be presented consistently (with red rectangles for qPCR primers and maybe like chromosomes in Fig4A), otherwise it is rather confusing.

- We have changed the figure as requested.

- FigS4 is never referenced in the text - if it stays in it should be, but it may also be removed as it shows additional close-ups of PAU genes. Also, for clarity the same color code should be used for the time course as in Fig.3 (the same colors could also be used also in Fig7A).

- We have now referenced Figure S4A in the text. We prefer not to change the color coding simply given the effort required, but if the editors prefer we will change it if needed.

- FigS2: As stated, overexpressed Sir3-HA and wt Sir3 have overall quite similar binding sites. However, at telomere 7L there seems to be a big stretch not bound by Sir3-HA that is bound by wt Sir3 - is there any explanation?

- This region is absent in the pGAL-Sir3-HA strain but is present in the wild-type, as noted in the text.

- The reference "Barsoum et al" lacks the year (2010).

- Thank you, we have corrected this.