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Sir3 Heterochromatin Protein Promotes Non-Homologous End Joining by Direct Inhibition of Sae2

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Thank you for submitting your manuscript on Sir3-Sae2 interplay for our editorial consideration. I have now heard back from three reviewer, and also further discussed it with an expert editorial advisor of our journal. In light of their combined feedback, we would be interested in pursuing this study further for EMBO Journal publication, pending satisfactory revision of a number of major and minor issues raised by the referees (whose reports are copied below) and by our advisor.

In particular, it will be important to address the following key points:

- As requested by the advisor, supporting the proposed Sir4/Sae2 binding competition by strengthening the evidence for direct Sir3-Sae3 interaction in the absence of Sir4 or possible other intermediary proteins. Ideally via in vitro binding studies or comparative pull-down/mass spec analyses of wt and mutant Sir3; alternatively by validating Sir3-Sae2 two-hybrid interactions in sir4Δ strains.

- Adding stronger support for a physiological (overexpression-independent) Sir3 role in Sae2 inhibition (refs 1 and 2) - e.g. by assessing Sae2 binding at DSBs in the absence of Sir3.

- More directly showing that Sir3 prevents Sae2-MRX interaction (refs 1 and 2).

- I realize that survival as readout for NHEJ capacity, criticized by referee 1, remains frequently used in the field and difficult to replace; testing whether plasmid re-joining is increased in Sir3-overexpressing cells as observed for sae2 mutants, and decreased in the sir3 mutant, might nevertheless provide a nice complementation to these readouts.

Please be reminded that it is our policy to allow only a single round of major revision, making it important to comprehensively and carefully respond to all the points raised by the referees and our advisor at the time of resubmission. Should you require extra time for this in light of the present pandemic situation, or have any particular questions regarding the referees' comments and how to best address them, please do not hesitate to contact me for further discussion already during the early stages of your revision. Our scooping protection (meaning that competing work appearing elsewhere in the meantime will not affect our considerations of your study) remains of course valid also during an extended revision period.

Referee #1:

In this manuscript by Bordelet and colleagues, the authors show that Sir3 interacts with Sae2 and this interaction impairs Sae2 function in DSB resection and NHEJ inhibition.

I think that the manuscript suffers of two main problems:

1) The biochemical data that Sir3 interacts with Sae2 is convincing. The problem is that most (if not all) of the experiments supporting the functional role of this interaction have been done upon Sir3 overexpression. Therefore, whether Sir3 controls Sae2 activity when is present in physiological amount is not known and for this reason I think that the manuscript, as it is, is more suitable for a biochemical journal.
2) In all the experiments, the NHEJ efficiency is measured by determining the percentage of survival after generation of an irreparable I-SceI DSB. Under this condition, survival depends on error prone NHEJ events that generate a sequence that cannot be cut by I-SceI. These events are rare and for this reason the percentage of survival is extremely low (less than 1% in wt cells). I think that it is important to measure whether canonical NHEJ events, that are more efficient, are also under the same control.

Major points
1. In Figure 1B, 1C, 1H, 5A, 5B, 6A, 6B % survival is extremely low because the error-prone NHEJ events measured are rare. The differences in survival % are very subtle (from 0.2 to 3 %). The p-values are not reported and I am not convinced about the significance of many differences. There are several other assays (also molecular assays) that can be used to measure the ability of cells to perform correct and more efficient NHEJ repair events.
2. Figure 1E: this experiment has to be repeated at different distances from DSB because, as it is, is very poor and not convincing.
3. line 131: how do the authors know that Sir3 is not bound to euchromatin DSB sites when it is overexpressed? It is hard to me to imagine that Sir3 interacts with Sae2 but it is not recruited at DSBs. If Sir3 interacts with Sae2 that is not bound at DSBs, how it can control DSB resection?
4. Lane 14: "this interaction impairs Sae2 interaction with MRX": This is an overinterpretation because it has not been tested directly.
5. lines 14-16, 297-299, "Sir3 limits Mre11-mediated resection, delays MRX removal and promote NHEJ. The authors show that when Sir3 is OVEREXPRESSED, it could inhibit Sae2 function. Whether it exerts the same effect when is present at physiological levels has not been investigated.

6. lines 300-302: "Sae2 inhibition is not due to a sequestration that prevents its recruitment to DSB, but....seems to impair the interaction between Sae2 and MRX. There are no evidences that Sir3 does not inhibit Sae2 recruitment at DSBs and prevents Sae2-MRX interaction.
8. I missed the authors' demonstration that Sir3 inhibits DSB resection independently of its heterochromatin promoting function. Is it because Sir3 overexpressed is not bound at euchromatin DSBs? This has not been shown.
9. Lines 74-76: the authors reported previously that Sir3 overexpression favors DSB repair by inhibiting DSB resection (by inhibiting Sae2?). Now they show that Sir3 promotes NHEJ by inhibiting Sae2 function. How can it be? I am confused.

Referee #2:

In this manuscript, the authors address the important question of how double-strand breaks (DSBs) in heterochromatin are repaired. In budding yeast, heterochromatin is restricted to telomeres and the HMR and HML loci, and requires the Sir3 and Sir4 proteins. Using specific assays to measure non-homologous end joining (NHEJ) repair in heterochromatin or euchromatin contexts, the authors describe a Sir3-mediated inhibition of Sae2, which results in a higher frequency of NHEJ. Notably oeSir3 increases NHEJ at a euchromatic break, phenocopying loss of Sae2. Additional mechanisms further increase NHEJ at a heterochromatic DSB. The authors demonstrate direct binding of Sir3 to Sae2, and suggest that Sir3 competes with the MRX complex for Sae2 binding, thereby reducing resection initiation. As a result, NHEJ is increased under conditions where Sir3 binds Sae2. This interaction is mediated by a portion of the Sir4 binding domain on Sir3, and the C-terminus of Sae2 and as a result, Sir4 competes with Sae2 for Sir3 binding. The experiments are thorough and support the conclusions that are drawn. The text is well-written and figures are clear for the most part. Several of the comments below can serve to improve the manuscript.

Comments:
1. The data showing Sae2-Sir3 interaction/co-localization are done in the absence of a DSB. It would be interesting to know whether Sae2 localization is increased near a DSB in the absence of Sir3. This would get at the question of whether Sir3 is sequestering Sae2 in a physiological setting. Additionally, it would be interesting to know if Sir3-OE would remove Sae2 near a DSB. Such an experiment would complement results shown in Figure 2B (in which there are however no error bars for the sir3 mutant data).
2. The authors suggest Sir3 competes with MRX for Sae2 binding but provide no direct evidence to support this idea. The C-terminal region of Sae2 that interacts with Sir3 includes the site for phosphorylation by CDK that directs interaction with Rad50. From the GST pulldown experiment, phosphorylation of Sae2 would appear to be dispensable for interaction with Sir3 but could potentially inhibit it. Unfortunately, the Sae2 interaction with MRX has been difficult to detect by IP with native proteins or two-hybrid assay so this might be difficult to test experimentally. If Sir3 binding to Sae2 prevented phosphorylation it might be possible to bypass with the S267E mutation.

Minor Points:
1. Be aware of several grammatical errors (Ex. On page 4, line 69: "Sir3 does not only promote genome stability..." should be "Sir3 not only promotes genome stability..."). There are other errors, mostly with subject-verb agreement. One such error in the Abstract: "We show that SIRs promotes..." should be "We show that SIRs promote..."
2. In Figure 1H, consider shifting the 29x and 9x expression levels for each of the promoters that are shown below the graph to the figure legend. Currently, these numbers are awkwardly placed.
3. In Figure 3B and 3C, the fragment colors should remain consistent with the colors in the full-length protein. Since there are white and gray portions of the full-length proteins, it can be a little confusing when the fragments are also white and gray. The +/- to the side is enough to indicate interaction.
4. It is worth noting why X-Gal colonies for SIR4C-SIR3SaID and SIR4C-sir3SaIDT557I are not dark blue in Figure 4C. It seems they have a slight blue color. One sentence in the text to address this result would be helpful.

Referee #3:

Review of Bordelet et al, Embo J

This manuscript by Bordelet et al describes the interaction between the heterochromatin protein Sir3 and Sae2 from S. cerevisiae. Sae2 usually stimulates the activity of the MRX complex, which resects DNA at double-strand breaks and thus promotes homologous recombination. Hence, when Sae2 is inhibited (here by interaction with Sir3), the repair of DSBs is channeled towards non-homologous end-joining.
The work is based on earlier work from the same group showing that the overexpression of Sir3 increases non-homologous end-joining (NHEJ) in subtelomeric regions (Batté et al, Embo J 2017). Here, the further characterization of this observation leads them to discover an interaction between part of the AAA+ domain of Sir3 and the C-terminal region of Sae2. This interaction is shown using multiple methods: Yeast two-hybrid, co-immunoprecipitation and in vitro interaction of recombinantly expressed, purified components. Based on the phenotypes, the authors conclude that Sir3 interaction inhibits resection by inhibiting Sae2, which in turn then no longer can stimulate the MRX endonuclease. Interestingly, this function of Sir3 is independent of its

function within the SIR complex, which silences the telomeres and the silent mating-type loci. The region of Sae2 interaction with Sir3 overlaps with that of Sir3's interaction to Sir4, indicating that there is competition of Sir4 and Sae2 for Sir3 binding. The authors further develop the work and isolate a mutant in Sir3 that has lost interaction with Sae2, but whose interaction with Sir3 is intact. Using this mutant, they validate their model that free Sir3 (i.e. that is not bound in the SIR complex) has a function in inhibiting Sae2 function.

In my opinion, this study provides very strong evidence for this novel and interesting function of Sir3. The authors present a very complete set of experiments - I really have nothing to add; all the controls that I would have wished for are there. The data are convincing, both from the aspect of biochemistry as well as of genetics/ molecular biology.

Minor comment:
The sentence in the abstract "How DNA repair occurs in heterochromatin remains poorly described." does not reflect very well the content of the ms.

Thank you for giving us the opportunity to submit a revised version of our manuscript. We would also like to thank the editorial advisor and referees for their assessment of this work and for their insightful comments to improve it. As detailed below, we have responded to comments and/or suggestions of the three referees. In many cases this has resulted in performing additional experiments now presented in 3 revised figures (Figures 1, 5, 6), 1 new figure (Figure 7) and 1modified EV figure.

As requested by the editorial advisor we added data to strengthen Sir3-Sae2 direct interaction which is now supported by colocalization (Figure 2A), ChIP data (Figure 2B and 7C-D) co-immunoprecipitation (including cells with native Sir3 levels, Figure 2C-D, and sir3 mutant, Figure 3F), two-hybrid interaction (including in sir4Δ cells, Figure 3B-D and sir3 mutant, Figure 4C) and *in vitro* pull-down of purified proteins (including with the sir3T557I mutant, Figure 3E and 5E). The Sir4/Sae2 binding competition is supported by localization experiments (Figure 6D), two hybrid experiments (Fig 6E), co-immunoprecipitation experiments (Figure 6F) and NHEJ assays (Figure 6A and 6C). We also performed a ChIP experiment to assess Sae2 binding to DSB in Sir3 overexpressing cells and in sir3Δ cells (Figure 7C-D). Finally we complemented our NHEJ assay at I-SceI DSB by testing NHEJ plasmid rejoining (Figure 6B).

Taking into account these and all the other points described in detail below, we believe that we have now positively responded to all the comments of the reviewers, and significantly improved our manuscript.

We are at your disposal for any questions that might arise, or any additional information that might be required. I am looking forward to hearing from you about the status of our manuscript.

Following is our point-to-point response to the referees' comments:

Editorial advisor:

Support the proposed Sir4/Sae2 binding competition by strengthening the evidence for direct Sir3-Sae3 interaction in the absence of Sir4 or possible other intermediary proteins. Ideally via in vitro binding studies or comparative pull-down/mass spec analyses of wt and mutant Sir3; alternatively by validating Sir3-Sae2 two-hybrid interactions in sir4Δ strains.

The direct Sae2-Sir3 interaction is supported by Sir3-Sae2 two-hybrid interactions in strains lacking Sir4 (Figure 3D) and by *in vitro* pull down using proteins expressed and purified from bacteria (Figure 3E). The loss of interaction between Sae2 and the sir3-T557I mutated fragment is now also shown by *in vitro* pull down using proteins expressed and purified from bacteria (Figure 5F).

We also strengthened the Sir4-Sae2 binding competition by showing that overexpression of Sir4 released Sae2 from the Sir3-induced cluster (Figure 6D) and decreased Sir3-Sae2 interaction detected by two-hybrid and co-immunoprecipitation *in vivo* (Figure 6E and 6F).

Referee #1:

1) The biochemical data that Sir3 interacts with Sae2 is convincing. The problem is that most (if not all) of the experiments supporting the functional role of this interaction have been done upon Sir3 overexpression. Therefore, whether Sir3 controls Sae2 activity when is present in physiological amount is not known and for this reason I think that the manuscript, as it is, is more suitable for a biochemical journal.

We thank the reviewer for the positive assessment of our biochemical data. The functional role of Sae2-Sir3 interaction in cells expressing WT level of Sir3 was already supported by showing that 1) this interaction occurs in WT cells (co-immunoprecipitation shown in Figure 2D), 2) Sae2 binds telomeres in a SIR3 dependent manner (Figure 2B), 3) deletion of *SIR3* decreases NHEJ (Figure 6A) and 4) deletion of *SIR3* lengthens telomeres in a *SAE2* dependent manner in *tel1Δ* cells (Appendix Figure S2). We added data showing that deletion of *SIR3* also decreases plasmid rejoining (Figure 6B).

2) In all the experiments, the NHEJ efficiency is measured by determining the percentage of survival after generation of an irreparable I-SceI DSB. Under this condition, survival depends on error prone NHEJ events that generate a sequence that cannot be cut by I-SceI. These events are rare and for this reason the percentage of survival is extremely low (less than 1% in wt cells). I think that it is important to measure whether canonical NHEJ events, that are more efficient, are also under the same control.

Sae2 is not involved in canonical NHEJ, in the sense that Sae2 only promotes error-prone Ligase 4 dependent NHEJ, and as such its inhibition cannot be tested in canonical NHEJ assays. As stated above we also measured NHEJ by plasmid rejoining assays in which NHEJ relative efficiency is higher (80% for WT). In this assay deletion of SIR3 also significantly decreases NHEJ, whereas deletion of *SAE2* or overexpression of Sir3 significantly increases it in an epistatic manner (Figure 6B).

Major points

1. In Figure 1B, 1C, 1H, 5A, 5B, 6A, 6B % survival is extremely low because the error-prone NHEJ events measured are rare. The differences in survival % are very subtle (from 0.2 to 3 %). The p-values are not reported and I am not convinced about the significance of many differences. There are several other assays (also molecular assays) that can be used to measure the ability of cells to perform correct and more efficient NHEJ repair events.

We now added the statistical tests showing the significance of differences in survival for all figures and assayed NHEJ by plasmid rejoining assays (see above and Figure 6B)

2. Figure 1E: this experiment has to be repeated at different distances from DSB because, as it is, is very poor and not convincing.

We performed this experiment by measuring DNA amount 0.2 kb from the DSB. Consistent with the involvement of MRX-Sae2 in short range resection and the proposed inhibition of Sae2 by Sir3, the effect of sae2Δ and of Sir3 overexpression were stronger at 0.2 kb than at 1 kb. These data are now presented in Figure 1E, and replace the measurement at 1kb, now shown in Figure EV1.

3. line 131: how do the authors know that Sir3 is not bound to euchromatin DSB sites when it is overexpressed? It is hard to me to imagine that Sir3 interacts with Sae2 but it is not recruited at DSBs. If Sir3 interacts with Sae2 that is not bound at DSBs, how it can control DSB resection?

This is a very important point. Overexpressed Sir3 is not present at the euchromatic I-SceI site prior to DSB induction contrarily to I-SceI site at subtelomere. Indeed, several previous studies (Hocher et al 2018, Strahl-Strahl-Bolsinger et al. 1997, Hecht et al. 1996) have shown that in absence of DNA damage Sir3 does not bind at euchromatic sites even when overexpressed. Hence a DSB induced at the intrachromosomal LYS2 locus occurs in chromatin devoid of Sir3.

For more clarity, we removed 'where Sir3 is not bound' from the sentence.

The data presented in the original manuscript were not directly addressing the behavior of Sir3 and Sae2 at the broken site. To assess this, we performed ChIP experiments and observed that Sae2 recruitment at DSB is impaired upon Sir3 overexpression. Since Mre11 is still recruited at DSB upon Sir3 expression (DSB-induced foci are increased, see Figure 1F-G), this suggests that Sae2-MRX interaction is impaired upon Sir3 overexpression possibly as the result of a trapping of Sae2 by Sir3 in the telomere hypercluster.

In contrast, the overexpression of the minimal interaction domain Sir3$^{\infty}$ which is sufficient to inhibit Sae2 did not impair Sae2 recruitment to DSB, suggesting that Sae2-MRX interaction is not prevented by Sir3$^{\infty}$-Sae2 interaction when Sae2 is not trapped in the telomere cluster.

Altogether these results reinforce our proposal that Sir3-Sae2 interaction directly inhibits Sae2 activity. They also show that Sae2-Sir3 uncouples Sae2 binding at DSB and MRX activation. These data are now presented in Figure 7.

4. Lane 14: "this interaction impairs Sae2 interaction with MRX": This is an overinterpretation because it has not been tested directly.

The microscopy experiments presented in the original manuscript, showing that MRX does not cluster with Sir3 whereas Sae2 does, suggested that most of the MRX complex is not interacting with Sir3-bound Sae2 when Sir3 is overexpressed and telomere bound. Consistently, Sae2 recruitment to DSB decreased upon Sir3 overexpression. However as stated above this is not the whole story and overexpressed Sir3$^{\infty}$ inhibits Sae2 without affecting its binding to DSB and hence interaction with MRX.

5. lines 14-16, 297-299, "Sir3 limits Mre11-mediated resection, delays MRX removal and promotes NHEJ. The authors show that when Sir3 is OVEREXPRESSED, it could inhibit Sae2 function. Whether it exerts the same effect when is present at physiological levels has not been investigated.

As detailed above, we present numerous data showing that Sir3 and Sae2 interact at physiological level (ChIP in Figure 2B, CoIP in Figure 2D) and supporting a physiological role for this interaction (NHEJ assays in Figure 6A and 6B, Telomere length in Appendix Figure S2).

6. lines 300-302: "Sae2 inhibition is not due to a sequestration that prevents its recruitment to DSB, but....seems to impair the interaction between Sae2 and MRX. There are no evidences that Sir3 does not inhibit Sae2 recruitment at DSBs and prevents Sae2-MRX interaction.

We now added ChIP data showing that overexpressed Sir3 sequesters Sae2 at telomeres and prevents its recruitment at DSBs, likely impairing its interaction with MRX. This is in agreement with the microscopy data showing that Sae2 colocalized with overexpressed Sir3 whereas Mre11 does not. We also show that the free Sir3$^{\infty}$ fragment, that does not sequester Sae2, allows its recruitment to DSBs while inhibiting its activity.

8. I missed the authors' demonstration that Sir3 inhibits DSB resection independently of its heterochromatin promoting function. Is it because Sir3 overexpressed is not bound at euchromatin DSBs? This has not been shown.
To assemble heterochromatin Sir3 needs Sir4 and Sir2. The fact that Sir3-oe promotes NHEJ in absence of Sir4 (Figure 1A) demonstrates an activity independent of its heterochromatin promoting function.

9. Lines 74-76: the authors reported previously that Sir3 overexpression favors DSB repair by inhibiting DSB resection (by inhibiting Sae2?). Now they show that Sir3 promotes NHEJ by inhibiting Sae2 function. How can it be? I am confused.
We indeed previously showed that Sir3 overexpression inhibits resection and favors DSB repair by homologous recombination at subtelomeric DSB (Batté et al. 2017). The strong resection inhibition we saw was mainly caused by Sir3-mediated silent chromatin and was not observed to the same extent when a Sir3 mutated form (sir3A2Q) unable to assemble silent chromatin was used (Batté et al. 2017). However, already in Batté et al. (see Figure 6D) the overexpression of the silencing deficient sir3A2Q mutant increased GC to some extent suggesting that resection was in part inhibited in absence of silent chromatin assembly.
We now show that resection inhibition by Sir3 overexpression occurs in part through inhibition of Sae2. Sae2 function is to trigger MRX resection activity and numerous previous studies have shown that resection is the cornerstone of DNA repair pathway choice. Indeed, NHEJ will act on unresected DNA ends whereas HR will use resected DSB ends. Accordingly, sae2Δ and mre11-nd mutants that impair resection and increase the half-life of unresected DNA ends have been shown to promote NHEJ in the same pathway (Figure 1C and Lee and Lee 2007, Huertas et al. 2008, Huertas and Jackson, 2009, Emerson et al. 2018). Increased NHEJ is thus one of the expected outcomes if Sae2 is inhibited.

Referee #2:

Comments:
1. The data showing Sae2-Sir3 interaction/co-localization are done in the absence of a DSB. It would be interesting to know whether Sae2 localization is increased near a DSB in the absence of Sir3. This would get at the question of whether Sir3 is sequestering Sae2 in a physiological setting. Additionally, it would be interesting to know if Sir3-OE would remove Sae2 near a DSB. Such an experiment would complement results shown in Figure 2B (in which there are however no error bars for the sir3 mutant data).
We apologize for the missing error bars for the sir3 mutant data. They are now added on Figure 2B.
As suggested by the referee, we assessed Sae2 binding at DSB and at telomere in response to DSB by ChIP (Figure 7A-7B). As detailed above, Sir3-OE impairs Sae2 binding to DSB, supporting a sequestration of Sae2 in the telomere cluster impairing Sae2-MRX interaction at DSB. We could not detect a significant increase in Sae2 binding in absence of Sir3. This is not surprising since Sae2-OE has been shown to decrease MRX binding to DSB (Clerici et al. 2006; Yu et al. 2018) that could in turn limit Sae2 recruitment to DSB. In addition Sae2 DSB-binding is slightly increased upon overexpression of the Sir3$^{\infty}$ fragment which is sufficient to inhibit Sae2 function. These results suggest that Sir3-Sae2 interaction can impair Sae2 activity without affecting its recruitment at DSB and its interaction with MRX when Sae2 is not trapped in the telomere cluster. These results reinforce our proposal that Sir3-Sae2 interaction is sufficient to inhibit Sae2 activity. However they also show that Sae2-Sir3 interaction uncouples Sae2 binding at DSB and MRX activation. This is reminiscent of the rad50S mutant in which Sae2 is strongly recruited to DSB (Yu et al 2018) but is deficient for Sae2-mediated activation. These data are now presented in Figure 7.

2. The authors suggest Sir3 competes with MRX for Sae2 binding but provide no direct evidence to support this idea. The C-terminal region of Sae2 that interacts with Sir3 includes the site for phosphorylation by CDK that directs interaction with Rad50. From the GST pulldown experiment, phosphorylation of Sae2 would appear to be dispensable for interaction with Sir3 but could potentially inhibit it. Unfortunately, the Sae2 interaction with MRX has been difficult to detect by IP with native proteins or two-hybrid assay so this might be difficult to test experimentally. If Sir3 binding to Sae2 prevented phosphorylation it might be possible to bypass with the S267E mutation.
We thank the reviewer for this suggestion and performed experiments to test this prediction. The sae2-S267E mutant localized in telomere clusters upon Sir3 overexpression, showing that both proteins interact (Figure 7E). In addition, NHEJ assay showed that sae2-S276E is inhibited by Sir3 overexpression (Figure 7F) and is thus unable to bypass Sir3 inhibition.

Minor Points:
1. Be aware of several grammatical errors (Ex. On page 4, line 69: "Sir3 does not only promote genome stability..." should be "Sir3 not only promotes genome stability...").
This has been corrected.
There are other errors, mostly with subject-verb agreement. One such error in the Abstract: "We show that SIRs promotes..." should be "We show that SIRs promote..."
This has been corrected.
2. In Figure 1H, consider shifting the 29x and 9x expression levels for each of the promoters that are shown below the graph to the figure legend. Currently, these numbers are awkwardly placed.
This has been corrected.
3. In Figure 3B and 3C, the fragment colors should remain consistent with the colors in the full-length protein. Since there are white and gray portions of the full-length proteins, it can be a little confusing when the fragments are also white and gray. The +/- to the side is enough to indicate interaction.
This has been corrected.
4. It is worth noting why X-Gal colonies for SIR4C-SIR3SalD and SIR4C-sir3SalDT557I are not dark blue in Figure 4C. It seems they have a slight blue color. One sentence in the text to address this result would be helpful.
We indeed observed a lighter blue color when assessing SIR4C-SIR3SalD interaction than for Sae2C- SIR3SalD. However, as this could result from numerous indirect effects (different protein stability, different folding stability), we don't think it enables us to draw meaningful comparisons between the two setups.

Referee #3:
We thank the reviewer for their very positive assessment of our manuscript.
Minor comment:
The sentence in the abstract "How DNA repair occurs in heterochromatin remains poorly described." does not reflect very well the content of the ms.
We thank the referee for their remark. We changed the sentence to “How heterochromatin proteins regulate DNA repair remains poorly described”.

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by original referees 1 and 2, whose comments are copied below. Since both are satisfied with your revision and have no further scientific concerns, we shall be happy to accept the study following final modifications of several presentational concerns in text and figures, as listed by referee 2.

I am therefore returning the manuscript to you for a final round of minor revision, to allow you to make these adjustments and upload any modified files. Once we will have received them, we should be ready to swiftly proceed with formal acceptance and production of the manuscript.

Referee #1:

The manuscript is improved. The authors addressed my concerns.

Referee #2:

The revised manuscript adequately addresses the main concerns raised at the initial review.

Minor comments:

1. In Fig 1C, the line indicating Sir3 OE is too long and should stop under the first sae2Δ.
2. In Fig 3B, the SAE2 label should be above the +/- and same thing for the SIR3 label in Fig 3C.
3. In Fig 5F, "In vitro pull-down" is off-center.
4. On page 11 of the text, it is stated that Sae2 binding to telomeres is not significantly affected by Sir3 overexpression (Fig 7B), however it is clear that there is an increase in Sae2 binding at telomeres under these conditions. This text should be changed to reflect the fact that Sir3 OE leads to an increase in Sae2 binding at telomeres, with an associated decrease in Sae2 binding at the DSB.
5. In Fig 7D, add "NHEJ at euchromatic DSB" above the graph to be consistent with all other figures.
6. On page 11: "and lead to a greater enrichment..." should be led not lead.
7. At the bottom of page 12, there is a space missing: "binding.Interestingly"

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dubrana Karine
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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	A minimum of 3 biological repeats were performed for each experiment. Survival was analysed on about 500 cells each time. Unpaired test were used to ask if the differences were statistically significant. See Material and Methods for specifics on each type of experiments . For statistical analysis of the data, the Prism9 software has been used.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	yes, calculated standard error to the mean or standrad deviation are shown by error bars
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes, a t test with a confidence limit of 95% were used
Is there an estimate of variation within each group of data?	N/A

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Anti-Sir3 antibody was characterized and described in Ruault et al. 2011
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All experiments have been done using the budding yeast Saccharomyces cerevisiae as a model organism. All strains used in this work were derivates of the W303 background and are described in Table EV1. No animal models were used during the realization of this work.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A