Comments and Suggestions for Authors

The authors have addressed many of the scientific limitations of the manuscript in this first revision. The Sir3-biochemistry in the new figure 1 helps, particularly the ITC. The quantitation of the images in the new figure 4 are welcomed. However, additional revision is required for the new figure 3d, and new figure 4 requires some additional controls, as stated below. The improvement in the English was appreciated but there are still issues that must be rectified, as itemized below.

We appreciate reviewer's recognition on our revised manuscript. We have now added some more figures and the corresponding description in our new revise version of manuscript. We have now also reedited some other paragraphs regarding to reviewer's commends.

Issues with the science:

Figure 3d – The authors claim that the ChIP-qPCR in the figure matches the composite ChIP-on-ChIP data in figures 3a-c. However, there is too much data in figure 3d to easily reach this conclusion. Moreover, the major point of figures 3a-c is that Sir3 and AAR are more enriched at proximal locations in the ysa1 mutant, and that both spread farther from the chromosome end in the ysa1 mutant. The authors don’t discuss this for figure 3d, and it is hard to tell from the data that this is really the case. This paper is supposed to be about the impact of AAR on Sir3! Unfortunately, the only thing the authors describe is that Sir3 spreads farther than Sir2 at Tel6R, and that AAR has no impact on Sir2. Thus, figure 3d does not really recapitulate the important data of figures 3a-c.

We thank for reviewer's criticism. We have now reedited the Figure 3d-3e as the new Figure 3a-3i and the Figure 3a-3c as the new Figure 4a-4c. We have also added some paragraphs of the corresponding description (lines 196-200, page 6 & lines 208-230, page 8). Yes, the reviewer's comment is right. We addressed the issue regarding to the impact of AAR on Sir3 and we just focused on the AAR promotes the Sir3 spreading further along the telomeric region in our current present story.
The error bars are suspiciously small and consistent from sample to sample. The authors need to define exactly what the error bars represent (which trials were used to calculate error and how was the error calculated). This is critical because the magnitudes of the differences they report are very, very small. For example, Sir2 levels drop only ~5% at the 0.6 kb site relative to the chromosome end. Similarly, Sir3 levels increase only ~10% at the 0.6 kb site relative to the chromosome end. Do the measurements of error validate a discussion of 5-10% differences by ChIP-qPCR?

We thank to reviewer mentions this. The error bars were generated from 1 standard deviation as the common statistical used. In fact, we was a little surprised to the data of standard deviation in the qPCR test. We calculated the averages and their standard deviations using the classical formula/function in the microsoft office excel software. The data of standard deviation are shown as below:

<table>
<thead>
<tr>
<th>Ct-SD</th>
<th>Wt Sir2-ChIP</th>
<th>Wt Sir3-ChIP</th>
<th>Wt AAR-ChAP</th>
<th>Dy sa1 Sir2-ChIP</th>
<th>Dy sa1 Sir3-ChIP</th>
<th>Dy sa1 AAR-ChAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Act in</td>
<td>0.14</td>
<td>0.11</td>
<td>0.17</td>
<td>0.17</td>
<td>0.04</td>
<td>0.20</td>
</tr>
<tr>
<td>YAL068C</td>
<td>0.03</td>
<td>0.04</td>
<td>0.03</td>
<td>0.08</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>YAL067C</td>
<td>0.03</td>
<td>0.11</td>
<td>0.10</td>
<td>0.35</td>
<td>0.02</td>
<td>0.12</td>
</tr>
<tr>
<td>YAL065C</td>
<td>0.05</td>
<td>0.37</td>
<td>0.09</td>
<td>0.02</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>YBR302C</td>
<td>0.02</td>
<td>0.04</td>
<td>0.03</td>
<td>0.04</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>YBR301W</td>
<td>0.06</td>
<td>0.00</td>
<td>0.04</td>
<td>0.14</td>
<td>0.03</td>
<td>0.20</td>
</tr>
<tr>
<td>YBR299W</td>
<td>0.57</td>
<td>0.09</td>
<td>0.02</td>
<td>0.20</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>Chr6_TEL0.07</td>
<td>0.11</td>
<td>0.03</td>
<td>0.16</td>
<td>0.12</td>
<td>0.06</td>
<td>0.07</td>
</tr>
<tr>
<td>Chr6_TEL0.6</td>
<td>0.14</td>
<td>0.02</td>
<td>0.20</td>
<td>0.08</td>
<td>0.07</td>
<td>0.11</td>
</tr>
<tr>
<td>YFR056C</td>
<td>0.07</td>
<td>0.08</td>
<td>0.14</td>
<td>0.12</td>
<td>0.10</td>
<td>0.04</td>
</tr>
<tr>
<td>YKL224C</td>
<td>0.10</td>
<td>0.05</td>
<td>0.10</td>
<td>0.11</td>
<td>0.01</td>
<td>0.11</td>
</tr>
<tr>
<td>YKL222C</td>
<td>0.04</td>
<td>0.05</td>
<td>0.07</td>
<td>0.15</td>
<td>0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>YKL221W</td>
<td>0.05</td>
<td>0.05</td>
<td>0.07</td>
<td>0.07</td>
<td>0.28</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Since only one of four telomeres is discussed (Tel6R), I recommend that only that only Tel6R be shown in the figure and the rest of the data be moved to the supplement.
We thank for reviewer's suggestion. We have now reedited the Figure 3d-3e as the new Figure 3a-3i and the Figure 3a-3c as the new Figure 4a-4c. We have also added some paragraphs of the corresponding description (lines 196-200, page 6 & lines 208-230, page 8).

Figure 4 – Can long ropes be formed with just Sir3C, nucleosomes and AAR? Can they be formed with just Sir3N, nucleosomes and AAR? Are nucleosomes even required for these ropes? These controls are easy and need to be included. It is likely that the images already exist.

We thank for reviewer's criticism. We have now added the Sir3N+Nucleosomes and Sir3C+Nucleosomes in absent or present AAR as other control tests (new Figure 5f-5i) and reedited the corresponding text as "....these effects were not observed when the full-length Sir3, Sir3N or Sir3C was used (Figure 5d-5i)...." (line274-275, page 10). And since the AAR does not interact with nucleosome and Nucleosome+AAR can not form the filament structure (Onishi et al., 2007; Tung et al., 2017), so we did not re-examine and add the control of Nucleosome+AAR in this paragraph.

Issues with English:

Lines 3 and 85 – what does "relative" mean?

We thank for reviewer's criticism. AAR is generated by Sir2 during its action on a histone epigenetic modification. That is, the production of AAR is an epigenetic action-dependent/relative event. So, originally, we call AAR is an epigenetic metabolic small molecule. Then after another reviewer's criticism, we consulted with an English science editor, so we change to call AAR as an epigenetic relative metabolic small molecule. In fact, both "an epigenetic relative small molecule" and "an epigenetic relative metabolic small molecule" should be acceptable. And we prefer to call AAR as "an epigenetic metabolic small molecule". So, we now correct back to "AAR is an epigenetic metabolic small molecule".
We thank to reviewer points out this. We have now reedited them as "....O-acetyl-ADP-ribose (AAR), an epigenetic metabolic small molecule, is generated by NAD-dependent Sir2/Class III histone deacetylase...." (lines 46-48, page 2), ".... assembly system of SIR-nucleosome pre-heterochromatin filaments that has been established. All purified yeast nucleosomes, Sir2, Sir3, Sir4, and AAR are required for this assembly, and the formation of these filaments is modulated...." (lines 52-54, page 2), and ".... AAA ATPase-like domain in the Sir3 C-terminal region that may interact with ATP [26], and AAR exhibits the structural similarity to ATP...." (lines 57-59, page 2).

Line 184 – “expression” levels?

We thank to reviewer points out this. We have now re-written it as "....expresional levels were...." (lines 116-117, page 3).

Line 314 – needs corrections.

We thank to reviewer points out this. We have now re-written it as "....arrows were pointed out the detected individual signal on the dot positions of Sir3 but no signal on the dot positions...." (lines 138-139, page 4).

Lines 362-363, 364-365 and 373-376 are redundant.

We thank to reviewer mentions this. To avoid the redundancy, we have now deleted the sentence: "Moreover, both Sir3 and AAR were colocalized over regions that clearly extended further beyond the chromosome ends compared with Sir2."

Lines 455-464 – It is still not at all clear what the authors are trying to say. Does a ysa1 null influence telomere clustering?
We thank for reviewer's criticism. In this paragraph, we discussed that the mechanism through the mitochondrial activity results in a nuclear effect of telomere cluster and tried to make an explanation for that. Sir3-dependent telomere clustering can be attributed to the mitochondrial activity of reactive oxygen species (ROS). AAR affects the electron transport chain and ysa1-deletion cells display higher resistance to exogenous ROS. Our present results may offer a potential answer. The amount of AAR is increased by approximately 50% in ysa1-deletion cells. And the increased level of AAR may protect cells from stress through its effects on ROS and the electron transport chain. AAR associates with Sir3 and increases their further spreading along the silent heterochromatin, thus, to form Sir3-dependent telomere clustering that may be a protective response to starvation/stress. In summary, these AAR effects may induce a more obvious phenomenon of telomere clustering and be involved in the phase-separated liquid-like droplet organization of heterochromatin.

Lines 478-485 – The speculation described here is difficult to follow. Too confusing.

We thank for reviewer's criticism. In this part, firstly, we described an open question: "Sir2 produces AAR, which accompanies the structural rearrangement of SIR complexes but the biological role of this conformational change is still unclearly". Then we discussed a potentially answer according to our data. So, we proposed that this conformational change may correlate with the structural or conformational alteration of the Sir3 BAH domain and C-terminal region to create a stable association space for AAR binding. It is likely that this results in stabilization of the intermolecular interactions between the Sir3 BAH domain and the C-terminal region through association with AAR.

Lines 484-485 – How does stabilizing a Sir3 dimer lead to polymers? Are the authors suggesting that AAR strengthens dimers of dimers? Are the authors suggesting that there are intermolecular interactions between the Sir3N domain of one Sir3 with the Sir3C of another Sir3? This section is too confusing.

Yes, reviewer is right. We proposed that the stabilization of intermolecular interactions between the Sir3 BAH domain and the C-terminal region through
association with AAR. We are now working to solve the high resolution of the Sir3-AAR structure via cryo-EM. We believe it is beyond the scope of the present story. However, we have a model as below:

![Diagram of protein-protein interactions]

Line 723 – Protein-protein interactions? Which proteins?

We thank reviewer points out this. The "protein-protein interactions" means any kind interaction that is able to promote the longer SIR-nucleosome filament formation, such as, Sir3-Sir3 interaction and Sir3-nucleosome interaction, however, other potential interactions (ex: nucleosome-nucleosome interaction) cannot be totally ruled out.

Lines 727-731 – These lines do not describe the figure. If the paragraph is supposed to be a conclusion, it does not read like it.

We thank for reviewer's criticism. We have now reedited this paragraph as "The ability of AAR for modulating the formation of the telomere silent heterochromatin regions that may be not totally mediated by Sir2. Another non-Sir2 AAR interaction protein, such as Sir3 may also form polySir3-AAR in the silent heterochromatin regions. As illustrated in the proposed model (Figure 6), AAR plays a critical role in stabilizing Sir3 interactions on the telomere silent heterochromatin domain, and AAR remains associated with Sir3 (in the apparent absence of Sir2 and Sir4) in the extended silent heterochromatic region" (lines 302-307, page11).
Issues with figures:

Figure 1 - The panels are not aligned with one another

The figure 1 looks OK, but figure 2 (on for reviewer version made by journal/publisher) looks something wrong. Here is the Figure 1 & 2 as below:

![Figure 1 & 2](image)

Figure 5 – My version of this figure is still distorted. Something must be causing the distortions in preparation of the reviewer's copy.
Here is the figure 6 (figure 5 of old version):