nature research

Peer Review File

Manuscript Title: Tracking break induced replication reveals its stalling at roadblocks

Redactions – unpublished data

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1:

In this report, the authors used the AMBER assay to track DNA synthesis during BIR. They showed that the migration of the D-loop proceeds slower than S-phase replication. The synthesis of the lagging strand is needed to stabilize the nascent leading strand. In the absence of Pif1 and Pol32, D-loop extension initiates, but does not proceed past 20 kb. Repetitive telomere DNA and highly transcribed regions, known to block DNA replication, also halt BIR replication. All these findings are interesting and provide useful information to understand the details of BIR, but do not represent a breakthrough. The AMBER assay is adapted from the DLE (D-loop extension) assay developed in Heyer's lab (Mol. Cell, 73:1255) with only minor modifications.

1. Since the AMBER assay does not significantly advance the DLE assay conceptually and technically, it should be called a modified DLE assay instead of giving it a new name. In Heyer's paper, DLE was also used to monitor DNA synthesis of the D-loop during BIR. Similar observations of initiation of DNA synthesis and the arrival of synthesis peak are reported in this study and in Heyer et al.'s report.

2. It would be better to use the DLC assay (D-loop capture assay), also developed in Heyer's lab (Mol. Cell, 73:1255), for monitoring strand capture (invasion) than to use disappearance of the Ya flap. Both Haber's lab (Gene & Dev., 23:291) and Heyer's lab (Mol. Cell, 73:1255) showed a delay of DNA synthesis after strand invasion, which is not mentioned in this study.

3. BIR synthesis seems to be much slower than S-phase replication. Since asynchronized cells were used in this study, reduced BIR DNA synthesis could result from a high percentage of cells that are not in S phase. One possibility is that BIR replication is slower than S-phase replication only when outside of S phase. It would be meaningful to compare BIR synthesis rate when cells are in S phase with S-phase replication rate.

4. The observation in this study that Pif1 and Pol32 mutants have a pronounced defect for long-range DNA syntheses is expected based on the study from Ira's group (Nature, 502:393). The DLC assay needs to be used to show that stand invasion is normal in the Pif1 and Pol32 mutants.

5. It is an interesting observation that impairment of lagging-strand synthesis limits leading strand synthesis to 20 kb. In a recent paper, Symington's lab showed that Polδ, but not Polε, is required for BIR DNA synthesis (Mol. Cell, 76:371). It would be informative to test whether Polε has any effect on BIR DNA synthesis using the DLE assay.

6. Since repetitive sequences and transcription (R-loop) stall general S-phase DNA replication, it is not surprising to see that they also suppress BIR replication. The current study provides

descriptive observations in this direction. It will be more impactful if the authors can demonstrate the mechanistic regulation of how fork blockage halts BIR in a way that may be similar or different from S-phase replication blockage. For instance, what are the proteins/signaling pathways that are involved in connecting the suppression of BIR-replication with fork blockage (by repetitive sequences and R-loops)? Are there different signaling mechanisms used for suppression of S-phase replication and BIR replication upon fork blockage? Does BIR replication also influence transcription efficiency?

7. It will be helpful to use a table to compare the frequencies of mutagenesis and GCR caused by BIR, transcription-associated BIR, and transcription-associated replication.

Referee #2:

Liu et al. developed a simple but powerful strategy to interrogate the strand invasion and repair synthesis steps of break-induced replication (BIR) and the role of known BIR factors in budding yeast that operate at a site-specific DSB on the truncated chromosome III using ddPCR and the monitoring of Ya fragment integrity. The authors also analyzed the effect of interstitial telomere sequences (ITS) and highly transcribed regions on BIR synthesis and the associated mutagenesis and chromosomal rearrangements. The results highlight the unique features of BIR synthesis that proceeds without coupling of leading and lagging synthesis and the potential of BIR to induce chromosomal instability and mutagenesis. Overall, the paper offers excellent insights into the DNA synthesis step of BIR and the in-depth analysis of known BIR mutants including pol32, pif1 and pri2-1. Application of ddPCR to detect BIR synthesis is clever and innovative. The effects of ITS and a highly transcribed region on the track of BIR synthesis are well justified and carefully described. Besides BIR synthesis and Ya integrity monitoring, the authors determined the types of repair outcomes and mutagenesis to comprehensively examine the BIR process and intermediates. The experiments are well executed with the proper controls and the results are presented clearly with insightful conclusions. Together, the paper should be an important addition to the field and provide an interesting paradigm to the repair synthesis steps in BIR and other homologous recombination events. It also likely raises many new questions regarding BIR and gene conversion mechanisms like all other exciting works do. I outlined below some of these questions, many of which should be clarified and resolved to generate a clear picture of BIR and BIR steps for publication.

1. To perform ddPCR, I wonder if cells were arrested at G2 prior to HO induction to limit the effect of DNA replication on BIR synthesis measurement. However, I could not find anywhere about G2 arrest (except those in pri2-1) in the paper including the Method section. Does it mean that most experiments were performed in asynchronous cells? If so, the authors should explain how the effect of DNA replication within a 90-kb BIR track on the ddPCR outcomes were minimized. If most experiments were performed in G2-arrested cells, such information should be clearly described in the text and the procedures.

2. Monitoring the integrity of Ya fragment to analyze the strand invasion event sounds logical. However, it was shown that strand invasion is not immediately followed by 3' flap removal. Therefore, the authors' assertion that BIR synthesis initiates immediately after strand invasion might be inaccurate and should be further confirmed by the timing of Rad51 ChIP, a more accurate indicator of strand invasion at the donor-specific DNA sequence. One can possibly insert a unique sequence next to the donor molecule to generate donor-specific DNA.

3. The effect of highly transcribed regions on BIR are interesting and worth analyzing further. Given the super-high expression the galactose promoter induces, one wonders what is the level of transcription that is sufficient to alter the BIR event. It can be determined by altering the level of galactose in the media and monitoring the level of HIS3 mRNA steady-state level and BIR. It could be that the transcription level and BIR are inversely correlated with each other or alternatively, a

certain threshold might exist that is co-incident with the level of R-loops. Can the authors examine the R-loop formation by ChIP using the R-loop-specific antibody at the Gal-HIS3 locus?

4. I am concerned that the ~20 kb of leading-only DNA synthesis in the pri2-1 mutant could be due to the hypomorphic nature of the mutation, instead of the intrinsic ability of cells to tolerate such a long ssDNA or a unique replication fork lacking lagging-strand synthesis up to 20 kb. One way to resolve this issue is to generate a pri2 degron and determine if the AMBER pattern is still similar to pri2-1 at the non-permissive condition.

5. Given that the telomere repeat sequence can form G4 DNA, is the effect of ITS on BIR and BIR synthesis specific to the telomere sequence or can it occur with other G4 DNA as well? Is there any effect on RRM3 deletion in BIR across ITS? Is there any size threshold of ITS to block BIR synthesis?

6. I am puzzled why the AMBER could not detect the apparent difference in BIR frequency at ITS in TLC1 and a tlc1 mutant. Should we interpret that BIR beyond ITS takes place in tlc1 after 10 h post-HO expression? If so, can the authors examine BIR synthesis by AMBER at later time points to match them with BIR frequency?

7. Given that BIR is not efficient at highly transcribed regions, one can deduce that BIR is not efficient in restarting stalled or collapsed forks at highly transcribed regions or at R-loops. The authors need to clarify these points in the paper.

8. Does SIN3 deletion impede BIR at GAL-HIS3 6 kb distal to the break?

9. In Fig. 3f, why are there two peaks at telomeric regions showing two copies of DNA?

Referee #3:

The authors examine BIR-associated DNA synthesis by developing a novel method based on droplet PCR, termed AMBER, using a well-established BIR assay in the budding yeast Saccharomyces cerevisiae. The authors are able to define a number of critical differences between an S-phase replication fork and the mode of DNA synthesis during BIR. First, they confirm and extend previous observations that BIR is slower and likely multi-phasic with early Pol32- and Pif1-independent phases, while extensive DNA synthesis become increasingly dependent on these proteins. Second and particularly insightful is the analysis of the primase mutant pri2-1, showing that leading and lagging strand are separated by about 20 kb of ssDNA. Thirdly, they show that interstitial telomeric repeats represent a significant obstacle to BIR, leading to telomere addition at the site of the interstitial repeat and loss of the distal sequence. Fourth, the authors define the conflict between transcription and BIR-associated DNA synthesis by measuring DNA synthesis, mutagenesis, and repair outcomes. Overall, this work provides important new insights into the mechanisms of BIR and how BIR may contribute to genome instability.

Addressing the following points will further strengthen this manuscript.

Specific comments:

1) The title should be reworded, by using 'details' the authors undersell their work.

2) The conclusion that DNA synthesis starts immediately after DNA strand invasion directly contradicts earlier work published by the Haber laboratory (refs. 21, 22). The authors should discuss this discrepancy, probably best in the Extended Data section.

3) In Figs 1, 3 and 4, the experiments are conducted in cycling cells, so that HO cleavage can

occur in G1 and post-S cells, with likely both sister chromatids being cleaved. This aspect should be discussed. Does BIR happen in G1? Is it different from post-S BIR?

4) Fig. 1: In a, the black triangle needs to be defined.

In c, why is there a copy number decrease at -150 kb in the rad52 strain?

In e, the authors show a difference between the phenotype of pol32 and pif1, which begs the analysis of the double mutant.

Is the removal of the Ya sequence affected in pol32 and pif1? This is another landmark that can be established.

5) Fig. 2: The authors deduce a distance of ~20 kb between the leading and lagging strand but the error in this number appears to be very large (in my estimate ± 10 kb). Please calculate and error for the value and provide it in the text.

In b, consider using the same scale for both graphs.

In d, the copy number in wild type starts higher than in the pri2-1 mutant. Is the difference significance and what is the explanation?

6) Figs 3 and 4: The stalling of the BIR DNA synthesis is inferred and should be directly demonstrated by 2D gel analysis.

7) Fig. 4 and line 169 & throughout: HO is a confusing abbreviation for the head-on collision in this text because the authors use the HO endonuclease to induce DSBs.

In a, why italics for leu- and ade-? They are phenotypes not genotypes.

In b, c, e and f, please provide in the figure the information, which pGAL construct and orientation is shown that the reader does not need to refer to the legend.

Line 206: Fig. 4a only shows ura3-29 under control of the GAL1 promoter, not the native URA3 promoter.

In Fig. 4h, the no-break control with the head-on construct shows elevated mutagenesis. Is this consistent with the known replication direction in this region? In lines 209-211, it is unclear, how the no-break control can relate to BIR.

8) Extended Data Fig. 1: Why is there a decrease in the Ya copy number in rad51? More detail on the assay for Ya cleavage could be provided. What is the placement of the primers?

9) Extended Data Fig. 5: To strengthen the conclusion about the involvement of R-loops, the authors should measure R-loop levels directly under these conditions.

Does sin3 affect BIR stalling after the BIR-associated DNA synthesis is established?

Line 175: Relative to the data in Extended Data Fig. 1b, it appears that Ya disappearance (i.e. cleavage) in Extended Data Fig. 5b is affected by ongoing transcription. Authors claim that ongoing transcription only affects the synthesis step of BIR, but it appears to also interfere with stable D-loop formation and flap cleavage.

Extended Data Fig. 5d,e: It would be helpful if the authors can label these plots.

10) Lines 72-74: Flap cleavage is presented here and throughout as the time of the invasion/D-loop formation, but the flap likely triggers multiple rounds of invasion/D-loop formation followed by disruption. Moreover, I am not sure that we know that flap cleavage itself does not trigger D-loop disruption.

11) Lines 219-221: The sentence "it is possible that BIR contributes to the genetic instabilities reported in relation to fragile sites." may have to be qualified as not fragile sites derive from DNA synthesis/transcription conflicts.

Minor issues:

12) Lines 93, 195: "wt" or "wt" is used as an abbreviation for wild type without previously defining. Italics/non-italics should be applied consistently to this term.

13) Line 103: "longer-range"; "long-range" instead?

14) Line 104: "almost no increase of synthesis" -- "almost no increase in synthesis" instead?

15) Line 116: "products by CHEF measuring the" -- "products by CHEF gel electrophoresis measuring the" instead?

16) Lines 122, 123: May need to insert a sentence in between these lines to explain the experimental rationale.

17) Line 147, 154: "CHEF analysis" -- "CHEF electrophoresis analysis" instead?

18) Line 228: References 15 and 34 may not strongly support the claim that BIR is involved in recovery of collapsed replication forks at fragile sites.

19) Line 311: The title of reference 30 is missing.

20) Line 472: The authors do not indicate the yeast strain background (i.e. W303, CEN-PK, S288c, etc.).

21) Lines 527 and 528: Typo "zymolase" should be "zymolyase".

Author Rebuttals to Initial Comments:

Responses to Reviewer 1 comments

1. We thank Reviewer 1 for his/her positive assessment of our manuscript. We hope that our additional work described here convinces Reviewer 1 that our manuscript is important for the field, based not only on the development of a new quantitative method to study DNA synthesis during BIR, but also based on numerous novel findings, including:

1. Determining the kinetics of BIR with high precision, which uncovered that the rate of BIR synthesis is ~6 times slower than S-phase replication.
2. The identification of BIR-specific roles for various proteins that revealed important mechanistic features of BIR. For example, our evaluation of a primase-deficient mutant enabled us to quantify the asynchrony between leading and lagging strand BIR synthesis.
3. By identifying the difficulty for BIR to initiate in the vicinity of highly transcribed units, we predicted the need for regulation of BIR-mediated replication restart,

as well as described genetic instabilities resulting from conflicts between BIR and transcription units.

4. We defined the mechanism of BIR interruption at ITSs, yet another genetic instability associated with BIR.

2. Reviewer1 comment*: "Since the AMBER assay does not significantly advance the DLE assay conceptually and technically, it should be called a modified DLE assay instead of giving it a new name. In Heyer's paper, DLE was also used to monitor DNA synthesis of the D-loop during BIR. Similar observations of initiation of DNA synthesis and the arrival of synthesis peak are reported in this study and in Heyer et al.'s report.*

Answer: We thank the Reviewer for this question. In response, we now mention in the revised manuscript that our method represents "a derivative of a recently developed DLE method measuring D-loop extension (Piazza et al., 2018; Piazza et al., 2019)". However, we would like to emphasize that, although DNA preparation for our AMBER method was definitely inspired by DLE, it differs from DLE not only in technical details, but also conceptually. Unfortunately, due to space constraints, we are unable to include an expanded discussion of this within the revised manuscript. Instead, we discuss this in detail below, and we have added a more brief clarification of this issue in the revised manuscript.

We agree that the DLE assay developed by Heyer's lab represents a breakthrough, as it was used to demonstrate that the initial BIR synthesis product can be captured both in wild type cells and in some BIR mutants (*e.g.*, *pol32Δ*)(Piazza et al., 2018; Piazza et al., 2019). This result was important for us, because it suggested that intermediate BIR products can be successfully stabilized and detected. In the DLE assay, this detection relies on quantitative PCR (qPCR), which requires digestion of the DNA by restriction enzymes followed by proximal ligation. Digestion and ligation are required for the DLE assay to create new junctions that could be formed only after BIR synthesis (not before), thus limiting the initial background signal so these new junctions could be detected by qPCR.

Our AMBER method employs droplet digital (dd) PCR, which is much more sensitive than qPCR and allows detection and quantification of the newly synthesized DNA without the need to eliminate the background signal of the original donor chromosome. Our goal was to preserve all newly synthesized DNA, including persistent ssDNA, and to quantify it in absolute numbers instead of as relative fold-changes. To achieve this, we used two buffers described for the DLE protocol (lysis buffer and DNA dissolving buffer (Piazza et al., 2018)), which seemed to be amenable to isolating and preserving ssDNA, particularly because they were devoid of SDS. We also developed procedures to remove detergents from the solutions to make the prepared DNA compatible with ddPCR. As a result, our AMBER method offers the following advantages:

1. Avoiding the high inter-sample variability that is characteristic of DLE (from variations in cutting and ligation efficiencies).

2. Detecting BIR synthesis by copy number changes at any position, whereas DLE can only detect DNA synthesis at the very beginning of BIR, due to limited restriction sites and the length requirement for full ligation.
3. Eliminating non-BIR signal by using of fluorescent probes in addition to primers (non-specific binding of primers to other locations can occur in DLE assays).
4. Allowing DNA synthesis to be quantified as copy number, so that both increases and decreases (*e.g.*, clipping of *Ya* flaps, degradation in *rad52Δ*, see our response #24 to the Reviewer 3) in DNA can be detected. The current DLE protocol has no means to identify elimination of DNA.

We thus believe that our method warrants a different name, as AMBER is not simply a combination of DLE with ddPCR. Our detailed protocol for DNA preparation for AMBER contains all these additional comments in the revised manuscript.

3. Reviewer 1 comment: *"It would be better to use the DLC assay (D-loop capture assay), also developed in Heyer's lab (Mol. Cell, 73:1255), for monitoring strand capture (invasion) than to use disappearance of the Ya flap. Both Haber's lab (Gene & Dev., 23:291) and Heyer's lab (Mol. Cell, 73:1255) showed a delay of DNA synthesis after strand invasion, which is not mentioned in this study".*

Answer: We thank the Reviewer for this suggestion. We agree that the DLC assay represents a more direct way to follow strand invasion compared to monitoring the kinetics of *Ya* disappearance. As suggested by the Reviewer, we analyzed strand invasion by DLC, which showed strand invasion initiates between ~1 and 1.5 hours after DSB induction. As an orthogonal approach, we assessed strand invasion by detecting the time of Rad51 loading on the donor chromosome using CHIP followed by qPCR using donor-specific DNA primers, as suggested by Reviewers #2 and #3. Rad51 ChIP data indicated strand invasion initiates between ~1 and 1.5 hours after DSB induction, consistent with the DLC assay. This is approximately 1 hour earlier than the beginning of BIR synthesis that we detected using AMBER, indicative of an approximately 1-hour delay between strand invasion and DNA synthesis, which is similar to the results obtained by Heyer's lab (Piazza et al., 2018; Piazza et al., 2019). This finding is also in line with the 40-minute delay previously observed for the initiation of DNA repair synthesis during MAT switching (Hicks et al., 2011). Importantly, though, we did not observe a long (3-4 hour) delay between strand invasion and DNA synthesis that was previously reported (Donnianni and Symington, 2013; Jain et al., 2009; Jain et al., 2016; Malkova et al., 2005). Based on our findings, we hypothesize that the 3- to 4-hour delay likely included not only the time required to initiate leading strand BIR synthesis, but also the time for the nascent ssDNA to be copied to dsDNA, consistent also with (Donnianni and Symington, 2013). In the revised manuscript, we have tried to clarify that the distinction we are making regarding the timing of initiation of BIR synthesis is between our data and the reported 3- to 4-hour delay reported by the Haber lab (Jain et al., 2009; Jain et al., 2016; Malkova et al., 2005).

Our response to this comment includes addition of data describing strand invasion kinetics analyzed by Rad51-CHIP and the DLC assay in Extended Data Fig. 1a,b. We made the following change to the abstract, which now states: “Here, we developed an assay to monitor repair specific DNA synthesis and demonstrate that BIR synthesis initiates ~1 hour following strand invasion……”. In addition, the following sentences have been included in the Results section: “BIR synthesis of the first 500 bp was detected by ddPCR as early as 2.5 hours post-DSB induction (Fig. 1b), about 1 hour after ChIP detection of Rad51 loading onto the donor Chromosome III, which was used as a readout of strand invasion (Extended Data Fig. 1a), or by D-loop capture (DLC) assay(Piazza et al., 2019) (Extended Data Fig. 1b)”.

4. Reviewer 1 comment*: “BIR synthesis seems to be much slower than S-phase replication. Since asynchronized cells were used in this study, reduced BIR DNA synthesis could result from a high percentage of cells that are not in S phase. One possibility is that BIR replication is slower than S-phase replication only when outside of S phase. It would be meaningful to compare BIR synthesis rate when cells are in S phase with S-phase replication rate”.*

Answer: In our system, DSBs are indeed initiated in asynchronous cells. However, by the time these cells initiate BIR synthesis, the culture is synchronized in G2/M arrest. In fact, in practically all nuclease-induced yeast BIR experimental systems described to date, BIR synthesis occurs in G2 (Donnianni and Symington, 2013; Donnianni et al., 2019; Jain et al., 2009; Jain et al., 2016; Malkova et al., 2005; Wilson et al., 2013). Other studies of BIR in yeast, such as events induced by telomere erosion, over-replication, etc., are also likely evaluating events that occur outside of S phase. Moreover, the examples of BIR-like events documented in human cells, which include MiDAS, ALT synthesis, etc, also refer to events occurring outside of S phase (Bhowmick and Hickson, 2017; Bhowmick et al., 2016; Dilley and Greenberg, 2015; Minocherhomji et al., 2015) . In sum, based on the information accumulated to date, BIR appears to be an event that occurs primarily after S phase, during G2 or even M stage of the cell cycle. Thus, we can confidently say that there is no known system where BIR occurs and can be followed during S phase. However, we agree with the Reviewer that BIR synthesis in the context of repairing broken replication forks could be different.

5. Reviewer 1 comment*: The observation in this study that Pif1 and Pol32 mutants have a pronounced defect for long-range DNA syntheses is expected based on the study from Ira’s group (Nature, 502:393). The DLC assay needs to be used to show that stand invasion is normal in the Pif1 and Pol32 mutants.*

Answer: Based on the studies from Ira’s group (Wilson et al., 2013) and others (Deem et al., 2008; Lydeard et al., 2007; Saini et al., 2013; Smith et al., 2009) we know that both *pol32Δ*

and *pif1Δ* mutants are defective in BIR that requires synthesis of 30-100 kb. We used AMBER to gain additional information about these enzymes. Our goal is to convey that, by using AMBER, we can now determine the extent of synthesis that is possible in the absence of *POL32*, *PIF1*, or any other BIR-related protein. We believe this is very important, because it allows us to diagnose the specific defect of any BIR mutant with high precision. To respond to the Reviewer's comments, we have included new data in the revised manuscript that demonstrate that no BIR synthesis is initiated in *pol32Δ pif1Δ* double mutants (Fig. 1e), and we also performed DLC assays in *pol32Δ* and *pif1Δ* mutants, which determined that they are proficient for strand invasion (Extended Data Fig. 3e). In addition, the following sentences have been included into the text: "Additionally, neither of these mutations affected the efficiency of strand invasion (Extended Data Fig. 3e)."; and also: "Interestingly, in *pol32Δ pif1Δ* double mutants, no synthesis occurred, even at the 200bp position (Fig. 1e), suggesting that Pol32 can promote at least some bubble migration in the absence of Pif1 and *vice versa."*

6. Reviewer 1 comment*: It is an interesting observation that impairment of lagging-strand synthesis limits leading strand synthesis to 20 kb. In a recent paper, Symington's lab showed that Polδ, but not Polε, is required for BIR DNA synthesis (Mol. Cell, 76:371). It would be informative to test whether Polε has any effect on BIR DNA synthesis using the DLE assay.*

Answer: In response to this question, we used AMBER to follow BIR synthesis in cells where Polε was inactivated by AID-degron. Interestingly, we observed no significant decrease in the amount of synthesis upon Polε inactivation 0.5 and 20 kb from the DSB (which is compatible with observations obtained by both the Symington and Haber labs (Donnianni et al., 2019; Lydeard et al., 2007)), as well as a mild, but statistically significant decrease in synthesis at the 90-kb position, which is compatible with observations made by Haber's lab (Lydeard et al., 2007). Together, these findings suggest that Polε indeed plays a role in BIR that travels long distances, although a significant amount of 90-kb product can be detected even in the absence of Polε. While the mechanism that may explain this phenomenon is not currently known, these results are consistent with data reported by the Symington and Haber research groups. Our new data are included in the revised manuscript in Extended Data Fig. 3f. In addition, the following sentence has been included in the text: "In addition, we observed only a mild defect that was statistically significant only at 90 kb following AID-degron inactivation of Polε (Extended Data Fig. 3f), consistent with the primary role of Polδ in BIR".

7. Reviewer 1 comment*: "Since repetitive sequences and transcription (R-loop) stall general S-phase DNA replication, it is not surprising to see that they also suppress BIR replication. The current study provides descriptive observations in this direction. It will be more impactful if the authors can demonstrate the mechanistic regulation of how fork blockage halts BIR in a way that may be similar or different from S-phase replication blockage.*

For instance, what are the proteins/signaling pathways that are involved in connecting the suppression of BIR-replication with fork blockage (by repetitive sequences and R-loops)? Are there different signaling mechanisms used for suppression of S-phase replication and BIR replication upon fork blockage Does BIR replication also influence transcription?

Answer: We agree that distinctions between the impact of road blocks on S-phase replication versus BIR are highly interesting and relevant. It is important to note that there are hundreds of manuscripts describing the impact of different impediments on DNA replication, while ours is the first manuscript to address these questions for BIR. Clearly, much more work is needed to comprehensively compare the nature and magnitude of the effects of replication impediments between BIR and regular replication; but, from our new data, we can now make a first comparison between these processes at a high level. The overall picture emerging is that BIR is far more sensitive than regular replication to at least the two obstacles tested here: high transcription and ITS. Below, we have divided our response by these two obstacles and added new mechanistic insights we gathered with additional experiments.

Impact of high transcription on BIR

Our main finding regarding the effect of highly transcribed sites on BIR is that head-on transcription profoundly blocks BIR initiation. Approximately 50% of BIR is never completed when a highly transcribed gene is present next to the strand invasion site, and BIR becomes "stuck" at the strand invasion for several hours. No effect this dramatic for a single transcribed gene has ever been documented for normal replication to our knowledge. Some reports suggest a dramatic block of replication by head-on transcription in certain mutants in bacteria (for example, in strains deficient for R-loop processing (Lang et al., 2017)), but this may not reflect what occurs in wild type cells. Thus, we conclude that the phenotype we described is specific for BIR.

Our new experiments show that initiation of BIR immobilizes rPolII on DNA, thus not only BIR is blocked, but also transcription is paused. Specifically, while addressing this Reviewer's question regarding "*mechanistic regulation of how fork blockage halts BIR" and* whether "*BIR replication also influence transcription*", we found a significantly lower level of *HIS3* mRNA transcripts when P_{GAL1}-*HIS3* was oriented in the head-on (H-On) direction versus co-directional (Co-D), even though rPolII binding to the coding region was similar between the two. Experiments employing another high transcription unit, P_{TET}-*HIS3*, provided additional insight. Specifically, while we observed a robust induction of P_{TET}-*HIS3* transcription 1 h after addition of doxycycline, with the level of *HIS3* mRNA positively correlating with the amount of doxycycline added (Fig. 4e), the level of *HIS3* mRNA induced by high doses of doxycycline decreased following BIR induction and was 25-fold lower in strains with a DSB compared to no-DSB controls at the 10-hour time point (Fig. 4f). At the same time, the level of BIR synthesis initiated in these experiments, based on AMBER

analysis at the 10-hour time point, was inversely correlated with the level of transcription (Fig. 4e). Taken together, these new results support that H-On transcription and strand invasion during BIR inactivate each other.

To gain additional insight into the "*mechanistic regulation of how fork blockage halts BIR",* and "*how BIR affects transcription after BIR initiated"*, we performed RNPII CHIP-seq analysis at a time point when about half of cells in the culture have completed DNA synthesis through 30 kb from the strand invasion site. Interestingly, we found that RNPII accumulated at TES zones of H-On- but not Co-D-oriented genes within the BIR tract. Importantly, this result was specific to BIR, as it was observed in BIR wild type cells, but not in *rad51Δ* mutants. Also, RNPII accumulation was not observed from 30 kb downstream of the DSB through the end of the donor Chromosome III, which we postulate is because BIR had not yet passed through this region in the majority of the cells during that time frame. This result implied that the successful passage of BIR through high transcription units does not result from a "global" decrease of transcription along the whole BIR template. Rather, we propose that established BIR synthesis has less problem traversing transcription units, but still remains somewhat problematic. In support of this we report that the level of genetic instability resulting from encountering H-On transcribed genes is orders of magnitude higher compared to normal replication (see our new results in Supplementary Table 1 and the response to the question 8).

We wish to emphasize that the question regarding the ability of BIR to initiate near transcription sites remains important irrespective of what is known about S-phase replication because of the important role that BIR is predicted to play specifically under these circumstances. The conclusion that we came to is that BIR is not well suited for this role, and that initiation of BIR therefore must be highly regulated to make it possible. For example, we predict that extensive DNA resection and/or fork reversal has to occur following replication fork collapse to ensure that BIR can initiate at an adequate distance from transcription sites. Once initiated, BIR can traverse transcription regions, although traversing units in the H-On orientation promotes mutations and chromosomal rearrangements that are at least an order of magnitude greater than those associated with S-phase replication. This result suggests that BIR-mediated recovery of S-phase replication forks that collapse following H-On collisions with transcription units could contribute substantially to the genetic instabilities associated with fragile sites.

In response to this reviewer's question, we added new data into Fig. 4d-g and Extended Data Fig. 8d-f. In addition, the following sentences addressing the mechanism of interaction between high transcription and BIR were added to the text: "The apparent collision between the migrating D-loop and transcription bubble is supported by much lower accumulation of *HIS3* mRNA in strains with H-On- versus Co-D-oriented *HIS3* 8 h after DSB induction (Fig. 4 d), while ChIP of the Rpb1 subunit of the rPolII complex confirmed similar rPolII binding in both orientations (see Extended Data Fig. 6a, b for primer positions). Based on these results, we propose that immobilized rPolII on DNA, blocks the progression of both BIR and further transcription. To uncouple the timing of DSB induction and transcription, we inserted

$P_{TET\ (on)}$-*HIS3* in H-On orientation (Fig. 4a), which also enabled us to regulate the level of transcription with doxycycline.

We observed a robust induction of $P_{TET(on)}$-*HIS3* transcription 1hour after addition of doxycycline (right before galactose addition), and the level of *HIS3* mRNA positively correlated with the amount of doxycycline added (Fig 4e). As anticipated, the level of BIR synthesis in these experiments analyzed at the 10-hour time after DSB induction, was inversely correlated with the amount of doxycycline (Fig. 4e). Notably, the level of *HIS3* mRNA induced by high doses of doxycycline (5µg/ml) decreased following BIR induction and was 25-fold lower in strains with a DSB compared to no-DSB controls at the 10-hour time point (Fig. 4f). Taken together, we propose that initiation of BIR and H-On transcription reciprocally block one another."

In addition, to address the collision between head-on transcription and stabilized BIR, we added the following sentences to our text: "We next tested whether BIR can traverse highly transcribed genes farther from the strand invasion site. No evident BIR synthesis defect was observed with P_{GAL1}-*HIS3* inserted 6 kb from strand invasion in either orientation as examined using AMBER assay (Fig. 4a, c, Extended Data Fig. 7g). This could mean that either established BIR synthesis has less problem traversing transcription units or that initiation of BIR synthesis leads to a global decrease in transcription along the whole template, to allow BIR passage through the chromosome. To distinguish between these possibilities, we compared rPolII distribution on the donor chromosome III before and 6 hours after DSB induction in strains undergoing BIR and in BIR-deficient *rad51Δ* mutants. At 6 hours, when BIR copying of the first 30 kb was completed in more than a half of the cells, while only a limited portion of cells had completed 90 kb of synthesis (Extended Data Fig. 8d), rPolII had accumulated in the TES regions of several H-On transcription genes within the first 30 kb of BIR in wild type cells, but not in *rad51Δ* cells (Fig. 4 g (i, ii), Extended Data Fig. 8e(i), f). rPolII accumulation was not observed from 60 kb downstream of the DSB through the end of donor Chromosome III (Fig. 4g (iii), Extended Data Fig. 8e (ii)), which we posit is because BIR had not yet passed through this region in the majority of the cells during that time frame. Therefore, we propose that BIR has only a localized effect on transcription regulation, which likely results from a conflict between BIR and transcription. Importantly, rPolII accumulation was not observed on Co-D-oriented genes located within the first 30kb (Fig. 4g (iv), Extended Data Fig. 8e(iii)), and it was specific to chromosome III genes (Extended Data Fig. 8e (iv)). Taken together, these results suggest that BIR synthesis can progress through transcribed genes, but actively transcribed genes in the H-On orientation promote transient stalling of the migrating bubble."

<u>Impact of ITS on BIR</u>

The difference between replication and BIR is also very clear when we compared the impact of ITSs. ITSs are maintained relatively well in the strain investigated, suggesting that they are successfully replicated during S phase. Strikingly, the same ITS blocked BIR in nearly all cells. We found that ITS blocked BIR synthesis even in telomerase-deficient (*tlc1Δ*) strains. We interpret this finding to mean that interruption of BIR at ITSs is the primary event, while

capping of the newly synthesized DNA with a telomere via recruitment of telomerase represents a secondary event. In the revised manuscript, we include new experiments to demonstrate that it was not G4 structures within ITSs that stalled DNA synthesis during BIR, as we observed no impact of G4 forming sequences (Kim et al., 2011; Kim and Jinks-Robertson, 2011; Lippert et al., 2011) and G4-stabilizing agents (Ribeyre et al., 2009) or mutations that result in deficient processing of G4 structures on BIR synthesis. Instead, we propose that it is more likely that the BIR interruption was caused by proteins binding to ITSs, such as the essential for cell viability Tbf1 protein, which has been shown to bind human telomere sequences in yeast with a threshold of ~40-45 for the formation of capped telomere structures (Ribaud et al., 2012). In support of this, AMBER detected a similar threshold of BIR progression defect when it encountered $(ITS)_{40}$, while ITSs with ≤28 telomere repeats did not impede BIR progression.

The data described above are included in the revised manuscript in Extended Data Fig. 5a-j for ITS. In addition, a new paragraph describing these findings have been included in the text.

"The potency with which ITS hinder BIR progression is unlikely due to forming G4 structure because BIR easily progressed through another, non-ITS, G4-forming sequence(Kim and Jinks-Robertson, 2011) inserted in either orientation at the same location, even in the presence of the G4-stabilizing agent, Phen-DC3(Piazza et al., 2010) (Extended Data Fig. 5b-e). In addition, deletion of *RRM3*, which can unwind G4 structures(Geronimo and Zakian, 2016), did not exacerbate the difficulty for BIR to traverse either $(ITS)_{40}$ or $(ITS)_{28}$ (see below) (Extended Data Fig. 5h- j). Meanwhile, we observed that human telomeric repeat (≥ 40) interfered with BIR progression, while a shorter ITS (≤28 repeats) did not interfere with BIR (Extended Data Fig. 5f), suggesting that the threshold for the number of telomeric repeats that can interrupt BIR is similar to the one that is required to bind Tbf1 for the formation of capped telomeres(Ribaud et al., 2012). However, Sanger sequencing of BIR outcomes in strains containing shorter ITS determined that there was frequently a reduced number of telomeric repeats (15% (11 out of 71) in BIR events versus 0 out of 41 from no-DSB control) (Extended Data Fig. 5g), indicative of transient BIR stalling even at shorter ITS that was likely followed by template switching altering the length of ITSs. "

8. Reviewer 1 comment*: It will be helpful to use a table to compare the frequencies of mutagenesis and GCR caused by BIR, transcription-associated BIR, and transcription-associated replication.*

Answer: We thank the Reviewer for this suggestion, and we have added the recommended tables. The original manuscript already contained all of the S-phase and BIR mutagenesis frequency data for the transcription experiments. For the revised manuscript, we performed additional experiments where we allowed for extra 3 days for mutant formation because we

noticed that Ura$^+$ colonies in one of the strains appeared to be smaller size. The obtained data confirmed all of our original conclusions. The data clearly demonstrate that the frequency of mutagenesis is increased more than ~100x during BIR compared to S-phase replication (no-DSB control), even when high transcription was not induced, which is consistent with our previous publication describing BIR-associated mutagenesis. However, when transcription in H-On-oriented P_{GAL1}-*ura3-29* was induced, the level of BIR-associated mutagenesis increased by an additional 11-fold. The results of this comparison are presented as graphs in Fig.4h. The frequencies of mutagenesis for each of these situations and all statistical analyses are presented in Supplementary Table 2, as suggested by the Reviewer.

Further, in response to the Reviewer's comment GCR frequencies resulting from the passage of BIR vs S phase replication through transcription units were included in the revised manuscript. In particular, the GCR frequencies for BIR were already present in the original manuscript. In response to the Reviewer's comment, we constructed new strains to follow the frequency of similar GCRs (genetic instabilities) associated with S- phase replication. To achieve this, we created diploid strains containing P_{GAL1}-*HIS3* at the same positions along Chromosome III as in the BIR strains, but these diploids contained mutations at the *HO*-recognition sites that prevented breakage at the *MAT* loci. Cells were grown in YEP-Lac medium and subsequently plated on either YEP-Galactose or YEPD to induce or not induce *HIS3* transcription, respectively. We followed the loss of Chromosome III markers, including *ADE3* and *HPH,* which allowed us to estimate the frequencies of chromosome loss associated with-S phase replication. In addition, we used CHEF gel electrophoresis to determine the fraction of chromosomal rearrangements among outcomes that showed no change in any scorable markers. The obtained results demonstrated that the frequency of GCRs (genetic instabilities) during BIR is significantly higher compared to S phase replication, even without inducing the P_{GAL1}-*HIS3* construct. The frequency of BIR-associated GCRs was further increased when a high-transcription unit was present in the proximity of the BIR initiation site in either orientation, with H-On-oriented transcription inducing a more drastic increase compared to Co-D-oriented transcription. The effect of transcription on BIR-associated GCRs was lower when the P_{GAL1}-*HIS3* cassette was placed 6 kb centromere distal to *MAT*. However, transcription unit in the H-On orientation still significantly increased the frequency of aberrant BIR outcomes (consistent with our data reported in the original manuscript). Overall, our results suggest that the frequency of GCRs and mutations resulting from collisions between BIR and highly transcribed units is significantly higher compared to those resulting from collisions between S-phase replication and the same transcription units.

These results have been added in the revised manuscript by including of the new Supplementary Table 1 and by adding data to the Supplementary Table 2. In addition, the following statements have been added to the text:

"Taken together, these results suggest that BIR synthesis can progress through transcribed genes, but actively transcribed genes in the H-On orientation promote transient stalling of the migrating bubble. In further support of this, P_{GAL1}-*HIS3* placed 6 kb away from the strand

invasion site increased the level of abnormal DSB repair outcomes in the H-On orientation, but not in the Co-D orientation (Extended Data Fig. 8c, Supplementary Table 1). Also, analysis of the mutation rate using a *ura3-29* reporter (Elango et al., 2018) under the control of *GAL1* at the same position demonstrated that the H-On orientation increased the rate of Ura^+ revertants by 11-fold compared to Co-D orientation, and the rate of Ura^+ revertants in the Co-D orientation was similar to that of *ura3-29* under control of the native *URA3* promoter in either orientation (Fig. 4a, h, Supplementary Table 2).

Responses to the Reviewer 2 comments.

We are grateful to the Reviewer 2 for stating that our manuscript "provides excellent insights into the DNA synthesis steps of BIR", using "clever and innovative" techniques that represent "an important addition to the field and provide an interesting paradigm to the repair synthesis steps in BIR and other homologous recombination events". We also thank Reviewer 2 for great suggestions to improve the manuscript. Below, we list our point-by-point responses to Reviewer 2's comments.

9. Reviewer 2 comment*: To perform ddPCR, I wonder if cells were arrested at G2 prior to HO induction to limit the effect of DNA replication on BIR synthesis measurement. However, I could not find anywhere about G2 arrest (except those in pri2-1) in the paper including the Method section. Does it mean that most experiments were performed in asynchronous cells? If so, the authors should explain how the effect of DNA replication within a 90-kb BIR track on the ddPCR outcomes were minimized. If most experiments were performed in G2-arrested cells, such information should be clearly described in the text and the procedures.*

Answer: In our system, DSBs are indeed initiated in asynchronous cells. However, by the time these cells initiate BIR synthesis, the checkpoint is activated and the culture is synchronized in G2/M arrest. When checkpoint machinery is functional, the cells remain arrested until BIR is completed (Malkova et al., 2005; Sakofsky and Malkova, 2017; Vasan et al., 2014). (Please see additional details in answer #4 to Reviewer 1 for details.) To address Reviewer 2's concern further, we repeated our analysis of BIR kinetics in cells that were arrested at G2/M by nocodazole prior to DSB induction and analyzed the progression of BIR over time using AMBER. We found and report in the revised manuscript (Extended Data Fig. 2b) that the kinetics of BIR in nocodazole-arrested cells was similar to the kinetics we observed in cells that were arrested by the checkpoint at G2/M after induction of the *HO* break in asynchronous cells.

In addition, the following new sentences were included in the revised text: "The truncation leaves homology only on the centromere-proximal side of the DSB, resulting in a very high efficiency of BIR using the full-length Chromosome III (donor) as the template for repair (Fig.1a). DSBs are induced by HO endonuclease in asynchronous cell populations, which leads to G2/M cell cycle arrest within 2-3 hours. BIR then proceeds in a synchronized culture of G2/M-arrested cells." (Malkova et al, 2005). Also: "The BIR rate was similar in cells that

were pre-arrested at G2/M by nocodazole prior to DSB induction (Extended Data Fig. 2a, b).". Also, we included more information on the HO induction and cell asynchrony or synchronization into the "Methods" section of the revised manuscript.

10. Reviewer 2 comment: *Monitoring the integrity of Ya fragment to analyze the strand invasion event sounds logical. However, it was shown that strand invasion is not immediately followed by 3' flap removal. Therefore, the authors' assertion that BIR synthesis initiates immediately after strand invasion might be inaccurate and should be further confirmed by the timing of Rad51 ChIP, a more accurate indicator of strand invasion at the donor-specific DNA sequence. One can possibly insert a unique sequence next to the donor molecule to generate donor-specific DNA.*

Answer: We thank the reviewer for this important suggestion, and we agree that monitoring the integrity of the *Ya* fragment allows only indirect assessment of strand invasion, even though it has been successfully used before (Wilson et al., 2013). As we previously discussed in our response # 3 to the Reviewer 1, for the revised manuscript, we followed strand invasion by two other methods: Rad51 ChIP (as also suggested by Reviewer 3) and using the DLC assay (suggested by Reviewer 1). Both methods indicated strand invasion initiates between ~1 and 1.5 hours after DSB induction. This is approximately 1 hour earlier than the beginning of BIR synthesis that we detected using AMBER, indicative of an approximately 1-hour delay between strand invasion and DNA synthesis, which is similar to the results obtained by Heyer's lab (Piazza et al., 2018; Piazza et al., 2019). This finding is also in line with the 40-minute delay previously observed for the initiation of DNA repair synthesis during MAT switching. Importantly, though, we did not observe a long (3-4 hour) delay between strand invasion and DNA synthesis that was previously reported (Jain et al., 2009; Jain et al., 2016; Malkova et al., 2005). Based on our findings, we hypothesize that the 3- to 4-hour delay likely included not only the time required to initiate leading strand BIR synthesis, but also the time for the nascent ssDNA to be copied to dsDNA (the DNA preparation by the "old" method could not isolate ssDNA, please refer to our Extended Data Fig. 1c and answer #21). In the revised manuscript, we have tried to clarify that the distinction we are making regarding the timing of initiation of BIR synthesis is between our data and the reported 3- to 4-hour delay reported by the Haber lab (Jain et al., 2009; Jain et al., 2016; Malkova et al., 2005).

Our response to this comment includes addition of data describing strand invasion kinetics analyzed by Rad51-CHIP and the DLC assay in Extended Data Fig. 1a,b, 3e, and 7c. In addition, we made the following change to the abstract, which now states:

"Here, we developed an assay to monitor repair-specific DNA synthesis and demonstrate that BIR synthesis initiates ~1 hour following strand invasion.". In addition, the following sentences have been included in the Results section: "BIR synthesis of the first 500 bp was detected by ddPCR as early as 2.5 hours post-DSB induction (Fig. 1b), about 1 hour after ChIP detection of Rad51 loading onto the donor Chromosome III, which was used as a readout of strand invasion (Extended Data Fig. 1a), or by D-loop capture (DLC)

assay(Piazza et al., 2019) (Extended Data Fig. 1b). The longer delay between strand invasion and BIR synthesis previously reported(Donnianni and Symington, 2013; Jain et al., 2009; Lydeard et al., 2007; Malkova et al., 2005) are likely due to different method of DNA preparation that does not preserve early DNA synthesis intermediates (Piazza et al., 2018; Piazza et al., 2019) (Extended Data Fig. 1c).

11. Reviewer 2 comment: *The effect of highly transcribed regions on BIR are interesting and worth analyzing further. Given the super-high expression the galactose promoter induces, one wonders what is the level of transcription that is sufficient to alter the BIR event. It can be determined by altering the level of galactose in the media and monitoring the level of HIS3 mRNA steady-state level and BIR. It could be that the transcription level and BIR are inversely correlated with each other or alternatively, a certain threshold might exist that is co-incident with the level of R-loops.*

Answer: We thank the reviewer for this great suggestion. Because both the transcription unit and the *HO* break in our system are initiated by galactose, we engineered a strain in which we replaced the *GAL1* promoter in P_{GAL1}-*HIS3* with $P_{TET(on)}$, which contains the *TET* promoter and activator to allow induction of *HIS3* transcription by addition of doxycycline. We inserted the P_{TET}-*HIS3* construct at the *MATα-inc* locus in the H-On orientation. Using this system, we were able to regulate the level of *HIS3* transcription by addition of different amounts of doxycycline, and transcription levels were determined by measuring mRNA using *HIS3*-specific primers. BIR synthesis was followed by AMBER. Similar to our results obtained with P_{GAL}*1-HIS3*, H-On transcription of P_{TET}-*HIS3* interfered with BIR progression. This represents an important demonstration that the interference between transcription and BIR is not specific to one particular promoter. Moreover, using this P_{TET}-*HIS3* construct allowed us to demonstrate that the strength of this interference directly correlated with transcription level. An additional and important observation was that, 10 hours after DSB induction, the level of *HIS3* mRNA decreased in all experiments irrespective of the amount of doxycycline that was originally added. This was observed only in samples where the DSB (and BIR) was induced, which suggested that, not only did transcription interfere with BIR, but BIR suppressed transcription as well. We observed a similar phenomenon with P_{GAL1}-*HIS3*, where expression of *HIS3* mRNA was much lower in the H-On versus the Co-D orientation 8 hours after DSB induction, even though ChIP of Rpb1 indicated that RNPII binding was similar between the two orientations. We interpret these interesting findings to suggest that BIR may induce a RNPII backtracking-like mechanism to inactivate transcription. Data from our P_{TET}-*HIS3* construct are presented in the revised manuscript in Fig. 4e, f, and the detailed protocol for these experiments is included in the Methods section. Also, we added the following statements into the text:

“The apparent collision between the migrating D-loop and transcription bubble is supported by much lower accumulation of *HIS3* mRNA in strains with H-On- versus Co-D-oriented *HIS3* 8 h after DSB induction (Fig. 4 d), while ChIP of the Rpb1 subunit of the rPolII complex confirmed similar rPolII binding in both orientations (see Extended Data Fig. 6a, b for primer

positions). Based on these results, we propose that immobilized rPolII on DNA, blocks the progression of both BIR and further transcription. To uncouple the timing of DSB induction and transcription, we inserted $P_{TET\ (on)}$-*HIS3* in H-On orientation (Fig. 4a), which also enabled us to regulate the level of transcription with doxycycline.

We observed a robust induction of $P_{TET(on)}$-*HIS3* transcription 1hour after addition of doxycycline (right before galactose addition), and the level of *HIS3* mRNA positively correlated with the amount of doxycycline added (Fig 4e). As anticipated, the level of BIR synthesis in these experiments analyzed at the 10-hour time after DSB induction, was inversely correlated with the amount of doxycycline (Fig. 4e). Notably, the level of *HIS3* mRNA induced by high doses of doxycycline (5µg/ml) decreased following BIR induction and was 25-fold lower in strains with a DSB compared to no-DSB controls at the 10-hour time point (Fig. 4f). Taken together, we propose that initiation of BIR and H-On transcription reciprocally block one another."

12. Reviewer 2 comment: *"I am concerned that the ~20 kb of leading-only DNA synthesis in the pri2-1 mutant could be due to the hypomorphic nature of the mutation, instead of the intrinsic ability of cells to tolerate such a long ssDNA or a unique replication fork lacking lagging-strand synthesis up to 20 kb. One way to resolve this issue is to generate a pri2 degron and determine if the AMBER pattern is still similar to pri2-1 at the non-permissive condition".*

Answer: In response to this concern from the Reviewer, we tried to construct *PRI2*-degron, but IAA-induced degradation of Pri2 was not very efficient in our system. Therefore, we constructed a degron for another subunit of the Polα-primase complex, *POL1*. Western blot analysis confirmed a high efficiency of degron-mediated degradation of Pol1. Therefore, we analyzed BIR kinetics using the AMBER assay in *POL1*-degron strains, which uncovered highly similar kinetics of BIR synthesis compared to our experiments with a *pri2-1* mutant at non-permissive (37°C) conditions. In both POLα-primase mutants, our data convincingly demonstrate that cells can only tolerate up to about ~20-30 kb of uncoupled leading strand DNA synthesis within the BIR migrating bubble (Please see response #27 to Reviewer 3 for further details). In the revised manuscript, our new analysis of BIR progression following inactivation of Pol1 is presented in Extended Data Fig. 4b. In addition, the following sentences have been added to the text: "Similar results were obtained when another subunit of alpha-primase complex, Pol1, was inactivated using an AID-degron (Extended Data Fig. 4b)."

13. Reviewer 2 comment: *"Given that the telomere repeat sequence can form G4 DNA, is the effect of ITS on BIR and BIR synthesis specific to the telomere sequence or can it occur with other G4 DNA as well.*

Answer: To address this suggestion from the Reviewer, we inserted a G4-forming sequence, which was previously demonstrated to promote genetic instabilities due to

formation of G4-structures (Kim et al., 2011; Kim and Jinks-Robertson, 2011; Lippert et al., 2011), 6 kb centromere distal to *MATα-inc* in the donor chromosome in both orientations. Our data demonstrated that BIR synthesis (assessed by AMBER) was not affected by this G4 sequence. Moreover, the G4 sequence did not block BIR progression even in the presence of Phen-DC3, a chemical known to stabilize G4 structures (Ribeyre et al., 2009). These results make it unlikely that interruption of BIR by ITS is caused by formation of G4 structures, even though we cannot exclude mild effects that were not detected by AMBER. It is more likely that interruption of BIR by ITSs results from the binding of a protein to ITSs (likely by yeast Tbf1, which is known to bind human telomere repeats), which leads to formation of DNA-protein complexes that interrupt BIR progression. The new experimental data on BIR progression through G4-forming sequences are included in Extended Data Fig.5 b-e of the revised manuscript, and the following sentences were added to the text: "The potency with which ITS hinder BIR progression is unlikely due to forming G4 structure because BIR easily progressed through another, non-ITS, G4-forming sequence(Kim and Jinks-Robertson, 2011) inserted in either orientation at the same location, even in the presence of the G4-stabilizing agent, Phen-DC3(Piazza et al., 2010) (Extended Data Fig. 5b-e). In addition, deletion of *RRM3*, which can unwind G4 structures(Geronimo and Zakian, 2016), did not exacerbate the difficulty for BIR to traverse either $(ITS)_{40}$ or $(ITS)_{28}$ (see below) (Extended Data Fig. 5h- j)."

14. Reviewer 2 comment: *"Is there any size threshold of ITS to block BIR synthesis?"*

Answer: To answer this question, we constructed a series of strains containing ITS repeats of varying lengths. Specifically, we created a CRISPR tool targeting the position located 6 kb centromere distal to *MATα-inc* on the donor Chromosome III and a repair template consisting of a 200-bp oligo containing 20 repeats of (TTAGGG) human telomere sequence flanked by 40-bp homology sequences on both sides to target the 6-kb position of chromosome III. We analyzed transformants using Sanger sequencing and identified strains containing 28, 20, 14 and 2 repeats of the TTAGGG sequence. We investigated the effect of each of these insertions by AMBER, and none of them blocked BIR synthesis, suggesting that the full length of 40 repeats was required to block BIR. We propose that this difference between the effect of 40 and ≤ 28 repeats is due to a size threshold for the repeat that is likely explained by stable binding of protein(s) to human telomere sequence in yeast that interrupts BIR progression. In fact, the experiments performed by David Shore's lab demonstrated that binding of the essential for cell viability Tbf1 protein to the sequence containing 40-45 human telomere repeats in yeast leads to the formation of "capped" structures that were not formed when the number of repeats was significantly lower (Ribaud et al., 2012).

Even though AMBER did not detect BIR interruption by 28 telomere repeats, we decided to further analyze BIR repair outcomes in strains that traversed 28 telomere repeats by CHEF gel electrophoresis and Sanger sequencing. We observed that BIR was able to traverse these repeats and proceed to the end of the chromosome in the majority (29/30) of cases,

but ~15% (11/71) of the BIR outcomes were associated with instabilities (changes of ITS size) indicative of transient stalling of BIR at the short ITS_{28} locus, followed by re-invasion or template switching. When the same experiment was performed without DSB induction, no alterations in ITS copy number were observed (0/42 outcomes), thus suggesting that the frequency of ITS changes occur much more often in association with BIR compared to S-phase replication. Based on these results, we propose that shorter telomere repeats also interfere with BIR, but less strongly than longer repeats. These differential effects of $(ITS)_{28}$ versus $(ITS)_{40}$ could be explained by either milder effects resulting from the same mechanism (*e.g.*, less stable binding of the protein), or they may result from distinct mechanisms, when BIR encounters these different structures, for example from template switching. Data describing BIR progression through ITS repeats of varying lengths are included in the revised manuscript in Extended Data Fig.5f-g. In addition, the following statement has been added to the text:

"Meanwhile, we observed that human telomeric repeat (≥ 40) interfered with BIR progression, while a shorter ITS (≤28 repeats) did not interfere with BIR (Extended Data Fig. 5f), suggesting that the threshold for the number of telomeric repeats that can interrupt BIR is similar to the one that is required to bind Tbf1 for the formation of capped telomeres(Ribaud et al., 2012). However, Sanger sequencing of BIR outcomes in strains containing shorter ITS determined that there was frequently a reduced number of telomeric repeats (15% (11 out of 71) in BIR events versus 0 out of 41 from no-DSB control) (Extended Data Fig. 5g), indicative of transient BIR stalling even at shorter ITS that was likely followed by template switching altering the length of ITSs. "

15. Reviewer 2 comment: *"Is there any effect on RRM3 deletion in BIR across ITS?"*

Answer: We deleted *RRM3* in our strain containing ITS, and this did not change the BIR pattern based on either genetic or AMBER analyses. However, because we have determined that only a very small amount of BIR can traverse ITS, it is possible that it would be difficult to detect any further defect. Importantly though, when we repeated the same experiment in the strain containing only 28 telomeric repeats instead of 40, which does not cause such a strong interruption of BIR (see the answer to question #14), we did not observe any effect of *rrm3Δ* on BIR progression through $(ITS)_{28}$. Thus, Rrm3 plays no apparent role in BIR progression through ITSs. Data describing the effect of *rrm3Δ* on BIR progression through ITSs were added to Extended data Fig 5h-j in the revised manuscript, and the following sentences were added to the text: "In addition, deletion of *RRM3*, which can unwind G4 structures(Geronimo and Zakian, 2016), did not exacerbate the difficulty for BIR to traverse either $(ITS)_{40}$ or $(ITS)_{28}$ (see below) (Extended Data Fig. 5h- j)".

16. Reviewer 2 comment: *I am puzzled why the AMBER could not detect the apparent difference in BIR frequency at ITS in TLC1 and a tlc1 mutant. Should we interpret that BIR beyond ITS takes place in tlc1 after 10 h post-HO expression? If so, can the authors examine BIR synthesis by AMBER at later time points to match them with BIR frequency?*

Answer: Yes, we do believe that $(ITS)_{40}$ interrupts BIR progression and does not allow BIR to pass through for at least several hours. In the original experiments, we only took time points up to 10 hours post-DSB induction, because cells that have finished BIR start dividing and can "overtake" the culture after that time point. To assess whether BIR might progress through ITSs after this time, we added nocodazole to the culture 6 hours post-DSB induction to prolong the G2/M cell cycle arrest beyond the endogenous DNA damage checkpoint arrest, and this enabled us to take additional time points through 16 hours. AMBER analysis of these experiments detected a small, but significant elevation of BIR product past ITS at 16 hours post DSB in *tlc1Δ* mutant compared to *TLC1* strains, which suggested that the blockage of BIR at ITS persisted for a long time in both strains, but eventually BIR gets through in *tlc1Δ*. If one combines the results that we obtained by analyzing BIR progression through G4-forming sequences and through shorter ITSs (see our responses #13 and #14), we propose that the observed blockage is not caused by secondary DNA structures. Instead, we envision that it results from stable binding of a protein(s) (*e.g.*, Tbf1) to the long telomere repeat sequence. It is possible that there is a minimum threshold for ITS length that determines whether a stable complex forms to block BIR progression or to prevent the newly synthesized recipient end from further progression. It has been demonstrated by David Shore's lab that the number of (TTAGGG) repeats affects the biological properties due to binding to the repeats (Ribaud et al., 2012). Specifically, binding of Tbf1 to a sequence of 40-45 repeats led to formation of "capped" telomeres, while binding to sequences that were significantly shorter did not. Our observation of a threshold number of repeats required for the block of BIR is consistent with this model. Data characterizing BIR progression at later time points are included in Extended Data Fig. 5a of the revised manuscript, and the following sentences were added in the text: "Consistently, a higher copy number increase was observed beyond the ITS in *tlc1Δ* compared to *TLC1* strains 16 hours after DSB induction (Extended Data Fig. 5a):

17. Reviewer 2 comment: *"Given that BIR is not efficient at highly transcribed regions, one can deduce that BIR is not efficient in restarting stalled or collapsed forks at highly transcribed regions or at R-loops. The authors need to clarify these points in the paper".*

Answer: We thank the reviewer for this comment. In fact, we believe that this point constitutes one of the most important messages of our paper that we would like to convey to the community. Indeed, BIR has been implicated by multiple studies as the mechanism that recovers replication forks collapsed at highly transcribed regions (Beck et al., 2019; Costantino and Koshland, 2018; Hu et al., 2019; Li and Wu, 2020; Macheret et al., 2020; Macheret and Halazonetis, 2015; Minocherhomji et al., 2015; Sakofsky and Malkova, 2017; Wu, 2019). Yet, our data demonstrates that the outcome of such recovery by BIR totally depends on its structural characteristics. Among these, the most important characteristic is the distance between the respective locations of the transcription block and of the position of BIR initiation. Here, we demonstrate that, when BIR is initiated too close to a transcription block, it can lead to a "stuck" phenotype. On the other hand, when BIR is initiated at a distance from the high-transcription site, then our results suggest that it can pass through far more efficiently, even though this event still promotes a high frequency of genetic

rearrangements and mutations. Based on these observations we believe it is possible that resection of the nascent DNA following collision of an S phase replication fork with transcription machinery (and possibly fork reversal followed by DNA cleavage) may play an important role in allowing BIR to recover replication forks collapsed at common fragile sites. In addition, our observation that ongoing BIR synthesis across highly transcribed regions in the H-On orientation promotes GCRs and mutations at levels that are orders of magnitude higher than those observed for S-phase replication, suggests that many of the genetic instabilities associated with replication traversing highly transcribed areas may in fact result from BIR-mediated fork recovery.

In response to this suggestion from Reviewer #2, the following sentences were added to the text: “In summary BIR is slower and more susceptible to mutations and instability at roadblocks when compared to replication. Thus, the purported role for BIR in the recovery of collapsed replication forks at fragile sites (Costantino and Koshland, 2018; Hu et al., 2019; Li and Wu, 2020; Sakofsky and Malkova, 2017) would seem paradoxical. We propose that initiation of BIR can be altered by extensive DNA resection, 3’ end degradation or fork reversal to initiate BIR away from the highly transcribed site or other impediment. Once initiated, BIR can traverse transcribed regions, although traversing transcription units in H-On orientation promotes mutations and chromosomal rearrangements that are at least an order of magnitude greater than those associated with S-phase replication synthesizing through the same regions. Instability of the D-loop traversing transcription units and other barriers may also stimulate common for BIR template switches. Thus BIR-mediated recovery of S-phase replication forks that collapse following H-On collisions with transcription units could contribute substantially to the genetic instabilities associated with fragile sites. “

[Unpublished Data Redacted]

19. Reviewer 2 comment: *In Fig. 3f, why are there two peaks at telomeric regions showing two copies of DNA?*

Answer: The presence of two copies of DNA (two peaks) at pre-telomeric regions results from a mapping problem. Both of these peaks correspond to regions that are present in multiple copies throughout the genome. The first one (~300 kb position on Chromosome III) represents *TEF1* promoter and *TEF1* terminator sequences that are flanking *HPHMX* cassette inserted at this position as well as at two other positions in the genome (flanking *KANMX* located 6 kb position centromere distal to *MATα-inc* and at *NATMX* present ~30 kb centromere proximal to *MATa* in the recipient chromosome). Another pre-telomeric peak corresponds to the region between *YCR102C* and *PAU3 (YCR104W).* This sequence is present in multiple copies in the yeast genome, and, therefore, we believe that this peak results from a mapping error. To address this comment, the following sentence has been included in the legend to Fig 3f: “2x-coverage peaks near 300kb position result from the presence of additional copies of the corresponding sequences in the genome.”

Responses to the Reviewer 3 comments.

We are grateful to Reviewer 3 for noting that our method is “novel”, and that “this work provides important new insights into the mechanisms of BIR and how BIR may contribute to genome instability”. We also thank Reviewer 3 for great suggestions to improve the manuscript, which we address below.

20. Reviewer 3 comment: *“The title should be reworded, by using ‘details’ the authors undersell their work:.*

Answer: We thank the Reviewer for this suggestion. In the new title the word “details” is replaced with “insights”.

21. Reviewer 3 comment: *“The conclusion that DNA synthesis starts immediately after DNA strand invasion directly contradicts earlier work published by the Haber laboratory (refs. 21, 22). The authors should discuss this discrepancy, probably best in the Extended Data section”.*

Answer: We believe the differences between ours and previous researchers are due to the difference in DNA preparation methods. To address this comment from the Reviewer, we re-analyzed the same batch of samples that were collected from the BIR kinetics time course presented in the original Fig.1b using DNA extraction methods that were previously used by the Haber laboratory (Jain et al., 2009; Jain et al., 2016; Malkova et al., 2005). These DNA samples were analyzed by ddPCR using the same primer sets that were employed for the AMBER analysis presented in Fig. 1b. We observed that, when the original DNA purification methods were used, the beginning of DNA synthesis was significantly delayed by approximately 3 to 4 hours compared to the time of strand invasion, which was similar to the time reported by Haber’s lab. Of note, with this original DNA preparation method, the copy number increase indicative of BIR was observed at the same time for all primer positions along the donor chromosome which is similar to previously reported findings (Donnianni and Symington, 2013; Jain et al., 2009; Jain et al., 2016; Malkova et al., 2005). This result is consistent with the idea that ssDNA synthesized by leading strand DNA synthesis cannot be detected by the original DNA preparation method until it becomes double stranded. Based on these results, we propose that the 3- to 4-hour delay between strand invasion and the beginning of BIR synthesis likely includes not only the time required to initiate leading strand BIR synthesis, but also the time needed to make the newly synthesized DNA double stranded. We addressed this point in the revised manuscript by including new data in Extended Data Fig.1c (see also Fig.2 in this response for the results of additional independent experiments), and by including the following statements in the text: “The longer

delay between strand invasion and BIR synthesis previously reported (Donnianni and Symington, 2013; Jain et al., 2009; Lydeard et al., 2007; Malkova et al., 2005) are likely due to different method of DNA preparation that does not preserve early DNA synthesis intermediates (Piazza et al., 2018; Piazza et al., 2019) (Extended Data Fig. 1c). “

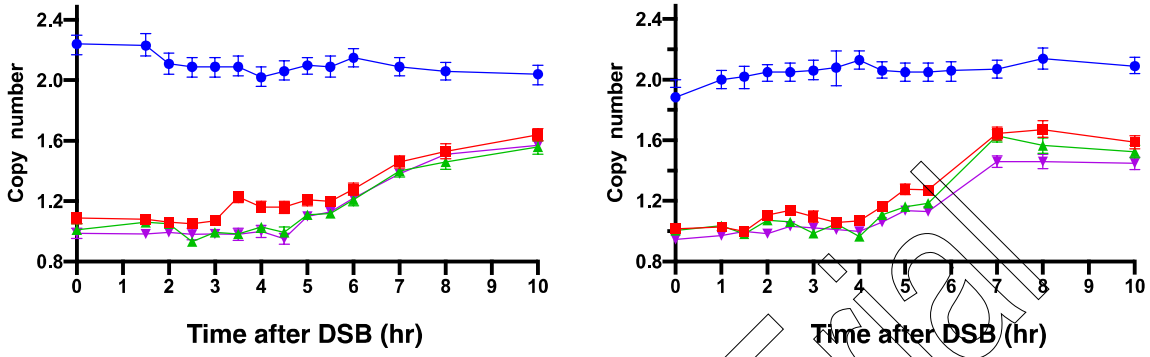

Figure 2. DNA synthesis detected by ddPCR in DNA samples purified by traditional (old) DNA preparation protocol. These panels present the results of two additional independent repeats of the experiment similar to presented in Extended Data Fig. 1c.

22. Reviewer 3 comment: *“In Figs 1, 3 and 4, the experiments are conducted in cycling cells, so that HO cleavage can occur in G1 and post-S cells, with likely both sister chromatids being cleaved. This aspect should be discussed. Does BIR happen in G1? Is it different from post-S BIR?*

Answer: As we discussed in our response to Reviewer 1 (see answer #4), in our system, DSBs are indeed initiated in asynchronous cells. However, by the time these cells initiate BIR synthesis, the culture is synchronized in G2/M arrest. In fact, in practically all nuclease-induced yeast BIR experimental systems described to date, BIR synthesis occurs in G2. To further address this comment from the Reviewer, we studied the *cdc7-as* derivative of our BIR strain in the presence of the kinase inhibitor NMPP1 (Ira et al., 2004), which arrested cells at G1, followed by induction of the *HO* break by addition of galactose to the medium. FACS analysis confirmed a uniform G1 arrest before galactose addition, but cells were unable to remain arrested for more than 3 hours after the break, and the majority of cells completed BIR in G2. Interestingly, a similar experiment performed in Gregory Ira’s lab using *cdc28-as ku70Δ* in the presence of kinase inhibitor showed no BIR product accumulated in G1 arrested cells. In sum, based on the information accumulated to date, BIR appears to be an event that occurs primarily after S phase, during G2 or even M stage of the cell cycle. Thus, there is currently no evidence that BIR occurs during G1, and similarly, there is no documented case of BIR completing within S-phase. Therefore, our paper is focused on BIR that occurs during G2.

23. Reviewer 3 comment: *"Fig. 1: In a, the black triangle needs to be defined".*

Answer: The black triangle in Figure 1a indicated the location of primers used for ddPCR to detect the time of *Ya* removal. In the revised manuscript, we have removed this from the main figures because we instead present Rad51 ChIP and DLC assays as more direct measurements of *Ya* cleavage to follow the kinetics of strand invasion (as suggested by all three reviewers). Kinetics of *Ya* removal are still shown in Extended Data Fig. 1d, but only for comparison of DNA strand degradation in *rad52Δ* versus *RAD52* strains.

24. Reviewer 3 comment: *"In Fig. 1c, why is there a copy number decrease at -150 kb in the rad52 strain?"*

Answer: We are glad the Reviewer mentioned this. Indeed, we observed a significant copy number decrease at the -150-kb position in *rad52Δ* cells at the 8-hour time point. We hypothesized that this may be the result of hyper-resection that was recently reported in *rad52Δ* by (Yan et al., 2019). To test this further, we analyzed the copy number at -50 kb and at -65 kb, which we predicted should degrade more quickly than -150 kb, and this is precisely what we observed. This finding is scientifically meaningful, and we believe this result also illustrates that, in addition to following DNA synthesis, AMBER can be used to measure DNA degradation.

Overall, in response to this comment we added the new data into the Extended Data Fig. 1d. and we state in the legend that: "In *rad52Δ* the rate of DNA degradation was >8kb/hr, much higher than in wt and *rad51Δ*."

25. Reviewer 3 comment: *"In Fig. 1e, the authors show a difference between the phenotype of pol32 and pif1, which begs the analysis of the double mutant".*

Answer: We thank the reviewer for this good suggestion. To address this question, we constructed a *pol32Δ pif1Δ* double mutant. Analysis of BIR in this strain by AMBER demonstrated no BIR synthesis even at the position located 0.2kb centromere distal to the HO break site. This result suggests that Pif1 can promote at least some bubble migration in the absence of Pol32 and vice versa, which is an exciting and new result that could not be obtained before without our AMBER method.

In response to the reviewer's comment, we added the new data into Fig. 1e. Also, we added the following sentence into the text: "Interestingly, in *pol32Δ pif1Δ* double mutants, no synthesis occurred, even at the 200bp position (Fig. 1e), suggesting that Pol32 can promote at least some bubble migration in the absence of Pif1 and *vice versa.*".

26. Reviewer 3 comment: *Is the removal of the Ya sequence affected in pol32 and pif1? This is another landmark that can be established.*

Answer: In response to this comment from the Reviewer, we investigated *Ya* removal kinetics in *pif1Δ, pol32Δ,* and *pif1Δ pol32Δ* mutants, and we compared these to our results in wild type cells (see Figure 3 in this response document). We observed similar kinetics of *Ya* removal in all three mutant backgrounds and in wild type. In addition, based on the suggestion of Reviewer 1, we assessed the kinetics of strand invasion in *pol32Δ* and *pif1Δ* mutants using the DLC assay, which confirmed successful strand invasion in wt, *pol32Δ,* and *pif1Δ* mutants. Since we believe (and based on reviewer's suggestions) that DLC is a better method to study strand invasion, we decided NOT to include these new data on Ya removal in this manuscript, but they are presented in the figure of this response to the reviewers.

In addition, the following sentences were added to the text: "Additionally, neither of these mutations affected the efficiency of strand invasion (Extended Data Fig. 3e)."

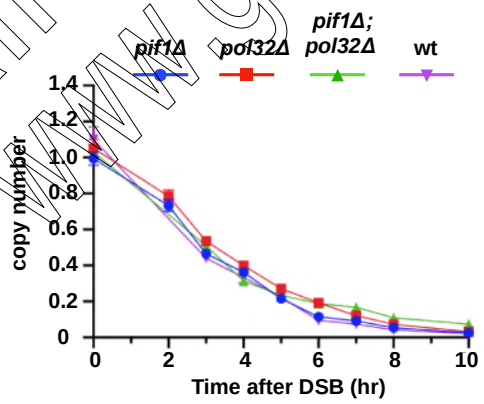

Figure 3. Kinetics of *Ya* clipping off in wt, *pif1Δ*, *pol32Δ* and *pif1Δpol32Δ* strains measured by AMBER.

27. Reviewer 3 comment: *"Fig. 2: The authors deduce a distance of ~20 kb between the leading and lagging strand but the error in this number appears to be very large (in my estimate ± 10 kb). Please calculate and error for the value and provide it in the text.*

Answer: We thank the Reviewer for this suggestion. In response, we designed two additional primer sets to evaluate BIR kinetics in the *pri2-1* mutant at 15- and 25-kb positions using AMBER analysis. We observed BIR synthesis at 15 kb, and a very small amount of synthesis at 20 kb and at 25 kb. Because we observed no synthesis at or beyond 30 kb in this genetic background, we believe that our estimation of maximum distance between leading and lagging strands falls between 25 and 30 kb, and our new data support this conclusion with higher resolution. We added these new data to the revised manuscript in Fig. 2b,c. Also, the following changes were made to the abstract: "Leading strand synthesis is initiated efficiently in primase-defective mutants strains, but fails to proceed beyond 30kb. We also made the following changes in the text: "Also, while the full-length BIR product was detected in wild type cells (1.43x at 90 kb position), the longest product detected in the *pri2-1* mutant cultures was 25 kb (~1.2x), and no DNA amplification was detectable at the 30 kb position (indistinguishable from 1.1x increase) (Fig. 2b, c).

28. Reviewer 3 comment: *"Fig.2 In b, consider using the same scale for both graphs".*

Answer: We do not think that using the same scale is practical, due to the smaller amount of synthesis observed in *pri2-1*. It would be very difficult to see the level of synthesis in *pri2-1* on the scale required to demonstrate wild type data. Notably, though, our bar graph in Fig. 2c shows the same data for *pri2-1* and wild type cells side-by-side on the same scale.

29. Reviewer 3 comment: *"Fig.2 d, the copy number in wild type starts higher than in the pri2-1 mutant. Is the difference significance and what is the explanation?"*

Answer: No, the difference in copy number between wild type and *pri2-1* at the beginning of synthesis is not significant. This was consistent in two additional biological repeats of this experiment. To avoid confusion, Fig. 2d in the revised manuscript presents one of these experiments.

30. Reviewer 3 comment: *"Figs 3 and 4: The stalling of the BIR DNA synthesis is inferred and should be directly demonstrated by 2D gel analysis".*

Answer: We would like to clarify that we use the word "stalling" to describe interruption of BIR at ITSs or at high-transcription units only in a very general sense, and we understand

that it could be misleading. What we observe by AMBER is that practically no BIR synthesis continues beyond ITS or the high-transcription unit when it is inserted near the site of strand invasion. In fact, we believe that the actual mechanisms and structures that emerge at these two replication obstacles differ from each other. It is likely that ITSs lead to interruption of BIR followed by immediate dissociation of the newly synthesized strand (and therefore no structure would be detected by 2D). In the case of a high-transcription unit, we indeed believe that a "stuck" structure is formed. However, this stuck structure is likely formed before BIR synthesis is even initiated, based on our data demonstrating that ongoing BIR synthesis is only minimally impacted by transcription. Our new data suggest the "stuck" phenotype may result from immobilization of rPolII, which blocks both BIR initiation and later transcription. Therefore, it is very likely no structures representing "stalled BIR synthesis" would be expected by 2D gel analysis. Also, the structures of stalled BIR synthesis have never been characterized by 2D; thus, it is hard to imagine what kinds of structures are expected, even if they were formed. With regard to intermediates of strand invasion formed before initiation of BIR synthesis, these structures have also never characterized by 2D gel analysis, and whether the structures of "frozen" strand invasion could be differentiated by 2D gel is unknown. In these situations, where there is no specific prediction for the structure(s) that would be formed or accumulated by BIR at ITSs or at a high-transcription unit, we believe that using AMBER is the most appropriate and direct way to document BIR interruption for now. In fact, a similar approach (deep sequencing) has been recently used by Houra Merrikh's group to document the "stuck phenotype" resulting from collision of DNA replication with R-loops in bacteria (Lang et al., 2017). However, we would like to thank the reviewer for the idea, and we will consider using 2D in the future to investigate BIR stalling at various obstacles, but we believe that this is beyond the scope of this manuscript.

31. Reviewer 3 comment: *"Fig. 4 and line 169 & throughout: HO is a confusing abbreviation for the head-on collision in this text because the authors use the HO endonuclease to induce DSBs".*

Answer: We agree. In response to this suggestion, we changed abbreviations for head-on (now called H-On) and for Co-directional (now called Co-D).

32. Reviewer 3 comment: *"In Fig.4a, why italics for leu- and ade-? They are phenotypes not genotypes".*

Answer: We agree. In response to this suggestion, we changed abbreviations for phenotypes.

Now it reads as: Ade^- and Leu^-.

33. Reviewer 3 comment: *"In Fig. 4 b, c, e and f, please provide in the figure the information, which pGAL construct and orientation is shown that the reader does not need to refer to the legend".*

Answer: We thank for this suggestion. We added the information to this figure, and also included similar information into all similar figures.

34. Reviewer 3 comment: *Line 206: Fig. 4a only shows ura3-29 under control of the GAL1 promoter, not the native URA3 promoter.*

Answer: We thank for this suggestion. We added *URA3* under its native promoter in the schematics in the Fig. 4a.

35. Reviewer 3 comment:" In Fig. 4h, the no-break control with the head-on construct shows elevated mutagenesis. Is this consistent with the known replication direction in this region? In lines 209-211, it is unclear, how the no-break control can relate to BIR."

Answer: We thank the reviewer for noticing this! Indeed, the P_{GAL1}*::ura3-29* construct is located ~6 kb centromere distal from *MATα-inc*, and there are two known highly active S phase replication origins located at a reasonable distance from this position. On the left (centromere proximal), ARS310 is located ~40 kb from P_{GAL}*1::ura3-29*. On the right (centromere distal), ARS315 is located ~18 kb from P_{GAL1}*::ura3-29*, and this is the most active origin in that region. Based on the distance, it is more likely that P_{GAL1}*::ura3-29* is replicated from ARS315, which means that the encounter between S phase replication and transcription of P_{GAL1}*::ura3-29* would occur in the opposite orientation from BIR. This might explain why the level of mutagenesis for the no-cut controls is higher for the Co-D-oriented reporter for BIR: because it was H-On with respect to S-phase replication. However, because we cannot be sure that S phase replication will always proceed through P_{GAL1}*::ura3-2 9* in the same direction, we chose not to indicate orientations for no-cut controls in Fig. 4h.

36. Reviewer 3 comment: "*Extended Data Fig. 1: Why is there a decrease in the Ya copy number in rad51? More detail on the assay for Ya cleavage could be provided. What is the placement of the primers?"*

Answer. We agree with the reviewer that some decrease in *Ya* copy number is observed in *rad51Δ.* Importantly, though, the kinetics of this decrease is very different from what we observed in the presence of Rad51. In wild type, *Ya* decreases due to strand invasion, and the change is much faster and more robust. Similar decreases in *Ya* copy number in *rad51Δ* with similar kinetics have been observed in previous studies (Wilson et al., 2013) and this is likely due to slow degradation of some *Ya* sequence in the absence of Rad51. In the revised manuscript, the details of our assay are provided in the Methods section, and primer sequences and placement are provided in Supplementary Table 4. Also, based on suggestions from all Reviewers, we have now used two additional, more precise methods to estimate the time of strand invasion: Rad51 ChIP and the DLC assay. Results of these new analyses are included in the revised manuscript in Extended Data Fig. 1a,b, 3e, and 7c.

Please also see also our responses #3, #21 and #38 to Reviewers 1, 2 and 3 for additional details.

[Unpublished Data Redacted]

38. Reviewer 3 comment: *"Line 175: Relative to the data in Extended Data Fig. 1b, it appears that Ya disappearance (i.e. cleavage) in Extended Data Fig. 5b is affected by ongoing transcription. Authors claim that ongoing transcription only affects the synthesis step of BIR, but it appears to also interfere with stable D-loop formation and flap cleavage.*"

Answer: We thank the Reviewer for this insightful comment. As we have mentioned, we agree with all three Reviewers that the removal of *Ya* might not reflect the strand invasion step *per se;* rather, it may represent the next step in BIR that occurs before the initiation of synthesis. The revised manuscript now presents Rad51 ChIP analysis to assess strand invasion in the presence of high-level transcription, which demonstrated no difference in strand invasion efficiency between strains with and without the induction high-level transcription. This new data is included in Extended Data Fig. 7c in our new manuscript. And the following sentences are added in our new manuscript: "AMBER analysis of BIR-specific DNA synthesis detected no increase in copy number within or beyond the H-On-orientated P_{GAL1}-*HIS3* sequence through the entire time course (10 hours post-DSB induction (Fig. 4b; Extended Data Fig. 7a,b), even though efficient strand invasion was confirmed (Extended Data Fig. 7c)."

Additionally, our data demonstrate that BIR is "blocked" only when strand invasion occurs very close to a high-transcription unit, and only in the H-On orientation, but not in situations where established BIR synthesis encountered the high-transcription unit. This makes it likely that formation of the "block" intermediates occurs after strand invasion, but before the beginning of BIR synthesis, which suggests that it at least partially coincides with the step of *Ya* removal. It will be interesting to further evaluate whether persistence of *Ya* could serve as a marker of the BIR "block" phenomenon.

39. Reviewer 3 comment: "Extended Data Fig. 5d,e: It would be helpful if the authors can label these plots".

Answer: We thank for this good suggestion. We labeled most of the plots in our new manuscript.

40. Reviewer 3 comment: *"Lines 72-74: Flap cleavage is presented here and throughout as the time of the invasion/D-loop formation, but the flap likely triggers multiple rounds of invasion/D-loop formation followed by disruption. Moreover, I am not sure that we know that flap cleavage itself does not trigger D-loop disruption.*

Answer: We thank the Reviewer for this suggestion, and we agree that flap removal alone cannot be used as a direct measure of strand invasion. In the revised manuscript, we now use two direct methods: Rad51 ChIP and the DLC assay. Please see answers #3, #21, and #38 for further details.

41. Reviewer 3 comment: *"Lines 219-221: The sentence "it is possible that BIR contributes to the genetic instabilities reported in relation to fragile sites." may have to be qualified as not fragile sites derive from DNA synthesis/transcription conflicts".*

Answer: If we understand the question correctly, the Reviewer is wondering whether our data suggest that the problems at fragile sites may result not from conflicts between S phase replication and transcription, but from problems between BIR and transcription. We believe that, in general, the accepted idea that conflicts between S phase replication and transcription lead to genome instability is correct. However, we propose that the pathway leading to this instability may include two steps: first, the interruption of S phase replication due to its conflict with transcription, and, second, the recovery of the interrupted S phase replication fork via BIR. Due to our demonstrated effect of high transcription on BIR, this second step may be responsible for many of the genetic outcomes that are associated with S phase replication and transcription. This point is clarified in the revised manuscript.

Minor issues:

42. Reviewer 3 comment: *"Lines 93, 195: "wt" or "wt" is used as an abbreviation for wild type without previously defining. Italics/non-italics should be applied consistently to this term".*

Answer: In response to the reviewer's comments all "wt" abbreviations were defined throughout the text and non-italics was used consistently.

43. Reviewer 3 comment: *"Line 103: "longer-range"; "long-range" instead?*

Answer: corrected as suggested by the reviewer.

44. Reviewer 3 comment: *"Line 104: "almost no increase of synthesis" -- "almost no increase in synthesis" instead?"*

Answer: corrected as suggested by the reviewer.

45. Reviewer 3 comment: *"Line 116: "products by CHEF measuring the" -- "products by CHEF gel electrophoresis measuring the" instead?*

Answer: corrected as suggested by the reviewer.

46. Reviewer 3 comment: *"Lines 122, 123: May need to insert a sentence in between these lines to explain the experimental rationale".*

Answer: In response to this comment the following sentence has been inserted:
"We hypothesized that new DNA detected in *pri2-1* mutant was single-stranded DNA accumulated during leading strand DNA synthesis."

47. Reviewer 3 comment:*"Line 147, 154: "CHEF analysis" -- "CHEF electrophoresis analysis" instead?"*

Answer: corrected as suggested by the reviewer.

48. Reviewer 3 comment: *"Line 228: References 15 and 34 may not strongly support the claim that BIR is involved in recovery of collapsed replication forks at fragile site*

Answer: In response to this comment we included several new references proposing that BIR is involved in recovery of collapsed replication forks at fragile sites. We also decided to leave the references that were used there originally since they also propose this idea. .

49. Reviewer 3 comment: *"Line 311: The title of reference 30 is missing.*

Answer: corrected.

50. Reviewer 3 comment: *"Line 472: The authors do not indicate the yeast strain background (i.e. W303, CEN-PK, S288c, etc.).*

Answer: The strains are derivatives of the JKM background that is used in the laboratory of Dr. James E. Haber (Brandeis University). However, JKM does not represent any classic yeast genetic background. In our table of strains (Supplementary Table 3), we provide references to the papers where the precursors of our strains are characterized.

51. Reviewer 3 comment: *"Lines 527 and 528: Typo "zymolase" should be "zymolyase".*

Answer: corrected as suggested by the reviewer.

References:

Beck, C.R., Carvalho, C.M.B., Akdemir, Z.C., Sedlazeck, F.J., Song, X., Meng, Q., Hu, J., Doddapaneni, H., Chong, Z., Chen, E.S., *et al.* (2019). Megabase Length Hypermutation Accompanies Human Structural Variation at 17p11.2. Cell *176*, 1310-1324 e1310.
Bhowmick, R., and Hickson, I.D. (2017). The "enemies within": regions of the genome that are inherently difficult to replicate. F1000Res *6*, 666.
Bhowmick, R., Minocherhomji, S., and Hickson, I.D. (2016). *RAD52* Facilitates Mitotic DNA Synthesis Following Replication Stress. Mol Cell *64*, 1117-1126.
Costantino, L., and Koshland, D. (2018). Genome-wide Map of R-Loop-Induced Damage Reveals How a Subset of R-Loops Contributes to Genomic Instability. Mol Cell *71*, 487-497 e483.
Deem, A., Barker, K., VanHulle, K., Downing, B., Vayl, A., and Malkova, A. (2008). Defective break-induced replication leads to half-crossovers in *Saccharomyces cerevisiae*. Genetics *179*, 1845-1860.
Dilley, R.L., and Greenberg, R.A. (2015). ALTernative Telomere Maintenance and Cancer. Trends Cancer *1*, 145-156.
Donnianni, R.A., and Symington, L.S. (2013). Break-induced replication occurs by conservative DNA synthesis. Proc Natl Acad Sci U S A *110*, 13475-13480.
Donnianni, R.A., Zhou, Z.-X., Lujan, S.A., Al-Zain, A., Garcia, V., Glancy, E., Burkholder, A.B., Kunkel, T.A., and Symington, L.S. (2019). DNA polymerase delta synthesizes both strands during break-induced replication. Molecular cell *76*, 371-381. e374.
Elango, R., Kockler, Z., Liu, L., and Malkova, A. (2018). Investigation of break-induced replication in yeast. In Methods in enzymology (Elsevier), pp. 161-203.
Geronimo, C.L., and Zakian, V.A. (2016). Getting it done at the ends: Pif1 family DNA helicases and telomeres. DNA Repair (Amst) *44*, 151-158.
Hicks, W.M., Yamaguchi, M., and Haber, J.E. (2011). Real-time analysis of double-strand DNA break repair by homologous recombination. Proc Natl Acad Sci U S A *108*, 3108-3115.
Hu, Q., Lu, H., Wang, H., Li, S., Truong, L., Li, J., Liu, S., Xiang, R., and Wu, X. (2019). Break-induced replication plays a prominent role in long-range repeat-mediated deletion. EMBO J *38*, e101751.
Ira, G., Pellicioli, A., Balija, A., Wang, X., Fiorani, S., Carotenuto, W., Liberi, G., Bressan, D., Wan, L., Hollingsworth, N.M., *et al.* (2004). DNA end resection, homologous recombination and DNA damage checkpoint activation require *CDK1*. Nature *431*, 1011-1017.
Jain, S., Sugawara, N., Lydeard, J., Vaze, M., Le Gac, N.T., and Haber, J.E. (2009). A recombination execution checkpoint regulates the choice of homologous recombination pathway during DNA double-strand break repair. Genes & development *23*, 291-303.
Jain, S., Sugawara, N., Mehta, A., Ryu, T., and Haber, J.E. (2016). Sgs1 and Mph1 helicases enforce the recombination execution checkpoint during DNA double-strand break repair in Saccharomyces cerevisiae. Genetics *203*, 667-675.
Kim, N., Huang, S.N., Williams, J.S., Li, Y.C., Clark, A.B., Cho, J.E., Kunkel, T.A., Pommier, Y., and Jinks-Robertson, S. (2011). Mutagenic processing of ribonucleotides in DNA by yeast topoisomerase I. Science *332*, 1561-1564.
Kim, N., and Jinks-Robertson, S. (2011). Guanine repeat-containing sequences confer transcription-dependent instability in an orientation-specific manner in yeast. DNA Repair (Amst) *10*, 953-960.
Lang, K.S., Hall, A.N., Merrikh, C.N., Ragheb, M., Tabakh, H., Pollock, A.J., Woodward, J.J., Dreifus, J.E., and Merrikh, H. (2017). Replication-Transcription Conflicts Generate R-

Loops that Orchestrate Bacterial Stress Survival and Pathogenesis. Cell *170*, 787-799 e718.
Li, S., and Wu, X. (2020). Common fragile sites: protection and repair. Cell Biosci *10*, 29.
Lippert, M.J., Kim, N., Cho, J.E., Larson, R.P., Schoenly, N.E., O'Shea, S.H., and Jinks-Robertson, S. (2011). Role for topoisomerase 1 in transcription-associated mutagenesis in yeast. Proc Natl Acad Sci U S A *108*, 698-703.
Lydeard, J.R., Jain, S., Yamaguchi, M., and Haber, J.E. (2007). Break-induced replication and telomerase-independent telomere maintenance require Pol32. Nature *448*, 820-823.
Macheret, M., Bhowmick, R., Sobkowiak, K., Padayachy, L., Mailler, J., Hickson, I.D., and Halazonetis, T.D. (2020). High-resolution mapping of mitotic DNA synthesis regions and common fragile sites in the human genome through direct sequencing. Cell Res.
Macheret, M., and Halazonetis, T.D. (2015). DNA replication stress as a hallmark of cancer. Annu Rev Pathol *10*, 425-448.
Malkova, A., Naylor, M.L., Yamaguchi, M., Ira, G., and Haber, J.E. (2005). *RAD51*-dependent break-induced replication differs in kinetics and checkpoint responses from RAD51-mediated gene conversion. Molecular and cellular biology *25*, 933-944.
Minocherhomji, S., Ying, S., Bjerregaard, V.A., Bursomanno, S., Aleliunaite, A., Wu, W., Mankouri, H.W., Shen, H., Liu, Y., and Hickson, I.D. (2015). Replication stress activates DNA repair synthesis in mitosis. Nature *528*, 286-290.
Piazza, A., Boule, J.B., Lopes, J., Mingo, K., Largy, E., Teulade-Fichou, M.P., and Nicolas, A. (2010). Genetic instability triggered by G-quadruplex interacting Phen-DC compounds in Saccharomyces cerevisiae. Nucleic Acids Res *38*, 4337-4348.
Piazza, A., Koszul, R., and Heyer, W.D. (2018). A Proximity Ligation-Based Method for Quantitative Measurement of D-Loop Extension in S. cerevisiae. Methods Enzymol *601*, 27-44.
Piazza, A., Shah, S.S., Wright, W.D., Gore, S.K., Koszul, R., and Heyer, W.-D. (2019). Dynamic processing of displacement loops during recombinational DNA repair. Molecular cell *73*, 1255-1266. e1254.
Ribaud, V., Ribeyre, C., Damay, P., and Shore, D. (2012). DNA-end capping by the budding yeast transcription factor and subtelomeric binding protein Tbf1. EMBO J *31*, 138-149.
Ribeyre, C., Lopes, J., Boule, J.B., Piazza, A., Guedin, A., Zakian, V.A., Mergny, J.L., and Nicolas, A. (2009). The yeast Pif1 helicase prevents genomic instability caused by G-quadruplex-forming CEB1 sequences in vivo. PLoS Genet *5*, e1000475.
Saini, N., Ramakrishnan, S., Elango, R., Ayyar, S., Zhang, Y., Deem, A., Ira, G., Haber, J.E., Lobachev, K.S., and Malkova, A. (2013). Migrating bubble during break-induced replication drives conservative DNA synthesis. Nature *502*, 389-392.
Sakofsky, C.J., and Malkova, A. (2017). Break induced replication in eukaryotes: mechanisms, functions, and consequences. Crit Rev Biochem Mol Biol *52*, 395-413.
Smith, C.E., Lam, A.F., and Symington, L.S. (2009). Aberrant double-strand break repair resulting in half crossovers in mutants defective for Rad51 or the DNA polymerase delta complex. Mol Cell Biol *29*, 1432-1441.
Vasan, S., Deem, A., Ramakrishnan, S., Argueso, J.L., and Malkova, A. (2014). Cascades of genetic instability resulting from compromised break-induced replication. PLoS Genet *10*, e1004119.
Wahba, L., Amon, J.D., Koshland, D., and Vuica-Ross, M. (2011). RNase H and multiple RNA biogenesis factors cooperate to prevent RNA:DNA hybrids from generating genome instability. Mol Cell *44*, 978-988.

Wahba, L., Gore, S.K., and Koshland, D. (2013). The homologous recombination machinery modulates the formation of RNA–DNA hybrids and associated chromosome instability. Elife *2*, e00505.
Wilson, M.A., Kwon, Y., Xu, Y., Chung, W.H., Chi, P., Niu, H., Mayle, R., Chen, X., Malkova, A., Sung, P.*, et al.* (2013). Pif1 helicase and Poldelta promote recombination-coupled DNA synthesis via bubble migration. Nature *502*, 393-396.
Wu, X. (2019). Replication Stress Response Links *RAD52* to Protecting Common Fragile Sites. Cancers (Basel) *11*.
Yan, Z., Xue, C., Kumar, S., Crickard, J.B., 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. Mol Cell *76*, 699-711 e696.

Reviewer Reports on the First Revision:

Referee #1:

The authors have performed new experiments and adequately addressed the reviewers' questions. The manuscript is much improved in this revised version. The authors cited references for DLC and DLE, and added in more details for the AMBER method. As suggested, they performed DLC combined with Rad51 ChIP to show the kinetics of strand invasion. They also provided evidence that strand invasion is normal in the Pif1 and Pol32 mutants and BIR synthesis is not changed when Polε is degraded by AID-degron. Regarding the comparison of BIR replication speed with S-phase replication, it needs to be clarified in the manuscript that the comparison is between BIR in G2/M and replication in S-phase.

Referee #2:

Overall, the paper was revised to fully address the reviewers' comments, and the authors added lots of new data to make the paper even more compelling. I still have a couple of questions/suggestions below but regardless, it is a great paper and will be impactful to the broad field of genome integrity research.

1. In Fig. 1c, I still could not explain why ~150 kb DNA is degraded but not 0.5 kb in the rad52 mutant?

2. I do not find it compelling without experimental results that the ITS effect is likely due to bound proteins, especially Tbf1.

3. Does BIR termination in pri-1 or other mutants coincide more frequently with any particular genes or DNA sequences within BIR tracks that could be explained by the level and/or direction of transcription or of protein binding tightly?

4. I am curious about the effects of checkpoints on some of the readouts. Will inactivate Mec1 yet arresting cells at G2 lead to more extensive uncoupling of leading and lagging?

Referee #3:

The revised manuscript contains many new data and significant clarifications that go a long way to address the comments by this and the other reviewers. My remaining issues are:

1) Abstract and text: The phenotype of pri2-1 is interpreted that at around 30 kb, lagging-strand

synthesis is needed to stabilize the leading strand. There could also be a requirement for re-initiation of the leading strand, as shown during DNA replication in E. coli by Marians.

2) Abstract line 42: Shouldn't it be 'Pif1 or Pol32' instead of 'and', as the single mutant shows some extensions, whereas the double mutant does not at all?

3) Abstract line 47: I do not understand 'first few kb of the strand invasion'. Do the authors mean first few kb of DNA synthesis?

Line 80: The addition of the Rad51 ChIP data is great, but the authors do not report on DNA strand invasion, they only report on proximity of Rad51 to the donor, which could also be an intermediate before DNA strand invasion. I suggest rephrasing this sentence.

Line 80 and Fig. S1b: The DLC data are poor and not helpful. The signal of wild type over rad52 is so low that it questions the validity. At 4 hr the difference is 2-2.5x; in comparison, in Piazza et al. the difference is about 100x. The data in Fig. S3e are a little better, but the difference between wild type and rad51 is still under 5x. I think the ChIP data suffice, but the interpretation has to be rephrased as discussed above.

Previous comments

1) The title should be reworded, by using 'details' the authors undersell their work.

Revision:
The new title is much improved.

2) The conclusion that DNA synthesis starts immediately after DNA strand invasion directly contradicts earlier work published by the Haber laboratory (refs. 21, 22). The authors should discuss this discrepancy, probably best in the Extended Data section.

Revision:
The explanation in the rebuttal and the clarification in the manuscript address this point well.

3) In Figures 1, 3 and 4, the experiments are conducted in cycling cells, so that HO cleavage can occur in G1 and post-S cells, with likely both sister chromatids being cleaved. This aspect should be discussed. Does BIR happen in G1? Is it different from post-S BIR?

Revision:
The explanation in the rebuttal and the clarification in the manuscript address this point well. The nocodazole experiment is a valuable addition.

4) Figure 1: In a, the black triangle needs to be defined.

Revision:
OK

In c, why is there a copy number decrease at -150 kb in the rad52 strain?

Revision:
The explanation in the rebuttal and the clarification in the manuscript address this point well.

In e, the authors show a difference between the phenotype of pol32 and pif1, which begs the analysis of the double mutant.

Revision:

The double mutant data are a valuable addition to the manuscript.

Is the removal of the Ya sequence affected in pol32 and pif1? This is another landmark that can be established.

Revision:
I suggest retaining the Ya flap removal data in the manuscript, as the DLC data, in particular in Figure S1b, are weak with very low signal in wt compared to the rad52 control (see below).

5) Figure 2: The authors deduce a distance of ~20 kb between the leading and lagging strand but the error in this number appears to be very large (in my estimate ± 10 kb). Please calculate and error for the value and provide it in the text.

Revision:
I accept the explanation in the rebuttal and the added data provide more resolution. The interpretation of the pri2-1 data has a potential problem as discussed below.

In b, consider using the same scale for both graphs.

Revision:
OK

In d, the copy number in wild type starts higher than in the pri2-1 mutant. Is the difference significance and what is the explanation?

Revision:
OK

6) Figures 3 and 4: The stalling of the BIR DNA synthesis is inferred and should be directly demonstrated by 2d gel analysis.

Revision:
I accept the explanation in the rebuttal.

7) Figure 4 and line 169 & throughout: HO is a confusing abbreviation for the head-on collision in this text because the authors use the HO endonuclease to induce DSBs.

Revision:
OK

In a, why italics for leu- and ade-? They are phenotypes not genotypes.

Revision:
OK

In b, c, e and f, please provide in the figure the information, which pGAL construct and orientation is shown that the reader does not need to refer to the legend.

Revision:
OK

Line 206: Figure 4a only shows ura3-29 under control of the GAL1 promoter, not the native URA3 promoter.

Revision:

OK

In Figure 4h, the no-break control with the head-on construct shows elevated mutagenesis. Is this consistent with the known replication direction in this region? In lines 209-211, it is unclear, how the no-break control can relate to BIR.

Revision:
I accept the clarification in the rebuttal.

8) Extended Data Figure 1: Why is there a decrease in the Ya copy number in rad51? More detail on the assay for Ya cleavage could be provided. What is the placement of the primers?

Revision:
I accept the clarification in the rebuttal, and the changes in the manuscript are helpful.

9) Extended Data Figure 5: To strengthen the conclusion about the involvement of R-loops, the authors should measure R-loop levels directly under these conditions.

Revision:

Does sin3 affect BIR stalling after the BIR-associated DNA synthesis is established?

Revision:
Omission of this part is fine.

Line 175: Relative to the data in Extended Figure 1b, it appears that Ya disappearance (i.e. cleavage) in Extended Figure 5b is affected by ongoing transcription. Authors claim that ongoing transcription only affects the synthesis step of BIR, but it appears to also interfere with stable D-loop formation and flap cleavage.

Revision:
The additional data with Rad51 ChIP are a good addition.

Extended Figure 5d, e: It would be helpful if the authors can label these plots.

Revision:
OK

10) Lines 72-74: Flap cleavage is presented here and throughout as the time of the invasion/D-loop formation, but the flap likely triggers multiple rounds of invasion/D-loop formation followed by disruption. Moreover, I am not sure that we know that flap cleavage itself does not trigger D-loop disruption.

Revision:
OK

11) Lines 219-221: The sentence "it is possible that BIR contributes to the genetic instabilities reported in relation to fragile sites." may have to be qualified as not fragile sites derive from DNA synthesis/transcription conflicts.

Revision:
OK

Minor issues:

12) Lines 93, 195: "wt" or "wt" is used as an abbreviation for wild type without previously defining. Italics/non-italics should be applied consistently to this term.

13) Line 103: "longer-range"; "long-range" instead?

14) Line 104: "almost no increase of synthesis"; "almost no increase in synthesis" instead?

15) Line 116: "products by CHEF measuring the"; "products by CHEF gel electrophoresis measuring the" instead?

16) Lines 122, 123: May need to insert a sentence in between these lines to explain the experimental rationale.

17) Line 147, 154: "CHEF analysis"; "CHEF electrophoresis analysis" instead?

18) Line 228: References 15 and 34 may not strongly support the claim that BIR is involved in recovery of collapsed replication forks at fragile sites.

19) Line 311, the title of reference 30 is missing.

20) Line 472: Authors do not indicate the yeast strain background (i.e. W303, CEN-PK, S288c, etc.).

21) Line527 and 528, type error "zymolase". It should be "zymolyase".

Revision:
All minor points were addressed.

Author Rebuttals to First Revision:

Reviewer 1

We thank Reviewer #1 for the positive assessment of the revised version of our manuscript. We are glad that s/he thinks that we "adequately addressed the reviewers' questions" and that "the manuscript is much improved in this revised version".

Reviewer 2

We are grateful for Reviewer #2 for the positive comments regarding our revised manuscript and for the statement that this revised manuscript is "even more compelling" and "is a great paper and will be impactful to the broad field of genome integrity research".

1. Reviewer 2 comments: "In Fig. 1c, I still could not explain why -150 kb DNA is degraded but not 0.5 kb in the rad52 mutant?"

Answer:

The yeast strain that we used has two copies of chromosome III (see Fig.1a of the manuscript). The truncated copy (recipient, top in Fig. 1a) contains the *MATa* allele that can be cut by *HO* endonuclease. The second, full copy of chromosome III (donor) contains *MAT*α*-inc*, which cannot be cut by *HO* endonuclease due to mutation. Before DSB induction,

the copy number detected by primer sets binding at -150 kb, -65 kb, or -50 kb are 2x (after normalization to the *ACT1* locus) because the regions at all of these positions are present in the original strain in two copies. Meanwhile, the Ya region is present in only one copy (1x), because it is specific to *MATa* (Extended Data Fig. 1d). The area recognized by the 0.5-kb primer is 1x as well, because it is present only in the donor (uncut) chromosome. Following galactose addition, the recipient copy of chromosome III is cleaved by *HO* endonuclease and undergoes degradation in *rad52Δ,* while the donor chromosome is not undergoing any change. Therefore, the copy number of chromosome regions that existed on the recipient chromosome before the cut will be decreased in *rad52Δ* due to degradation, while the copy number detected by the 0.5-kb primer that is not present in the recipient before the cut (and in the absence of BIR) will not change.

2. Reviewer 2 comments: I do not find it compelling without experimental results that the ITS effect is likely due to bound proteins, especially Tbf1.

Answer:

In response, we would like to point out that determining the role of Tbf1 binding in the disruption of BIR by ITSs did not represent our original goal. We observed that the disruption of BIR by ITSs seems to require ~40 telomeric repeats, which was similar to the requirement for the binding of Tbf1 to telomeres to form a "capped" structure. In addition, we observed that inserting another sequence capable of forming G4-quadruplex structures did not interrupt BIR, thus suggesting that binding of a protein might be more important that G4-structure formation. However, testing the role of Tbf1 binding on BIR directly proved difficult. In particular, *TBF1* is an essential gene, and it cannot be deleted. We attempted to replace the native promoter of *TBF1* with the TET-off promoter to shut down the transcription of *TBF1*. However, we could not produce any viable transformants, which we think could be due to a toxic effect of Tbf1 when its expression level changed. We then started constructing the *TBF1-AID* degron system. This process will take a significant amount of time, and we are not certain that the degron will be effective in degrading protein that is already bound to ITSs before auxin addition (and the beginning of BIR). We are concerned that the pre-bound Tbf1 will not be removed from the ITS and might still block BIR synthesis. All in all, we would like to agree with this reviewer that it is too early to write about the possible role of Tbf1 on the disruption of BIR by ITSs in this manuscript, and that this should rather become a focus of our future studies. Therefore, we replaced our original statement with the following one: "BIR interruption at ITSs might be promoted by either a protein bound to ITS or by formation of secondary DNA structures. The former is more likely because BIR easily progressed through another, non-ITS, G4-forming sequence inserted in either orientation at the same location, even in the presence of the G4-stabilizing agent, Phen-DC3 (Extended Data Fig. 6d-f). In addition, deletion of *RRM3*, known to unwind G4 structures, did not exacerbate BIR disruption upon encountering either $(ITS)_{\sim 40}$ or $(ITS)_{28}$ (Extended Data Fig. 6g-i)."

3. Reviewer 2 comments: Does BIR termination in *pri2-1* or other mutants coincide more frequently with any particular genes or DNA sequences within BIR tracks that could be explained by the level and/or direction of transcription or of protein binding tightly?

Answer:

This is a great question, but it is difficult to answer based on our current analysis. We observed that, in the absence of primase, BIR synthesis cannot traverse beyond 25 to 30 kb. In our current experimental system, this region contains 19 genes (10 in H-On and 9 in Co-D orientation in respect to BIR). The expression levels of these genes at the time of BIR varies, and none of these genes is highly expressed. Based on our current analysis, the amount of synthesis observed in *pri2-1* mutants is progressively decreased between *MAT* and the 30-kb position, and the pattern of this decrease appears even throughout the region. In other words, there are no sharp drops or clear blocking positions that would show a pattern similar to the one that we observed in our scenario of forcing BIR interruption by ITSs. The pattern that we observe in *pri2-1* does not suggest the existence of one particular "stopping point" in the primase mutants. However, we cannot exclude the existence of some "hot spots" that might have an increased impact on BIR for any of the reasons you mention, but these cannot be detected by our current study in which BIR occurs asynchronously and only in one chromosome. In the future, we believe this is an interesting topic to pursue, and it may require investigation of multiple chromosome regions and possibly also performing single-cell analysis.

4. Reviewer 2 comments: I am curious about the effects of checkpoints on some of the readouts. Will inactivate Mec1 yet arresting cells at G2 lead to more extensive uncoupling of leading and lagging?

Answer:

We thank the reviewer for this great suggestion. We agree that checkpoints may play a role in some of the readouts and that inactivation of *MEC1* (or some other checkpoint genes) might lead to changes in uncoupling of leading and lagging strands. However, we feel that it will be an interesting focus for another extensive study, as it will require construction of several checkpoint mutants that will also harbor additional mutations (for example *pri2-1*). This is certainly an area of significant interest for our future research.

Reviewer 3

We thank Reviewer 3 for his/her positive assessment of our revised manuscript. We are also thankful for the suggestions that allowed us to clarify several points and to make several grammatical and stylistic improvements.

5. Reviewer 3 comments: Abstract and text: The phenotype of *pri2-1* is interpreted that at around 30 kb, lagging-strand synthesis is needed to stabilize the leading strand. There could also be a requirement for re-initiation of the leading strand, as shown during DNA replication in *E. coli* by Marians.

Answer:

We agree with this reviewer that it is possible that the nascent leading strand may dissociate from its template within the first 30 kb and re-invade (re-initiate). However, this reinvasion would again create a 3'OH invading end that could serve as a primer, and, therefore, it would not require primase, as we demonstrate here. Thus, we believe that our results demonstrating primase-independent initiation of leading strand DNA synthesis also suggest that formation of Okazaki fragments is needed to stabilize the growing leading strand. We feel that we can keep this idea as our proposed interpretation of the results, but to address the Reviewer's point, our revised abstract now states: "Without primase, leading strand synthesis is initiated efficiently, but fails to proceed beyond 30 kb, suggesting that primase is needed for stabilization of the nascent leading strand." Our text has also been changed as follows: "We propose that primase-deficient cells can use the 3' invading strand as a primer to successfully initiate BIR leading strand synthesis; however, stable extension of the leading strand requires primase, likely for synthesis of Okazaki fragments (Extended Data Fig. 4c)."

6. Reviewer 3 comments: Abstract line 42: Shouldn't it be 'Pif1 or Pol32' instead of 'and', as the single mutant shows some extensions, whereas the double mutant does not at all?

Answer:

We corrected as proposed by the reviewer.

7. Reviewer 3 comments: Abstract line 47: I do not understand 'first few kb of the strand invasion'. Do the authors mean first few kb of DNA synthesis?

Answer:

We thank the Reviewer #3 for catching this. This sentence is removed from our new shortened version of the abstract.

8. Reviewer 3 comments: Line 80: The addition of the Rad51 ChIP data is great, but the authors do not report on DNA strand invasion, they only report on proximity of Rad51 to the donor, which could also be an intermediate before DNA strand invasion. I suggest rephrasing this sentence.

Answer:

We agree with the reviewer that Rad51ChIP does not perhaps measure the strand invasion *per se.* However, it is well established that association of Rad51 with the donor sequence specifically reflects strand invasion, and this assay has been used to measure strand invasion in multiple studies [1-3]. In fact, Rad51 ChIP represents a standard way of measuring

strand invasion efficiency and kinetics, as well as to study its genetic control. Thus, we believe that Rad51 ChIP is a good choice to track strand invasion. In response to the Reviewer's comment, we clarified our statement as follows: "…about 1 hour after beginning of strand invasion (detected by ChIP of Rad51 loading onto the donor chromosome III(reflecting strand invasion), or by D-loop capture (DLC)…".

9. Reviewer 3 comments: Line 80 and Fig. S1b: The DLC data are poor and not helpful. The signal of wild type over rad52 is so low that it questions the validity. At 4 hr the difference is 2-2.5x; in comparison, in Piazza et al. the difference is about 100x. The data in Fig. S3e are a little better, but the difference between wild type and rad51 is still under 5x. I think the ChIP data suffice, but the interpretation has to be rephrased as discussed above.

Answer:

We agree with this reviewer that the DLC signals measured in our system do not look as robust as the data in Piazza et al, 2019 [4]. We believe that the main reason for this is the difference between the two systems. In the Piazza *et al* study, the donor and recipient chromosomes share a limited amount of homology (2kb); in contrast, in our system, the donor and recipient chromosomes share about 100 kb of homology. While a significant fraction of strand invasion events occurs in the first 3 to 5 kb of the DSB [5], strand invasion can also happen further away. Thus, only a fraction of strand invasion events can be captured by the DLC method in our system, where the region available for strand invasion is much longer. Nevertheless, the increase in recombination-proficient strains is significantly higher compared to the recombination-defective control. In addition, the kinetics of strand invasion detected using the DLC method is similar to our results using Rad51 ChIP. Thus, we believe that showing the results obtained by both methods is practical.

10. Reviewer 3 comments: "Is the removal of the Ya sequence affected in pol32 and pif1? This is another landmark that can be established".

Revision:
I suggest retaining the Ya flap removal data in the manuscript, as the DLC data, in particular in Figure S1b, are weak with very low signal in wt compared to the rad52 control (see below).

Answer:

We included Ya flap removal data in our revised manuscript (Extended Data Fig. 3b).

11. We greatly appreciate that Reviewer #3 checked our responses to all other comments and found them acceptable.

1 Jain, S. *et al.* A recombination execution checkpoint regulates the choice of homologous recombination pathway during DNA double-strand break repair. *Genes & development* **23**, 291-303 (2009).

2 Sugawara, N., Wang, X. & Haber, J. E. In vivo roles of Rad52, Rad54, and Rad55 proteins in Rad51-mediated recombination. *Mol Cell* **12**, 209-219, doi:10.1016/s1097-2765(03)00269-7 (2003).

3 Hicks, W. M., Yamaguchi, M. & Haber, J. E. Real-time analysis of double-strand DNA break repair by homologous recombination. *Proc Natl Acad Sci U S A* **108**, 3108-3115, doi:10.1073/pnas.1019660108 (2011).

4 Piazza, A. *et al.* Dynamic processing of displacement loops during recombinational DNA repair. *Molecular cell* **73**, 1255-1266. e1254 (2019).

5 Deem, A. *et al.* Defective break-induced replication leads to half-crossovers in Saccharomyces cerevisiae. *Genetics* **179**, 1845-1860 (2008).