THE EMBO JOURNAL

Mechanisms restraining break-induced replication at two-ended DNA double-strand breaks

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DOI: 10.15252/embj.2020104847

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Review Timeline:

Submission Date:	27th Feb 20
Editorial Decision:	31st Mar 20
Revision Received:	16th Jan 21
Editorial Decision:	8th Feb 21
Revision Received:	4th Mar 21
Accepted:	9th Mar 21

Editor: Hartmut Vodermaier

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 31st Mar 2020

Thank you for submitting your manuscript on mechanisms preventing BIR at two-ended breaks. Three expert referees have now assessed it, and I am pleased to say that in light of their overall positive comments, we would be interested in pursuing this study further for EMBO Journal publication.

As you will see from the reports copied below, the main issues raised by all three reviewer are related to aspects of presentation, both of the general outset/questions/significance, as well as of the experimental details, results and interpretations. Furthermore, referees 1 and 2 also raise a few questions regarding the involvement of specific biochemical activities such as end resection or end tethering, which would probably be best-addressed with some additional separation-of-function experiments.

REFEREE REPORTS

Referee #1:

EMBOJ-2020-104847

Pham et al. report identification and analysis of genes important for regulating DNA double-strand break (DSB) repair pathways, specifically the choice between synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR). DNA replication associated with homologous

recombination (HR) mechanisms is known to be mutagenic, and cells are, in general, in favor of repairing DSBs through SDSA, thus minimizing long-range DNA synthesis. The authors found that Rad59 and Rad52, involved in ssDNA annealing, are important for suppressing BIR in budding yeast. The D-loop unwinding helicase Mph1, Mre11-Rad50-Xrs2 and the histone deacetylase Sir2 have also been found to be involved in the decision-making mechanism. Overall, their results indicate that disrupting capture of the second end is a critical determinant of BIR usage. The mechanism that ensures the engagement of two DSB ends per one repair event is of critical importance, especially when the organism has many repetitive elements such as humans. The work is well-conducted and technically sound, and overall, the presented data support their conclusions. However, little effort has been made to make the results accessible to a wider audience. The authors must present their results in a way that allows non-experts to more easily understand them. In addition, the following experimental concerns should be addressed.

Major points

(1) Involvement of the genes investigated has been clearly demonstrated although the wild-type proteins often exert multiple functions. Rad52 is involved in at least three functions: Rad51 loading, ssDNA annealing and suppressing end resection, the first of which was separated by employing R70A but other functions have not been investigated. DSB resection seems extremely relevant to me, so it would be interesting to include rad52-delC80 (Yan et al. 2019 Mol Cell). Overall, I suggest including rad52-null and rad52-delC80 on top of rad52-R70A. Another pleiotropic enzyme is MRX, which is involved in DSB end resection in addition to DSB end tethering. Employing a nuclease-negative, tethering-positive mutant along (and vice versa) will be informative.

(2) Related to (1), DSB end resection could be an important factor and some of the mutants employed might have affected this particular step along with what the authors imply. The impact of the employed mutants on end resection needs to be assessed using the same strains assayed for BIR efficiency except that the donor is deleted to prevent the repair of the DSBs that are formed.

(3) In general, little effort has been made to make the manuscript accessible to non-specialists. Particular examples include the lack of explanation or citation for why BIR does not proceed towards the centromere in these assays (page 11, 1st paragraph), the lack of explanation for what the different graphs actually show (e.g., "Repair efficiency among all cells by 6 h" and "Repair efficiency compared to WT by 6 h" are exceedingly similar), and the failure to mention that HMR has been deleted (and that MATa nevertheless preferentially utilizes HML), particularly relevant in the introduction to Fig. 5 (page 14, 2nd paragraph). Please ensure that the manuscript is accessible to a wider audience.

(4) There is a general tendency for DSB repair efficiency to be reduced as the amount of homology on the WXY-side increases. The DSB-end on this side would also interact with the donor, having the potential to initiate BIR towards CEN. These events could interfere with the Z-primed BIR or formation of products detected with the probe employed. Thus, it is important to examine the behavior of the DSB-end on this side with regards to how it is repaired (e.g., by Southern blotting). If some experiments have already been done, the results, even if they are negative, should be presented somewhere with proper annotations.

Minor points

(1) Fig. 1F: Why is the repair efficiency so low, even in WT? Please provide some clarification about this in the manuscript.

(2) The annealing activity of Rad59 and Rad52 is required to suppress BIR (Fig. 1). Cells are completely inviable in an assay where there is no homology on the non-invading side, consistent with the fact that all of the repair is by BIR (Fig. 2). Why then do the rad59d and rad52-R70A strains show reduced repair efficiency in this assay (~20% and ~45% reduction, respectively; Fig. S2D)? This reduction suggests that ssDNA annealing is required to some extent for BIR at two-ended DSBs, which seems contradictory to the authors' assertion that ssDNA annealing functions to suppress BIR (e.g., Fig. 1). Please provide some clarification about this in the manuscript.

(3) Related to (2) above, some effort is made to justify the results by demonstrating that deletion of MPH1 can partially suppress the reduced repair efficiency of the rad52-R70A strain (Fig. S2F). While the presented model (Fig. S2G) can explain this reduction, how do the authors explain the mild reduction observed in the rad59d strain (Fig. S2D), when it has been shown that, unlike Rad52-dependent annealing, annealing by Rad59 is strongly inhibited by RPA (Petukhova et al. 1999 JBC)?

(4) Again, related to (2) and (3) above, why do the authors not consider the simpler model, namely that this is a Rad51-independent event involving Rad52-dependent strand invasion? Such a mode of BIR has previously been observed (Malkova et al. 1996 PNAS) and should at least be discussed.

(5) Fig. S1C, D. Based on the graph (panel D), WT repairs the DSB via SSA ~4-fold more efficiently than rad59d and ~10-fold more efficiently then the rad52-R70A strains. However, this difference does not seem to be reflective of the band intensities in the representative gel image (panel C). I can only guess some normalization was involved, but there is no description of this. Please provide a clear explanation of how the results for each assay were quantified in the Methods and/or accompanying legend.

(6) Most of the assays involve identification of DNA fragments by Southern blotting based on differences in restriction digest patterns. While the current schematics are useful in demonstrating what is happening conceptually, they fail to translate into anything that can practically assist in interpreting the Southern blots. It would be much more helpful if the authors included additional schematics that displayed the relevant recombination event and the expected DNA fragment with its size indicated. For example, something similar to Fig. 1 of Hunter and Kleckner (Cell, 2001). This will make the work much more accessible to a non-specialist audience. At least, please include restriction enzyme sites employed in the current diagrams depicting the detection systems (Fig. 1B, 1G, 2A, 2F). Locations of the probes are meaningless without them.

(7) Fig. 1 experiments related to G. Please include there a graph for Repair efficiency among all cells by 6 h, as in other experiments.

(8) Fig. 1 and S1. In several panels, statistical comparisons are between rad59d and rad52-R70A (e.g., 1D, 1E, S1B, S1D). In others, comparisons are made between wild type and rad52-70A (e.g., 1I, 1J). The latter certainly seems more appropriate, unless the authors have a particular reason for comparing rad59d with rad52-R70A. If so, please explain, and if not, please correct this.

(9) The annotation of the RAD52 coding sequence has been modified such that the rad52-R70A allele actually corresponds to rad52-R37A (the N-terminal 33 residues were removed). This was described in Page 551 Line 5 from the bottom right column - Page 553 Line 7 on the left column by Mortensen et al. (2002 Genetics). Please also see the "History" section of the SGD webpage for the Rad52 sequence (https://www.yeastgenome.org/locus/S000004494/sequence). It may be helpful to readers if this is mentioned somewhere.

(10) Fig. 1 legend. It seems that the data used in the graph for Fig. 1L are the same data that were presented earlier in the figure (panels D and I). If so, please state this clearly in the legend so as not to give the impression that these data are experimental replicates of the earlier data.

(11) Page 9, 2nd paragraph. The authors conclude that BIR efficiency is "significantly increased" in the rad52-R70A strain when mph1 is deleted. As written, this suggests a substantial increase in BIR efficiency. While the difference is indeed statistically significant (Fig. S2F) and therefore likely "real", the increase itself is marginal. Please rephrase this part to more accurately reflect the result.

(12) Page 14. line 6 from the bottom. "However, the effect deleting SIR2 is not related to mating type per se, as repair profiles were the same in sir2D mutant cells that express both MATa1 and HMLa2 genes or in the system that expresses only a genes". Please cite the data/reference for this.

(13) There appears to be several typos and/or grammatical errors. Some examples are listed below (original sentence in quotation marks with a recommended revision beneath it). In addition to these examples, there are many more throughout the manuscript. Please try to address them.
i) Abstract
"...by Break-Induced Replication (BIR) that involve extensive and..."
...by Break-Induced Replication (BIR), which involves extensive and...
ii) Page 3, L2
"In most basic HR pathway by..."
In the most basic HR pathway by...
iii) Page 3, L3
"... loop (D-loop) and primes short-patch..."
...loop (D-loop) that primes short-patch...
iv) Page 3, L5
"... anneals to the second end of a DSB."
"... anneals to the second end of the DSB."
v) Fig. 4A, top of the third panel,
"rad5-2R70A" should read rad52-R70A
vi) Fig. 4D, top of the third panel,
"rad 59" should read rad59
vii) Fig. 4D, top of the third panel,
"rad 59" should read rad59
viii) Fig. S2B, top of the third panel,
"rad52R70A" should read rad52-R70A
ix) Fig. S3. Panel A-(II),
"BIR (Ade+, G418S)", should read BIR (Ade+, G418R).
x) Fig. 4A.
"HMRalpha-inc" should read "HMLalpha-inc".

(14) Many in-text citations include two authors followed by et al. The authors should adhere to the EMBO J format.

Referee #2:

Repair of double strand breaks (DSBs) by homologous recombination is key to maintaining genomic integrity. At two-ended DSBs, typically a short patch of repair synthesis, followed by capture of the

second end, repairs the break by gene conversion (GC). However, with a single-ended DSB, the repair synthesis is never captured by a second end and can continue to the end of the chromosome in a process called break-induced replication (BIR). Although BIR is the default mechanism for single-ended DSBs, it is not well understood how BIR is suppressed during two-ended DSB repair. Here, Pham et al. characterize several factors that influence the choice between GC and BIR at two-ended breaks.
The authors use multiple haploid intra-chromosomal recombination assays with different homologies, as well as a diploid allelic recombination assay, to assess the roles of Rad52 and Rad59 ssDNA annealing activity, Mph1 helicase, the MRX complex and heterochromatin in suppressing BIR at two-ended DSBs. Most of the data are clear and consistent with the proposed model, although there are some points that should be addressed to strengthen the findings, which are listed below.

1. There are some differences in repair efficiencies after 6 hr between the different assays. For example, the repair efficiency in the H2100-no gap assay (~40%) was lower than H1400 (~85%) or the H150 assay (~50%). Given that repair should be more efficient with more homology, it is worth explaining this observation in the text. Additionally, the repair efficiency seems to be more affected by the rad52 and rad59 mutants in the H150-no gap assay than the H150 assay. This point is also a bit contradictory to the expectation and would be useful to address.
2. In determining the outcomes in the allelic recombination assay, it is not immediately clear how a gene conversion with crossover (Trp+, Leu-, G418R) is distinguished from a BIR event with the same genotype. Were sectored colonies analyzed? This should be addressed in the Results text or in Methods.
3. Why does pif1 suppress the chromosome loss defect of the rad52-R70A mutant? I would expect there to be more half-crossover events in the pif1 rad52-R70A mutant; can they be detected in this assay?
4. In regards to the role Mre11 is playing in suppressing BIR, it is assumed here that the role is the end-tethering function. However it cannot be ruled out that resection at both ends is critical for GC, whereas resection at one would be sufficient for BIR. The authors do mention this possibility in the Discussion but do not explain why they favor the end-tethering role. Scully and colleagues showed that the ratio of long-tract (BIR?) to short tract GC events is changed in BRCA1 or CtIP-depleted cells. Furthermore, they found that elimination of 53BP1 restores STGC to BRCA1- cells, indicating that it is due to the end resection defect. The authors could address this question by deleting Ku, which is known to suppress the mre11 end resection defect, or by over-expressing Exo1. Alternatively, they could test mutants of the MRX complex that reduce end tethering but maintain end resection. These additional experiments would need to be done to determine the mre11 defect.
5. The sir2 results are interesting but seem a bit preliminary. All BIR assays in yeast require synthesis to the telomere and would require D-loop migration through heterochromatin in sub-telomeric regions. Does sir2 increase BIR efficiency in more typical BIR assays, e.g., the one reported by Lydeard et al. (2007)? How would heterochromatin affect survival of telomerase-negative cells which use BIR to maintain telomeres? This might be a point to add to the Discussion.

Minor points:
1. Figure 1B: How far would BIR have to synthesize to reach the telomere? The efficiency of BIR is negatively impacted over long distances and could contribute to the failure to detect BIR initiated at the other break end (Figure 2).
2. Not all southern blots have loading controls (missing from 2B, 2G, 4A, 4C).
3. Luke-Glaser and Luke (2012) were the first to show increased BIR in the mph1 mutant; their work should be cited.
4. P. 16: Maloisel et al. (2008) should be cited for the role of Pol delta in short patch synthesis.

5. Figure 5E: the 0hr time point for mre11 is missing.
6. Figure S3: the BIR category (II) should be G418s.
7. There are some typos in the Figures, e.g., rad5-2R70A in 4A.

Referee #3:

In this manuscript the authors address the issue of what factors promote two-ended repair, mainly being gene conversion, and prevent BIR one-ended repair when the DNA break is a two-ended break. This is important because most BIR outcomes are deleterious, resulting in lethal events or chromosome rearrangements, mutagenesis and extensive loss of heterozygosity. Although the studies are done in yeast, the findings are extended to human cells as most factors and pathways studied here are conserved. Thus the findings have broad implications. The short comings of the paper are that first, some of the mechanism were already known to some extent, although never analyzed in the systematic way they are in this paper, and second, that no single mechanism/gene/pathway has a profound effect on BIR vs SDSA when both are possible. Nonetheless, in toto the study reveals new information about regulation of two-ended break repair.

Introduction: I think the point here is to emphasize the deleterious consequences of BIR repair to make the case that cells would have mechanism(s) to prevent this type of repair if other options are available. This point gets lost in the extensive description of BIR and its outcomes. Some minimal rewriting here would really drive home the point that is brought up as the point of the paper at the start of the second paragraph of the introduction. At the end of the third paragraph it is important to emphasize the universality of the BIR process, its occurrence in human cells, and that all the proteins mentioned in this section save Rad59 exist in human cells and have the same activity as in yeast.

Results: The work is carefully done and uses assays and reporters developed by the authors. Several of these assays look at just one or a few outcomes, this should be noted. For example, as the authors do note, some assays have limited homology. Eventually the authors note this and indeed the length of homology turns out to be an important factor in preventing BIR. However, details of the assays, extensively described, may pass by many readers. Instead of presenting the experiments chronologically, it might be better to turn them around and point out that length of homology, previously known to have a role, was tested directly and out this at the front of the section rather than at the end. An example is at the end of the section on Rad52 and Rad59, at the end of the paragraph in the middle of page 7. Somehow this short homology could be more prominently noted earlier, although it is briefly mentioned at the bottom of page 6. This is just to guide the reader who is not intimately familiar with the assays. On a related note, the assays use constructs designed to measure a particular type of repair event. How close are these to events going on with breaks in cells? It might be useful to contextualize the types of events with breaks in human cells, those with limited homology in repeat sequences versus those with chromatid homology.

Additional comments:
Table S1 needs some type of legend to explain what the numbers are; the numbers plus those in parentheses. One can guess but it is best not to.

Referee #1:

EMBOJ-2020-104847

Pham et al. report identification and analysis of genes important for regulating DNA double-strand break (DSB) repair pathways, specifically the choice between synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR). DNA replication associated with homologous recombination (HR) mechanisms is known to be mutagenic, and cells are, in general, in favor of repairing DSBs through SDSA, thus minimizing long-range DNA synthesis. The authors found that Rad59 and Rad52, involved in ssDNA annealing, are important for suppressing BIR in budding yeast. The D-loop unwinding helicase Mph1, Mre11-Rad50-Xrs2 and the histone deacetylase Sir2 have also been found to be involved in the decision-making mechanism. Overall, their results indicate that disrupting capture of the second end is a critical determinant of BIR usage. The mechanism that ensures the engagement of two DSB ends per one repair event is of critical importance, especially when the organism has many repetitive elements such as humans. The work is well-conducted and technically sound, and overall, the presented data support their conclusions. However, little effort has been made to make the results accessible to a wider audience. The authors must present their results in a way that allows non-experts to more easily understand them. In addition, the following experimental concerns should be addressed.

We thank the reviewer for complementing our work. We have tried to make the results more accessible to non-experts.

Major points

(1) Involvement of the genes investigated has been clearly demonstrated although the wild-type proteins often exert multiple functions. Rad52 is involved in at least three functions: Rad51 loading, ssDNA annealing and suppressing end resection, the first of which was separated by employing R70A but other functions have not been investigated. DSB resection seems extremely relevant to me, so it would be interesting to include rad52-delC80 (Yan et al. 2019 Mol Cell). Overall, I suggest including rad52-null and rad52-delC80 on top of rad52-R70A. Another pleiotropic enzyme is MRX, which is involved in DSB end resection in addition to DSB end tethering. Employing a nuclease-negative, tethering-positive mutant along (and vice versa) will be informative.

These are all excellent questions that we have addressed.

1a. The reviewer asked whether the function of Rad52 in resection control can contribute to the choice between BIR and SDSA. The reviewer suggests testing *rad52*delC80 mutant in budding yeast that should

have normal DNA binding/annealing but much faster resection. *rad52DC80* was previously tested in fission yeast and was shown to have much faster resection than wild type cells. We note that the very C-terminal part of the Rad52 is not conserved between fission and budding yeast; therefore, we have not made *rad52*delC80 in budding yeast. In general, the role of Rad52 in restraining resection in budding yeast is less pronounced than in fission yeast (Yan *et al*, 2019). As the experiments here relevant to Rad52 were done in *rad52-R70A* DNA binding mutant, we first checked if this mutant has faster resection as its fission yeast counterpart, *rad52-R45A* mutant. Slightly faster resection was observed in *rad52-R70A* compared to wild type but not nearly as fast as observed in *rad52-R45A* mutant in fission yeast (**Appendix Fig S2A**). It is unlikely that a minor change in extensive resection is responsible for a dramatic change in BIR/SDSA contribution to DSB repair. However, to address the impact of faster resection on the choice between BIR and SDSA directly, we generated *dot1Δ* mutant cells that has much faster resection likely due to decreased loading of the protein inhibiting resection Rad9 (Chen *et al*, 2012). We note that the faster resection in *rad52Δ* and in mutants deficient in Rad9 recruitment depends on Sgs1-mediated resection in both budding and fission yeasts. As shown in new **Appendix Fig S2B**, no change in BIR/SDSA distribution was observed in *dot1Δ* cells. Therefore, we think it is unlikely that slightly faster resection in *rad52-R70A* alters the choice of BIR and SDSA.

In a revised manuscript we state:
*"Besides the well-established role of Rad52 in loading Rad51 and ssDNA annealing, Rad52 and its DNA binding domain also negatively regulate extensive resection, particularly in fission yeast (Yan et al., 2019). It is unlikely that the role of Rad52 in resection suppresses BIR because rad52-R70A mutant shows only a minor increase of extensive resection rate, less than observed in equivalent fission yeast DNA binding mutant rad52-R45A (**Appendix Fig S2A** and (Yan et al., 2019). Second, deletion of DOT1 that shows larger increase of extensive resection rate (Chen et al., 2012) does not alter SDSA and BIR contribution to DSB repair (**Appendix Fig S2B**)."*

1b. We also generated a *rad52*-null mutant as the reviewer suggested. As expected, we observed no repair by any mechanism as *RAD52* is essential for all homologous recombination pathways in budding yeast. This control experiment is added to the new **Appendix Fig S2C**.

1c. Finally, the reviewer asked the question whether the role of MRX complex in directing repair toward SDSA is related to the known function of this complex in ends tethering or resection. A similar question was asked by reviewer #2 (question 4). We are not aware of any published separation of function MRX mutant with normal resection and poor tethering or vice versa. We have not tested here *mre11* nuclease-dead mutant because it has negligible resection phenotype at HO induced DSBs while the complete deletion of *mre11* has severe resection phenotype. Thus, testing *mre11* nuclease-dead mutant would not address the question. However, we made progress in understanding the function of MRX in the regulation of BIR/SDSA, as explained below.

First, we note that in H150 no-gap system, 150 bp homology is present immediately next to DSB. Therefore, minimal resection is required to expose homologous sequences for HR. However, if resection is asynchronous on two ends in *mre11Δ* mutants, it could favor one of two ends in homology search and strand invasion while the other end (unresected) would not be available to participate in HR at the same time. This could lead to an increase of BIR at two-ended breaks. We carefully tested if two ends of a break are resected synchronously in wild type and *mre11Δ* mutants. It was done in a population of cells, so we would only detect asynchrony of resection if either telomere proximal or centromere proximal end was overall better resected in *mre11Δ* mutants. Resection was tested by Southern blots and the

DNA fragments tested on both sides of the break had comparable and short size (~400 bp). In WT cells, both centromere and telomere proximal ends, are resected synchronously (**Figure 6C**). In *mre11Δ* mutants, as expected, the initial resection is slower overall compared to WT. Interestingly the telomere proximal end (Z-end) is resected faster compared to centromere proximal end (Y-end). Therefore, we conclude that at least in some cells, the telomere proximal end is resected faster in *mre11Δ* cells. As resection was not tested in individual cells, we cannot exclude the possibility that in some *mre11Δ* cells, the centromere proximal end was resected faster.

Second, we made a double mutant *mre11Δ yku70Δ* as *yku70Δ* partially suppresses the resection defect of *mre11Δ* cells. In *mre11Δ yku70Δ* cells, the initial resection is faster on Y-end, and both ends are resected synchronously (**Figure 6C**). We further tested whether the elimination of Ku70 from *mre11Δ* also suppresses the higher level of BIR. Indeed, BIR is significantly decreased in H-150 no-gap and H-150 assays in *mre11Δ yku70Δ* cells when compared to *mre11* cells (**Figure 6A, B**). These results suggest that the synchrony of resection of two DSB ends is important to suppress BIR at two-ended DSBs. Finally, we want to add that we cannot exclude the possibility that tethering of DSB ends by MRX also contributes to the regulation of SDSA/BIR as possibly synchronous resection of two ends requires ends-tethering.

(2) Related to (1), DSB end resection could be an important factor and some of the mutants employed might have affected this particular step along with what the authors imply. The impact of the employed mutants on end resection needs to be assessed using the same strains assayed for BIR efficiency except that the donor is deleted to prevent the repair of the DSBs that are formed.

The role of resection was carefully discussed above with respect to Rad52 and MRX complex. In the case of Sir2, the change of BIR/SDSA distribution is observed only in case the donor is within heterochromatin, excluding the role of resection at broken locus.

(3) In general, little effort has been made to make the manuscript accessible to non-specialists. Particular examples include the lack of explanation or citation for

a. why BIR does not proceed towards the centromere in these assays (page 11, 1st paragraph),

Published literature suggests that BIR does not go through the centromere itself. We assume that the BIR specific synthesis is not possible through kinetochore bound centromere in G2/M cells. However, in this manuscript, we do not discuss DNA synthesis through the centromere but rather DNA synthesis in the centromere direction. In assays H-1400 or H-2100, where there is plenty of homology on both sides of the break, we do not see the synthesis of the first few kb in a direction toward the centromere. We made this point clear in the manuscript. Moreover, we have decided to explore this question by constructing a new strain where strand invasion occurs at Z-end within HMRa-inc; the second end has no homology with the template while HML was deleted. New DNA synthesis can go only in the centromere direction, but the centromere itself is cut off from recombining sequences by the HO break. We observed only 3% of the product (by 10 hrs after DSB induction) that monitors just the first few kb of dsDNA synthesis (**Figure EV3B**). This level of DNA synthesis is much lower than in BIR assays where synthesis goes toward the telomere (compare **Fig EV1B** and **Fig EV3B**). Thus Z-end sequence cannot prime efficient BIR synthesis in HMR in the direction of centromere. We exclude that the centromere itself has a long-range effect on DNA synthesis as it is separated from recombining sequences by HO break. It remains to be determined in the future how polarity of synthesis and/or what local features determine whether BIR is efficient.

b. *the lack of explanation for what the different graphs actually show (e.g., "Repair efficiency among all cells by 6 h" and "Repair efficiency compared to WT by 6 h" are exceedingly similar),*

Repair efficiency among all cells shows how efficient the repair is among all cells that induced the break. Here the intensity of bands corresponding to repair products is compared to the band corresponding to an uncut parental locus at time "0" that is set to 100%.
Repair efficiency compared to WT simply compares the repair product of mutant cells to that of wild type, which is set to 100 %. Here we also indicate the contribution of BIR and SDSA to the repair. We modified the legend of the figure 1F and others to make it clear. We thank the reviewer for this suggestion.

c. *and the failure to mention that HMR has been deleted (and that MATa nevertheless preferentially utilizes HML), particularly relevant in the introduction to Fig. 5 (page 14, 2nd paragraph). Please ensure that the manuscript is accessible to a wider audience.*

We mentioned within the results section of the revised manuscript that *HMR*a was deleted.

(4) There is a general tendency for DSB repair efficiency to be reduced as the amount of homology on the WXY-side increases. The DSB-end on this side would also interact with the donor, having the potential to initiate BIR towards CEN. These events could interfere with the Z-primed BIR or formation of products detected with the probe employed. Thus, it is important to examine the behavior of the DSB-end on this side with regards to how it is repaired (e.g., by Southern blotting). If some experiments have already been done, the results, even if they are negative, should be presented somewhere with proper annotations.

The explanation provided by the reviewer is very likely true, and this is how we interpreted the data as well. We agree that the strand invasion of "second" end (Y-end) can interfere with BIR primed by Z-end in a direction of telomere, particularly in H-1400 or H-2100. In these two assays, the DNA probe used for Southern blots can detect BIR toward the centromere (Z probe), yet we do not see any BIR toward the centromere. In H1-50 assay, we designed another probe that can detect BIR toward the centromere, and we also did not detect any BIR (**Figure EV3A**). We clarified this point throughout the results sections. In addition, we constructed one more BIR system as described above, where DNA synthesis primed by Z-end is possible only toward the centromere, and again, nearly no DNA synthesis was observed (**Figure EV3B**).

Minor points

(1) Fig. 1F: Why is the repair efficiency so low, even in WT? Please provide some clarification about this in the manuscript.

Viability of the wild type strain is about 80% while repair by 6 hrs is about 50-55%. Low repair indicates that not all cells completed the repair by 6 hrs. Repair is faster in assays with homology next to the break (80%, **Fig. 1K**) or when homology is longer (90% **Fig. 2E**). It seems that these parameters (homology size, homology distance from DSB) dictate the kinetics of repair.

(2) The annealing activity of Rad59 and Rad52 is required to suppress BIR (Fig. 1). Cells are completely inviable in an assay where there is no homology on the non-invading side, consistent with the fact that

all of the repair is by BIR (Fig. 2). Why then do the rad59d and rad52-R70A strains show reduced repair efficiency in this assay (~20% and ~45% reduction, respectively; Fig. S2D)? This reduction suggests that ssDNA annealing is required to some extent for BIR at two-ended DSBs, which seems contradictory to the authors' assertion that ssDNA annealing functions to suppress BIR (e.g., Fig. 1). Please provide some clarification about this in the manuscript.

We note that data from **Figure S2** are now presented in **Fig EV1**.

BIR is mildly decreased in annealing mutants mostly when homology on the invading strand is short. In allelic BIR assay, where homology is extensive, annealing activity is nearly completely dispensable for BIR. Thus, we conclude that in *rad59* or rad52-R70A mutants, short homology at invading DSB end may interfere with either completion or kinetics of BIR. We proposed that annealing could enhance the D-loop stability (to be exact we proposed that it can extend heteroduplex DNA by a known three strand recombination), or it can promote formation of the D-loop that was unwound but retained RPA coated ssDNA (**Fig EV1G**). We note that Rad59 is needed for Pol32-dependent telomere recombination in yeast. The exact function of Rad59 in BIR at telomeres is not known, but it may be related to the function identified here.

(3) Related to (2) above, some effort is made to justify the results by demonstrating that deletion of MPH1 can partially suppress the reduced repair efficiency of the rad52-R70A strain (Fig. S2F). While the presented model (Fig. S2G) can explain this reduction, how do the authors explain the mild reduction observed in the rad59d strain (Fig. S2D), when it has been shown that, unlike Rad52-dependent annealing, annealing by Rad59 is strongly inhibited by RPA (Petukhova et al. 1999 JBC)?

We thank the reviewer for bringing to our attention Petukhova et al. work. We cite this work in a revised manuscript. Rad59 does not promote annealing by itself in the presence of RPA *in vitro,* and it does not promote annealing in cells by itself as cells carrying Rad59 but not Rad52 are SSA deficient. Later work showed that Rad59 promotes Rad52 mediated annealing by alleviating the inhibitory effect of Rad51 on annealing. Yet other published work showed that the role of Rad59 is particularly important for Rad52-mediated annealing between short ssDNA (Sugawara *et al*, 2000). Therefore, considering the role of annealing in BIR mediated by short homologous sequences, it is not surprising to see a small BIR defect in *rad59Δ* cells.

(4) Again, related to (2) and (3) above, why do the authors not consider the simpler model, namely that this is a Rad51-independent event involving Rad52-dependent strand invasion? Such a mode of BIR has previously been observed (Malkova et al. 1996 PNAS) and should at least be discussed.

The only Rad51-indepdent BIR was shown for recombination between repeats within plasmids, telomeric repeats, and in allelic recombination mediated by repetitive Ty elements. The fact that frequent Rad51-mediated repair in the allelic system is represented only by gross chromosomal rearrangements was published later by Malkova lab (VanHulle *et al*, 2007). However, to make sure that there is no Rad51-independent BIR in the assays used here, we deleted *RAD51* in H-150 assay. As expected, no repair was observed (new **Appendix Fig S2C**).

(5) Fig. S1C, D. Based on the graph (panel D), WT repairs the DSB via SSA ~4-fold more efficiently than rad59d and ~10-fold more efficiently then the rad52-R70A strains. However, this difference does not seem to be reflective of the band intensities in the representative gel image (panel C). I can only guess some normalization was involved, but there is no description of this.

Please provide a clear explanation of how the results for each assay were quantified in the Methods and/or accompanying legend.

The function of the DNA binding domain of Rad52 and specifically residue 70 (arginine) of Rad52 and Rad59 in ssDNA annealing is well established and published by several groups. We included these data just to confirm the mutants. Considering the overlap with published work and the fact that we needed to add many new figures and explanations, we removed all these data and simply cited published work.

(6) Most of the assays involve identification of DNA fragments by Southern blotting based on differences in restriction digest patterns. While the current schematics are useful in demonstrating what is happening conceptually, they fail to translate into anything that can practically assist in interpreting the Southern blots. It would be much more helpful if the authors included additional schematics that displayed the relevant recombination event and the expected DNA fragment with its size indicated. For example, something similar to Fig. 1 of Hunter and Kleckner (Cell, 2001). This will make the work much more accessible to a non-specialist audience. At least, please include restriction enzyme sites employed in the current diagrams depicting the detection systems (Fig. 1B, 1G, 2A, 2F). Locations of the probes are meaningless without them.

We added the **Appendix Figure S1** that shows all restriction fragments and probes used.

(7) Fig. 1 experiments related to G. Please include there a graph for Repair efficiency among all cells by 6 h, as in other experiments.

Figures were rearranged to fit the additional graph in Figure 1. The graph for Repair efficiency among all cells by 6hr was added and is now **Figure 1K**.

(8) Fig. 1 and S1. In several panels, statistical comparisons are between rad59d and rad52-R70A (e.g., 1D, 1E, S1B, S1D). In others, comparisons are made between wild type and rad52-70A (e.g., 1I, 1J). The latter certainly seems more appropriate, unless the authors have a particular reason for comparing rad59d with rad52-R70A. If so, please explain, and if not, please correct this.

We made all comparisons consistent with reviewer's suggestion. We thank the reviewer for the careful reading of our manuscript.

(9) The annotation of the RAD52 coding sequence has been modified such that the rad52-R70A allele actually corresponds to rad52-R37A (the N-terminal 33 residues were removed). This was described in Page 551 Line 5 from the bottom right column - Page 553 Line 7 on the left column by Mortensen et al. (2002 Genetics). Please also see the "History" section of the SGD webpage for the Rad52 sequence (https://www.yeastgenome.org/locus/S000004494/sequence). It may be helpful to readers if this is mentioned somewhere.

We explained the sequence arrangement in methods section:

"We note that *RAD52* contains multiple putative start codons (Mortensen *et al*, 2002). In this work, *RAD52* sequence starts from the first start codon, and the annealing mutants corresponds to R70A. In SGD database, *RAD52* sequence begins at the third ATG modified; it is shorter by 33 residues, and the annealing mutant corresponds to R37A."

(*10) Fig. 1 legend. It seems that the data used in the graph for Fig. 1L are the same data that were presented earlier in the figure (panels D and I). If so, please state this clearly in the legend so as not to give the impression that these data are experimental replicates of the earlier data.*

The reviewer is correct; we pooled together results from several assays to demonstrate the impact of the gap more clearly within a single graph. As there is no space for these data in modified figure 1, we decided to move them to the **Appendix Figure S3**. The legend of this figure provides the information on the data source.

(11) Page 9, 2nd paragraph. The authors conclude that BIR efficiency is "significantly increased" in the rad52-R70A strain when mph1 is deleted. As written, this suggests a substantial increase in BIR efficiency. While the difference is indeed statistically significant (Fig. S2F) and therefore likely "real", the increase itself is marginal. Please rephrase this part to more accurately reflect the result.

We changed the word “significantly” to “slightly”.

(12) Page 14. line 6 from the bottom. "However, the effect deleting SIR2 is not related to mating type per se, as repair profiles were the same in sir2D mutant cells that express both MATa1 and HMLa2 genes or in the system that expresses only a genes". Please cite the data/reference for this.

These are the systems used here, and the data were shown. We rephrased our statement to make it clear that we refer to the presented data:

“We note that *sir2*Δ cells in H-150 assay carrying *HML*α-inc and *MAT***a** express both mating type genes. Previous studies have shown that cells expressing both *MAT***a**1 and *HML*α2 genes exhibit some mating-type specific effects on DNA repair (Valencia-Burton *et al*, 2006). However, the effect of deleting *SIR2* is not related to mating-type *per se*, as BIR increase was observed in *sir2* mutant cells that express both *a* and α genes (H-150) or in the system that expresses only α genes (H-150 no-gap, **Fig 5A-B**).”

(13) There appears to be several typos and/or grammatical errors. Some examples are listed below (original sentence in quotation marks with a recommended revision beneath it). In addition to these examples, there are many more throughout the manuscript. Please try to address them.

We carefully edited the manuscript to eliminate any typos.

i) Abstract
"...by Break-Induced Replication (BIR) that involve extensive and..."
...by Break-Induced Replication (BIR), which involves extensive and...
ii) Page 3, L2
"In most basic HR pathway by..."
In the most basic HR pathway by...
iii) Page 3, L3
"... loop (D-loop) and primes short-patch..."
...loop (D-loop) that primes short-patch...
iv) Page 3, L5
"... anneals to the second end of a DSB."
"... anneals to the second end of the DSB."

v) Fig. 4A, top of the third panel,
"rad5-2R70A" should read rad52-R70A
vi) Fig. 4D, top of the third panel,
"rad 59" should read rad59
vii) Fig. 4D, top of the third panel,
"rad 59" should read rad59
viii) Fig. S2B, top of the third panel,
"rad52R70A" should read rad52-R70A
ix) Fig. S3. Panel A-(II),
"BIR (Ade+, G418S)", should read BIR (Ade+, G418R).
x) Fig. 4A.
"HMRalpha-inc" should read "HMLalpha-inc".

(14) Many in-text citations include two authors followed by et al. The authors should adhere to the EMBO J format.

We fixed all the citations. We thank the reviewer again for careful reading.

Referee #2:

Repair of double strand breaks (DSBs) by homologous recombination is key to maintaining genomic integrity. At two-ended DSBs, typically a short patch of repair synthesis, followed by capture of the second end, repairs the break by gene conversion (GC). However, with a single-ended DSB, the repair synthesis is never captured by a second end and can continue to the end of the chromosome in a process called break-induced replication (BIR). Although BIR is the default mechanism for single-ended DSBs, it is not well understood how BIR is suppressed during two-ended DSB repair. Here, Pham et al. characterize several factors that influence the choice between GC and BIR at two-ended breaks. The authors use multiple haploid intra-chromosomal recombination assays with different homologies, as well as a diploid allelic recombination assay, to assess the roles of Rad52 and Rad59 ssDNA annealing activity, Mph1 helicase, the MRX complex and heterochromatin in suppressing BIR at two-ended DSBs. Most of the data are clear and consistent with the proposed model, although there are some points that should be addressed to strengthen the findings, which are listed below.

We thank the reviewer for complementing our work.

1a. There are some differences in repair efficiencies after 6 hr between the different assays. For example, the repair efficiency in the H2100-no gap assay (~40%) was lower than H1400 (~85%) or the H150 assay (~50%). Given that repair should be more efficient with more homology, it is worth explaining this observation in the text.

Lower repair efficiency in H-2100 assay by 6 hrs is related to the mating type. H-2100 is recombination between *MAT*alpha and *HML*alpha-inc, and thus it is not enhanced by a so-called recombination enhancer (Wu & Haber, 1996). We provided this information in the results section. We note that in all other systems where the template is *HML*alpha-inc, recombination enhancer is active, and this information is provided in the results section as well.

1b. Additionally, the repair efficiency seems to be more affected by the rad52 and rad59 mutants in the H150-no gap assay than the H150 assay. This point is also a bit contradictory to the expectation and would be useful to address.

In general, the gap has an opposite impact on SDSA and BIR; gap inhibits SDSA and promotes BIR. This is clear in wild type cells where BIR is higher in H-150 with the gap (20%) when compared to a system without the gap (5%). The same pattern is observed in annealing mutants. In *rad59Δ* , in a system without the gap, BIR constitutes 40% of the product while with the gap, 80% of the product is the BIR. Finally, in *rad52R70A,* there is nearly only BIR product in both systems. The gap likely makes it more difficult to capture the second end that carries homology only at some distance from the DSB end.

When repair efficiency by BIR (SDSA nearly does not contribute) is compared in *rad52*-R70A annealing mutant in a system with or without the gap, repair is less efficient when there is no gap. It is very likely that both ends can invade the template in a system where there is no gap because both ends have homology with the template immediately next to the DSB end. Strand invasion of 150 bp Y sequence likely interferes with BIR initiated at "Z" end and going toward the telomere. We clarify these points within the results section. Finally repair efficiency (BIR+SDSA by 6 hrs) is not significantly different in WT and *rad59Δ* cells.

2. In determining the outcomes in the allelic recombination assay, it is not immediately clear how a gene conversion with crossover (Trp+, Leu-, G418R) is distinguished from a BIR event with the same genotype. Were sectored colonies analyzed? This should be addressed in the Results text or in Methods.

In the allelic recombination assay, the BIR event is scored as *Trp+*, *Leu-*, *G418**R*** colonies. Reciprocal colony sectors are used to detect gene conversion with crossover. After mitosis, gene conversion with crossover will generate *Trp+*, *Leu-/+ G418**R/S*** sectored colonies. This information is provided within the figure, clarified in results sections and is also addressed in more detail in the Methods section.

3. Why does *pif1* suppress the chromosome loss defect of the *rad52-R70A* mutant? I would expect there to be more half-crossover events in the pif1 rad52-R70A mutant; can they be detected in this assay?

Indeed, less chromosome loss is observed in *pifΔ rad52-R70A* (~0.5 %) when compared to *rad52-R70A* (~2 %) and we do not see half-crossovers as discussed below. This result suggests that chromosome loss in *rad52-R70A* is likely due to Pif1-mediated repair events that were likely initiated but not completed. Perhaps the D-loop intermediate in *pif1 rad52-R70A* cells is not extended efficiently, and spontaneous annealing with the other DSB end and repair are increased. This is only a very small fraction of the events. Therefore, we do not provide any extensive explanation for this result considering manuscript size constraints.

In the allelic recombination assay used here, both BIR and half-crossover events would correspond to *Trp+, Leu-, G418R* colonies. We note that to make sure that *Trp+, Leu-, G418R* events truly correspond to BIR in wild type or mutant cells, we analyzed them by CHEF and demonstrated that vast majority of them are really BIR events (**Figure EV5B**). Half-crossovers product would leave just single chromosome III 50% of the time while we don't see even single such event.

We did not find any half-crossovers in *pif1Δ rad52-R70A* products, among very rare Trp+, Leu-, G418R (7 colonies tested by presence of HML by PCR). It is possible that in *pif1Δ rad52-R70A* cells in allelic recombination, half-crossovers do not occur as frequently as in BIR assays tested previously. This could be because in allelic recombination, cleavage of the D-loop resulting in half-crossover still provides an opportunity to use two ends of a chromosome for the repair by gene conversion. We note that most of the annealing mutant *rad52-R70A* cells repair the break in allelic recombination by gene conversion. Considering limited space in this manuscript, we do not discuss the lack of half-crossover outcomes *pif1Δ rad52-R70A.*

4. In regards to the role Mre11 is playing in suppressing BIR, it is assumed here that the role is the end-tethering function. However it cannot be ruled out that resection at both ends is critical for GC, whereas resection at one would be sufficient for BIR. The authors do mention this possibility in the Discussion but do not explain why they favor the end-tethering role. Scully and colleagues showed that the ratio of long-tract (BIR?) to short tract GC events is changed in BRCA1 or CtIP-depleted cells. Furthermore, they found that elimination of 53BP1 restores STGC to BRCA1- cells, indicating that it is due to the end resection defect. The authors could address this question by deleting Ku, which is known to suppress the mre11 end resection defect, or by over-expressing Exo1. Alternatively, they could test mutants of the MRX complex that reduce end tethering but maintain end resection. These additional experiments would need to be done to determine the mre11 defect.

Avery similar question was asked by reviewer #1, question 1C. New experiments point at the asynchrony of resection as a likely major problem in *mre11Δ* cells that increases BIR contribution.

5. The sir2 results are interesting but seem a bit preliminary. All BIR assays in yeast require synthesis to the telomere and would require D-loop migration through heterochromatin in sub-telomeric regions. Does sir2 increase BIR efficiency in more typical BIR assays, e.g., the one reported by Lydeard et al. (2007)? How would heterochromatin affect survival of telomerase-negative cells which use BIR to maintain telomeres? This might be a point to add to the Discussion.

Our results show that deletion of *SIR2* affects on BIR/SDSA competition when homology on the second end is short. The reviewer asked whether *sir2Δ* has any impact on BIR when the template is not within a closed chromatin structure such as in *Lydeard et al. (2007).* In three assays used in our original manuscript, the *HML* template is within silent chromatin, and *SIR2* deletion leads to its unsilencing. In our revised manuscript, we further used an established assay where the template is within the unsilenced region of the genome so that deletion of *SIR2* should have no impact on the chromatin state of the template. Here an HO break at *URA3* gene is repaired with a partial *URA3* region ("*RA*") inserted on the other side of the chromosome by either gene conversion or BIR. The homology on each side of the break is 300 bp, and the BIR in wild type cells is only 0.8% (Anand *et al*, 2014). While we observed a over 30-fold increase of BIR in *rad52R70A* annealing mutant cells, we did not see any change in BIR frequency in *sir2Δ* cells in this system, suggesting that Sir2 role in BIR regulation is related to the chromatin state of the template **(Fig EV4A, B**).

Closed chromatin structure within the template could promote SDSA by slowing down the D-loop migration and/or making the D-loop more prone to unwinding. We favor the second possibility as BIR kinetics in *sir2Δ* cells, tested in BIR H-0 system, are only modestly increased when compared to wild type cells (new data in **Fig EV4C**).

To address the question on telomere recombination that occurs likely via BIR in a normally silenced region, the effect of *sir2Δ* on telomere recombination was tested previously in yeast. The absence of Sir2 indirectly affected telomere recombination (Lowell *et al*, 2003). Increased telomere recombination was observed but this increase was related to the mating type effect only. Finally, we note that HML silencing is stronger than the silencing of telomeres (Haber, 2012). Therefore, repressed chromatin structure at the HML locus might have a stronger effect on BIR compared to telomeric regions.

Minor points:

-1. Figure 1B: How far would BIR have to synthesize to reach the telomere? The efficiency of BIR is negatively impacted over long distances and could contribute to the failure to detect BIR initiated at the other break end (Figure 2).

BIR would need to synthesize about 12 kb to reach the telomere. This information is provided in results section. We measure DNA synthesis of just a few kb in the direction of telomere or centromere and only the former is observed, so the long distance is not the sole reason for lack of DNA synthesis toward centromere. We set up a new system to look at BIR that is possible only toward centromere and still observed only very weak DNA synthesis (**Fig EV3B**). This result is discussed in response to referee #1.

2. Not all southern blots have loading controls (missing from 2B, 2G, 4A, 4C).

When the probe used to detect DNA fragments that recombine also detected the template DNA, we also used it as a loading control. We indicated the band as "template/loading control" in the revised figure in such cases.

3. Luke-Glaser and Luke (2012) were the first to show increased BIR in the mph1 mutant; their work should be cited.

We quoted this work in a revised manuscript.

4. P. 16: Maloisel et al. (2008) should be cited for the role of Pol delta in short patch synthesis.

We quoted this work in a revised manuscript.

5. Figure 5E: the 0 hr time point for mre11 is missing.

We repeated the experiment to add time 0.

6. Figure S3: the BIR category (II) should be G418s.

We corrected the mistake.

7. There are some typos in the Figures, e.g., rad5-2R70A in 4A.

We checked the figures carefully to eliminate such typos.

Referee #3:

In this manuscript the authors address the issue of what factors promote two-ended repair, mainly being gene conversion, and prevent BIR one-ended repair when the DNA break is a two-ended break. This is important because most BIR outcomes are deleterious, resulting in lethal events or chromosome rearrangements, mutagenesis and extensive loss of heterozygosity. Although the studies are done in yeast, the findings are extended to human cells as most factors and pathways studied here are conserved. Thus the findings have broad implications. The short comings of the paper are that first, some of the mechanism were already known to some extent, although never analyzed in the systematic way they are in this paper, and second, that no single mechanism/gene/pathway has a profound effect on BIR vs SDSA when both are possible. Nonetheless, in toto the study reveals new information about regulation of two-ended break repair.

We thank the reviewer for complementing our work and for providing constructive criticism.

1. Introduction: I think the point here is to emphasize the deleterious consequences of BIR repair to make the case that cells would have mechanism(s) to prevent this type of repair if other options are available. This point gets lost in the extensive description of BIR and its outcomes. Some minimal rewriting here would really drive home the point that is brought up as the point of the paper at the start of the second paragraph of the introduction. At the end of the third paragraph it is important to emphasize the universality of the BIR process, its occurrence in human cells, and that all the proteins mentioned in this section save Rad59 exist in human cells and have the same activity as in yeast.

We added these important points on the significance of the study.

2. Results: The work is carefully done and uses assays and reporters developed by the authors. Several of these assays look at just one or a few outcomes, this should be noted. For example, as the authors do note, some assays have limited homology. Eventually the authors note this and indeed the length of homology turns out to be an important factor in preventing BIR. However, details of the assays, extensively described, may pass by many readers. Instead of presenting the experiments chronologically, it might be better to turn them around and point out that length of homology, previously known to have a role, was tested directly and out this at the front of the section rather than at the end. An example is at the end of the section on Rad52 and Rad59, at the end of the paragraph in the middle of page 7. Somehow this short homology could be more prominently noted earlier, although it is briefly mentioned at the bottom of page 6. This is just to guide the reader who is not intimately familiar with the assays.

We modified the text and provided information on the role of homology in the first paragraph of the results section. We note that previously the homology tested was either 50 or 150 bp, and all assays had a gap between homologous sequences that also stimulate BIR (Mehta *et al.*, 2017). Here we test far more extensive homology ranging from 150 bp to over 100 kb, and some assays had a gap while others did not.

The result section begins with the following paragraph:
"Initial steps of both SDSA and BIR involve strand invasion of one DSB end that results in formation of a displacement loop (D-loop). Gene conversion via SDSA is completed when the D-loop is extended, and the newly synthesized strand is displaced from its template and anneals to the second DSB end (**Fig 1A**). In BIR, the D-loop migrates even to the end of the chromosome. Previous work showed that 46, 50 bp or

even 150 bp homology on the second end is too short for efficient second end capture and for completion of SDSA, resulting in high level of BIR (Deem *et al*, 2008; Mehta *et al*, 2017). In these assays, an additional potential impediment for second end capture was the presence of a 700 bp gap between homologous sequences (Mehta *et al.*, 2017). Here, we have used a number of new assays with much longer homology ranging from 150 bp to over 100 kb, and assays with or without the gap to study the role of annealing and other enzymes in the competition between SDSA and BIR for DSB repair."

On a related note, the assays use constructs designed to measure a particular type of repair event. How close are these to events going on with breaks in cells? It might be useful to contextualize the types of events with breaks in human cells, those with limited homology in repeat sequences versus those with chromatid homology.

We added more information in the discussion section on possible consequences of BIR-SDSA choice in the context of the human genome. Also, we added a phrase in the abstract that indicates the significance of the study for recombination between shorter repeats.

In discussion we state:
"The lack of the enzymes regulating BIR frequency is partially (e.g., annealing mutants) or entirely (e.g., *mph1*) mitigated by the increased homology. This observation suggests that the control of BIR at two-ended breaks by enzymes coordinating the engagement of two DSB ends is essential in recombination between short repeated sequences and, thus, in genomes carrying many repetitive elements, such as humans."

Additional comments:
Table S1 needs some type of legend to explain what the numbers are; the numbers plus those in parentheses. One can guess but it is best not to.

Table S1 is now Appendix Table S1 and a legend was added to the table.

Anand RP, Tsaponina O, Greenwell PW, Lee CS, Du W, Petes TD, Haber JE (2014) Chromosome rearrangements via template switching between diverged repeated sequences. *Genes Dev* 28: 2394-2406
Chen X, Cui D, Papusha A, Zhang X, Chu CD, Tang J, Chen K, Pan X, Ira G (2012) The Fun30 nucleosome remodeller promotes resection of DNA double-strand break ends. *Nature* 489: 576-580
Deem A, Barker K, Vanhulle K, Downing B, Vayl A, Malkova A (2008) Defective break-induced replication leads to half-crossovers in Saccharomyces cerevisiae. *Genetics* 179: 1845-1860
Lowell JE, Roughton AI, Lundblad V, Pillus L (2003) Telomerase-independent proliferation is influenced by cell type in Saccharomyces cerevisiae. *Genetics* 164: 909-921
Mehta A, Beach A, Haber JE (2017) Homology Requirements and Competition between Gene Conversion and Break-Induced Replication during Double-Strand Break Repair. *Mol Cell* 65: 515-526 e513
Mortensen UH, Erdeniz N, Feng Q, Rothstein R (2002) A molecular genetic dissection of the evolutionarily conserved N terminus of yeast Rad52. *Genetics* 161: 549-562

Sugawara N, Ira G, Haber JE (2000) DNA length dependence of the single-strand annealing pathway and the role of Saccharomyces cerevisiae RAD59 in double-strand break repair. *Mol Cell Biol* 20: 5300-5309.
Valencia-Burton M, Oki M, Johnson J, Seier TA, Kamakaka R, Haber JE (2006) Different mating-type-regulated genes affect the DNA repair defects of Saccharomyces RAD51, RAD52 and RAD55 mutants. *Genetics* 174: 41-55
VanHulle K, Lemoine FJ, Narayanan V, Downing B, Hull K, McCullough C, Bellinger M, Lobachev K, Petes TD, Malkova A (2007) Inverted DNA repeats channel repair of distant double-strand breaks into chromatid fusions and chromosomal rearrangements. *Mol Cell Biol* 27: 2601-2614
Wu X, Haber JE (1996) A 700 bp cis-acting region controls mating-type dependent recombination along the entire left arm of yeast chromosome III. *Cell* 87: 277-285
Yan Z, Xue C, Kumar S, Crickard JB, Yu Y, Wang W, Pham N, Li Y, Niu H, Sung P *et al* (2019) Rad52 restrains resection at DNA double strand break ends in yeast. *Molecular Cell* in press

1st Revision - Editorial Decision 8th Feb 2021

Thank you for submitting your revised manuscript for our consideration. The three original reviewers have now assessed it once again, finding their initial concerns satisfactorily addressed and only retaining a few minor, mostly presentational issues. Once these remaining points have been incorporated in a final version of the study, we shall therefore be happy to accept it for EMBO Journal publication.

When re-revising the manuscript, please also pay attention to incorporate the following editorial points in the final version of the study.

REFEREE REPORTS

Referee #1:

Re: EMBOJ-2020-104847R

In the revised manuscript, authors strengthened the results reported in the original manuscript and further extended their findings in response to criticisms/comments raised by this and other reviewers. Their findings provided a novel insight into how potentially highly deleterious Break-Induced Replication (BIR) is suppressed when a DNA double-strand break (DSB) produces two ends. The data in the revised manuscript argue for the importance of the three steps of homologous recombination in suppressing BIR: ssDNA annealing involved in second end capture, D-loop unwinding and resection of DSB ends. They also identified a strong polarity in the direction of BIR when a DSB is formed at the Y/Z boundary. The result is solid and the phenomenon is very intriguing. The paper is now of enough quality and importance which warrants publication in this journal. I only have a few issues to be addressed before publication.

1. The authors put a lot of emphasis on the importance of synchronous resection of two DSB ends. It is certainly true that resection of the two ends is differently affected when MRN is defective, which is partially suppressed, with respect to its bias and quantity, by mutating KU70. However, how can they really conclude that the lack of synchrony, as opposed to a general reduction in resection, caused the elevated BIR seen in mre11? In other words, isn't it possible that a simple reduction in resection also accounts for BIR elevation seen in mre11? In my opinion, it is necessary to make a comparison between a mutant showing biased, reduced resection versus the one with unbiased, reduced resection to make the point. I found their emphasis on synchrony to be a bit too overstated.

2. I found the CHEF results presented in EV5B to be very confusing. Among the isolates from mre11, the bands come with a variety of migration levels. Just as an example, the two bands for sample#20 and those for #21, are they supposed to be different? Maybe they are meant to be different because they look different. The same is true for #7 and #8. I didn't find any particular explanation regarding where the difference comes from. A bit more explanation would help.

minor points

3. page 10, Fig EV2A, "extensive homology", specifying roughly how long (the distance between DSB at MATa and kanMX) would help.

4. page 16, line 3, it is claimed that the elimination of Mph1 along with rad59-del or rad52-R70A further increases the BIR contribution. While it is clearly so with rad59-del, I understand that BIR is already very high in rad52-R70A by itself in H-1400, and didn't find any substantial increase when it is combined with mph1 (Fig2B vs Fig4A).

5. Page 16, what does "High positioned nucleosomes" mean? Does it mean something like "densely-packed nucleosomes"?

6. Paragraph breaks are inconsistent.

Referee #2:

The authors have addressed most of my concerns from the initial review. I have a few suggestions for improving the text in places and there are some relevant citations that are missing.

p. 5, top: A recent paper confirmed suppression of BIR by Mph1 over-expression in a different assay system (E. Stivison et al 2020 Nucleic Acids Res).

p. 7, and p 8, first paragaphs: Y. Bai et al (1999, Genetics) showed synergism between rad59 and rad52-K70R for SSA and SDSA. Although the SDSA defect of the double mutant was not attributed to a switch between SDSA and BIR, the study should be cited.

p. 11, last paragraph: "However, annealing-deficient cells rad59Δ and particularly rad52-R70A increased BIR to ~30% and 60- 70%, respectively (Fig 2B, C)."
This sentence is awkward. I think replacing "cells" with "mutants" would help, or to just say: "However, rad59Δ and rad52-R70A mutants increased BIR to..."

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Referee #3:

The authors have performed several experiments based on the reviewers' comments. These results strengthen the paper and make clearer the regulation and prevention of BIR at two-ended

DSBs. As the genes involved and the mechanisms are conserved, the findings have important implications for break repair in mammalian cells. The work remains quite detailed, but for the reader willing to invest the time, they will come away with a much deeper understanding the repair processes shown in Figure 7.

First, we want to thank all of the reviewers for carefully reading our manuscript and for many excellent suggestions. Our responses to reviewer's questions are listed below.

Referee #1:

Re: EMBOJ-2020-104847R

In the revised manuscript, authors strengthened the results reported in the original manuscript and further extended their findings in response to criticisms/comments raised by this and other reviewers. Their findings provided a novel insight into how potentially highly deleterious Break-Induced Replication (BIR) is suppressed when a DNA double-strand break (DSB) produces two ends. The data in the revised manuscript argue for the importance of the three steps of homologous recombination in suppressing BIR: ssDNA annealing involved in second end capture, D-loop unwinding and resection of DSB ends. They also identified a strong polarity in the direction of BIR when a DSB is formed at the Y/Z boundary. The result is solid and the phenomenon is very intriguing. The paper is now of enough quality and importance which warrants publication in this journal. I only have a few issues to be addressed before publication.

1. The authors put a lot of emphasis on the importance of synchronous resection of two DSB ends. It is certainly true that resection of the two ends is differently affected when MRN is defective, which is partially suppressed, with respect to its bias and quantity, by mutating KU70. However, how can they really conclude that the lack of synchrony, as opposed to a general reduction in resection, caused the elevated BIR seen in mre11? In other words, isn't it possible that a simple reduction in resection also accounts for BIR elevation seen in mre11? In my opinion, it is necessary to make a comparison between a mutant showing biased, reduced resection versus the one with unbiased, reduced resection to make the point. I found their emphasis on synchrony to be a bit too overstated.

The best assay to address this question is a H-150 no-gap assay where homology is right next to DSB. In this system slow but synchronous resection would expose homologous sequences equally on both sides of the DSB. In *mre11 yku70* mutant, resection is decreased but synchronous while in *mre11* single mutant resection is slow and asynchronous (Fig. 6C). We observed increased BIR only in *mre11* but not in *mre11 yku70* suggesting that asynchrony of resection rather than slow resection changes BIR contribution.

2. I found the CHEF results presented in EV5B to be very confusing. Among the isolates from mre11, the bands come with a variety of migration levels. Just as an example, the two bands for sample#20 and those for #21, are they supposed to be different? Maybe they are meant to be different because they look different. The same is true for #7 and #8. I didn't find any particular explanation regarding where the difference comes from. A bit more explanation would help.

All bands for the lanes 8-20 migrated slightly faster compared to other lanes that resulted either from different PFG electrophoresis in these lanes or from spontaneous loss of some sequences (e.g. Ty) in parental strain used to construct the diploid. We favor the first possibility because both chromosomes involved in recombination migrate faster and in addition the longer chromosome above the bands in question also migrated faster. We also note that all across the gel there are tiny differences in migration pattern (e.g lane 6 and 7 on the gel on the left).

minor points
3. page 10, Fig EV2A, "extensive homology", specifying roughly how long (the distance between DSB at MATa and kanMX) would help.

In the disomic system, a double strand break is induced at MATa locus. The centromere-proximal end shares extensive homology (~200kb) while the centromere-distal (the arm with the KANMX marker) shares only 46bp homology with the template. This information is presented on line 5, paragraph 2 on page 10.

4. page 16, line 3, it is claimed that the elimination of Mph1 along with rad59-del or rad52-R70A further increases the BIR contribution. While it is clearly so with rad59-del, I understand that BIR is already very high in rad52-R70A by itself in H-1400, and didn't find any substantial increase when it is combined with mph1 (Fig2B vs Fig4A).

According to reviewer suggestion we modified text to explain the data more precisely.

5. Page 16, what does "High positioned nucleosomes" mean? Does it mean something like "densely-packed nucleosomes"?

We changed the text to “densely packed nucleosomes”

6. Paragraph breaks are inconsistent.

We adjusted the paragraph breaks.

Referee #2:

The authors have addressed most of my concerns from the initial review. I have a few suggestions for improving the text in places and there are some relevant citations that are missing.

p. 5, top: A recent paper confirmed suppression of BIR by Mph1 over-expression in a different assay system (E. Stivison et al 2020 Nucleic Acids Res).

This study is cited in a revised manuscript.

p. 7, and p 8, first paragaphs: Y. Bai et al (1999, Genetics) showed synergism between rad59 and rad52-K70R for SSA and SDSA. Although the SDSA defect of the double mutant was not attributed to a switch between SDSA and BIR, the study should be cited.

This study is cited in a revised manuscript.

p. 11, last paragraph: "However, annealing-deficient cells rad59Δ and particularly rad52-R70A increased BIR to ~30% and 60- 70%, respectively (Fig 2B, C)."
This sentence is awkward. I think replacing "cells" with "mutants" would help, or to just say: "However, rad59Δ and rad52-R70A mutants increased BIR to..."

We replaced “cells” with “mutants” according to the suggestion.

p. 16, Sir2 section: "High positioned nucleosomes could potentially affect the D-loop migration or D-loop unwinding and therefore impact BIR and SDSA choice." Change High to Highly.

We changed to ‘highly’ according to the suggestion.

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This study is cited in a revised manuscript.

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We thank the reviewer for this excellent suggestion. This study is cited in a revised manuscript.

Referee #3:

The authors have performed several experiments based on the reviewers' comments. These results strengthen the paper and make clearer the regulation and prevention of BIR at two-ended DSBs. As the genes involved and the mechanisms are conserved, the findings have important implications for break repair in mammalian cells. The work remains quite detailed, but for the reader willing to invest the time, they will come away with a much deeper understanding the repair processes shown in Figure 7.

We thank the reviewer for complementing our work.

Accepted **9th Mar 2021**

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Grzegorz Ira
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2020-104847

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

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 iustified
- ➔ 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 $\chi 2$ 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?	Experiments were conducted with at least 3 biological repeats or at least 500 colonies were analyzed for each experiment. The sample size was determined based on the previous similar experiments. This sample size allows to detect the change of frequency of the main DSB repair outcomes.
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?	No exclusions were done
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.	Genetic experiments use yeast colonies, which all are similar in size and phenotypes are not easy to distinguish. As a result, no blinding was needed.
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. Appropriate statistics were included for all figures and the type of tests are indicated in the figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Depending on the experiment assay, we used three different statistical tests: Welch's t test, chi-square test, and Mann-Whitney U-test. All the statistical tests were done using the software Prism 9 The data meet the assumptions of the chosen statistical tests for different assay.
Is there an estimate of variation within each group of data?	Yes. The mean with standard deviation as well as individual data points were plots.

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
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http://datadryad.org

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

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	No. Therefore the Welch's t test to account for the unequal variance between groups.

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).	No antibodies were used.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used.

* 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.	N/A
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