nature portfolio

Peer Review File

53BP1 deficiency leads to hyperrecombination using break-induced replication (BIR)

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors elucidate a novel role of 53BP1 in inhibiting Break-Induced Replication (BIR) in addition to its function in suppressing end resection, through the modulation of PIF1 and PCNA recruitment. They demonstrate that 53BP1 employs distinct mechanisms to suppress the recruitment of PIF1 and PCNA at broken replication forks and at double-strand break (DSB) sites. Specifically, at DSB sites, 53BP1 inhibits PCNA ubiquitination, whereas it prevents the re-localization of ubiquitinated PCNA at broken forks. Additionally, the protein SMARCAD1 counteracts and displaces 53BP1 at broken forks to facilitate BIR. This study presents intriguing findings; however, several issues need to be addressed prior to publication.

Comments:
1. In the model depicted in Figure 7f (left), the authors suggest, "We propose that 53BP1 deficiency disrupts the balance of end fill-in DNA synthesis on ssDNA overhangs by interfering with the coordination between Polα-primase-directed priming and subsequent DNA synthesis elongation (Fig. 7f, left)." Given that synthesis in Polα-primase-directed priming occurs using ssDNA overhangs as templates, whereas BIR-like synthesis utilizes ssDNA overhangs as primers in a contrary direction, it is challenging to reconcile these mechanisms.

The authors might also explore an alternative scenario. It is established that 53BP1 is essential for maintaining the stability of reversed arms at stalled replication forks. Similarly, 53BP1 could be crucial for stabilizing the ends at double-strand break (DSB) sites. In the absence of 53BP1, the resulting instability at these deDSB sites—akin to that observed in the reversed arms of forks—may lead to significant gaps. These gaps could necessitate a greater reliance on PCNA-PIF1-dependent synthesis for repair. This is my personal speculation, and the authors are not obliged to accept this viewpoint.

2. In several Proximity Ligation Assays (PLAs), such as those shown in Figures 4b-g and 5a-g, it is claimed that recruitment of PCNA or PIF1 is augmented when the PLA signal (of PCNA/gH2AX or PIF1/gH2AX) increases. How can it be ascertained that the enhanced signal is not merely due to an increase in gH2AX levels?

3. “With our reporter system, we observed local jumping/template switching in the BIR-EJ events in WT U2OS cells (14.6%), with an increase in 53BP1-KO cells (22.6%).” is this difference statistically significant?

4. The abstract states, "We demonstrated that loss of 53BP1 induces BIR-like hyperrecombination, which depends on Polα-primase-mediated end fill-in DNA synthesis on ssDNA overhangs at DSBs." There is no evidence provided that Polα-primase-mediated DNA synthesis specifically occurs on ssDNA overhangs as opposed to other structures, such as D-loops.

5. On page 9, it is mentioned, "By performing PLA of stably expressed Flag-PIF1 with endogenous

PCNA, we found that human PIF1 interacts with PCNA in U2OS cells without treatment, and this interaction is enhanced upon HU treatment (Fig. 5e left and middle)." The PLA experiments demonstrate proximity between PIF1 and PCNA but do not definitively show interaction between them.

Reviewer #2 (Remarks to the Author):

In this work titled “53BP1 deficiency leads to hyperrecombination using break-induced replication (BIR)” by Shah and colleagues, new insights are shown that clarify how 53BP1 is involved in choice of repair pathways following single- and double-ended DNA breaks. The importance of this pathway choice is mostly seen in BRCA-deficient cancers that rely on mutagenic pathways to restore complicated DNA lesions, induced by perturbed replication or chemotherapeutics. One of these mutagenic pathways is break-induced replication, which, similar to HR, uses the sister-chromatid as template for repair. Using a specialized reporter-system, the authors clearly show that loss of 53BP1 results in an increase in HR but also in BIR. Surprisingly, proteins essential for BIR, PIF1 and PolD3, seem to be required for this increase, both for HR and BIR, and independent of BRCA-status. The authors continue to explore this phenotype mostly for PIF1, demonstrating that a requirement of ubiquitinated-PCNA and SMARCAD1 to drive BIR in absence of 53BP1. Lastly, 53BP1-deficient cells seem to rely strongly on PIF1, as determined by an observed synthetic lethal growth inhibition. This suggests that in absence of 53BP1, the pathway choice is not dominantly HR, but might be driven by BIR or a pathway utilizing BIR-proteins.

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Major points of revision

While the conclusions derived from the data are sound, I do have a number of major concerns that I feel should be addressed to consider publication in Nature Communications.

- To start, what surprises me in the analyses of the data and discussion, no mention is made of the BIR(-like) repair used in mitotic DNA synthesis pathway, which is PIF1 and PolD3 dependent, as previously shown by the authors themselves for PIF1 (ref.13). Furthermore, MiDAS is RAD51 (BRCA1)-independent, similar to the data shown in this study, and dependent on RAD52. Given that the BIR/LTGC assay takes 5 days, it would not surprise me that the repair of this reporter could take place at the transition of G2/M when MiDAS is shown to be executed. As no mention is made in this study of RAD52, or MiDAS besides the introduction, this should be addressed, preferably experimentally, that BIR observed in the BIR/LTGC assay is different from MiDAS and what the effect of RAD52 loss is in these reporter assays.
If it is the case that 53BP1 normally limits MiDAS to G2/M transition, but in 53BP1-deficient cells is allowed during S-G2, this would be a very promising addition to the current hypothesis.

- In line with this, Spies et al. in 2019 showed how 53BP1-nuclear bodies can determine repair of under-replicated DNA via Rad51 or Rad52 (https://www.nature.com/articles/s41556-019-0293-6).

Can the authors comment how they see their model of 53BP1 limiting BIR in this context? It could be possible that 53BP1-loss shifts repair of ur-DNA from Rad51-dependent HR, to Rad52-dependent BIR, due to the loss of 53BP1-NB formation.

- Regarding data quality, it is not clear from the plotted data, especially in figures 4, 5, and 6, whether these are from single biological replicates or multiple. At least, in the way currently shown, it seems all data points are treated as a single experiment and tested as such for significance. These should be properly tested across the medians/means of each experiment, and preferably the data points of each biological replicate should be shown individually, for clarification.

- Statements on BIR involvement on broken replication forks seem to be derived from a few experiments where IR is exchanged for HU. To claim that these are indeed broken forks it would strengthen the data to show this experimentally besides PLA-based assays, i.e. DNA fiber assays to show replication stress or stalling.

- "BIR utilization in 53BP1-deficient cells results in a synthetic lethal interaction between the 53BP1 and BIR pathways, providing new opportunities for targeted cancer treatment."
While the synthetic lethality effects seem very clear, TP53BP1 mutation or loss, similar for TIRR amplification, is a low frequency event in cancers. The frequencies should be properly addressed in the discussion besides the current phrase “TIRR amplification is frequently observed in a wide range of cancers, with the highest frequencies present in breast, prostate and pancreatic cancers”.

- The synthetic lethal effect observed in U2OS cells is very promising, is synthetic lethality similarly observed for shPOLD3? It would strengthen the synthetic lethality effect observed by repeating the 53BP1-KO +shPIF1 depletion in at least one other cell line model, possibly a model with inherent BRCA-deficiency to show the independence of this lethality to BRCA1/2.

- Fig2: "With our reporter system, we observed local jumping/template switching in the BIR-EJ events in WT U2OS cells (14.6%), with an increase in 53BP1-KO cells (22.6%). Most jumping/template switching events contain MH sequences at the jumping sites (WT: 72.0%; 53BP1-KO: 77.8%) (Fig2c)"
Is this data significant over multiple experiments? Seems a small increase and possibly due to variance in repair outcomes, and will probably level out after sufficient clones are analyzed. Otherwise, I suggest rephrasing the conclusions drawn on these data.

- Fig2a: "suggesting that MMEJ is the major mechanism for end joining to complete BIR-EJ (Fig. 2a)"
Do the authors have evidence of SSA as a method to complete BIR with this reporter? Or is that outcome not possible due to lack of an appropriate homology domain for SSA? If so, it would be fair to state that BIR-EJ works via MMEJ as can be judged by this reporter specifically.
Furthermore, larger deletions due to extensive end resection are generally repaired via SSA. PolQ-KO or depletion should be done to show it is really MMEJ that drives BIR-EJ events.

- Fig3a: 53BP1-KO cells show an increase in HR/STGC events of ~100%. Knockdown of PIF1 or POLD3 reduces this to WT levels again, suggesting the increase in HR is solely due to BIR repair events. The authors claim this is due to seDNA caused by nicks. Can the authors explain how this is possible with I-Sce1 induced breaks?

- In Fig4 and to some extent fig5 and 6, PLA is used to monitor localization of Flag-PIF1 to yH2AX or PCNA after 4Gy irradiation.
I have a number of concerns for these experiments. First of all, Flag-PIF1 is stably expressed, is this on top of endogenous Pif1 and how strong is the expression of Flag-Pif1 or EGFP-Pif1?
Second, with EGFP or Flag-tagged PIF1, I would expect to see standard immunofluorescence of localization to yH2AX. Why was PLA used for PIF1 staining?
Third, why was PLA used for PCNA and yH2AX localization in 4e, and 4f? A number of the PLA stainings could be done with conventional immunofluorescence, especially when analyzing DNA lesions induced by 4Gy irradiation.
Fourth, typically, loss of 53BP1 results in an increase in HR following irradiation at 4Gy or HU treatment, and measured by increased RAD51 foci at yH2AX. Is such an increase observed in these cells, and is that similarly overlapping with PCNA and PIF1? If not, this would indeed show there is a BRCA1/RAD51 independent pathway that utilizes BIR in absence of 53BP1.
Lastly, although as stated ~100 nuclei were measured for each sample, the experiments with PLA in figure 4 and 5 seem to be derived from one biological replicate in each panel. If this is not the case, it would be appropriate to show the medians of each biological replicate, and perform statistical testing on the repeated medians instead of taking all data points together. If these data do come from single biological replicates, ilt would be prudent to repeat the crucial panels at least with multiple experiments.

-Fig5 b,c,d These experiments use irradiation to produce deDSBs. Why are replication forks mentioned here (page9 "these data... ssdna overhangs."? If this related to the HU treatment used in Fig5a, then please show the effect of shPRIM or CD437 treatment on HU-treated cells, in both WT cells and 53bp1-deficient cells.

-Fig 5i: %EGFP positive cells in FIG5i is around 14% in BIR/LGTC for WT cells, this is much higher compared to previous assays in Fig1 and Fig3 where it's ~5% for WT cells, and this high percentage is seen only in 53BP1-KO cells. Can the authors explain this?

-Fig6a-e, Were these experiments not conducted in untreated cells? Furthermore, similar as in Fig4 and Fig5, it seems these are single experiments performed. Where the effect sizes in Fig4 and 5 are substantial, for panels such as PIF1-yH2AX PLA for example in fig6 the effect sizes are small and warrent repeated measurements.

Fig7. The synthetic lethality observed for 53BP1-KO cells with PIF1 depletion, where it is clear these cells no longer proliferate or die within two or three days of plating up until 5days. In the STGC or LTGC reporter assays, a 5 day incubation period is also present before measurement of %EGFP cells. Is a similar loss of viability or proliferation observed in those cells? As in these reporter assay experiments PIF1 depletion is similarly performed in 53BP1-KO cells, viability loss could seriously

affect the %EGFP outcomes.

Minor points:
- Can the authors state the aim of this study in the introduction, before summarizing the results?

- "However, considering that SSA is mediated by a deletion mechanism between repetitive sequences it may be too specialized to salvage a general HR defect."
Which is the case for BIR as well, as the authors mention the 100x-1000x increases mutation rate for BIR. Therefore, I dont see the relevance of this statement on SSA. It would seem both SSA and BIR are not refered repair pathways unless specifically needed.

- "Notably, long tract gene conversion (LTGC) generally employs the BIR mechanism" The mention of LTGC on page3 first paragraph seems out of the blue. It is unclear if LTGC is a repair mechanism on its own or if its part of BIR. Can the authors clarify what this sentence refers to?

- Throughout the text the authors make use of BIR-mode, BIR mode, BIR mechanism, BIR pathway. Please use a consistent term for BIR.

- Multiple mentions in results of "green cells", please change to "EGFP+cells" which is what is measured.

- "and we determined the role of 53BP1 for BIR in BRCA1-deficient cells."Unclear statement, please rephrase.

- In this study, we showed that deficiency in 53BP1 leads to uncontrolled assembly of BIR-like replisomes"
If with "in this study" the authors mean their previous study (ref13), then this should be more clearly stated.

- "this data suggests that while generating ssDNA overhangs is necessary, their length is not the key determinant in triggering the overloading of PCNA and PIF1 to IR-induced DSBs in the absence of 53BP1."
This should be confined tot the discussion section, or experimentally addres why the ssDNA overhang length is relevant here.

- Fig4a: Please show quantification of the experiment where these images came from.

Re: NCOMMS-24-23322

We thank the reviewers for reviewing our manuscript NCOMMS-24-23322, by Sameer Shah and Youhang Li et al. We greatly appreciate the valuable comments and suggestions from the reviewers, which have significantly helped us improve our manuscript. We have performed a substantial number of new experiments and extensively expanded our study along the lines suggested by the reviewers. We have also revised the Introduction and Discussion sections in response to the reviewers' comments. Point-by-point responses to the reviewers' critiques are listed below. The changes in the manuscript are marked in blue.

Reviewer #1:

Comments:

1. In the model depicted in Figure 7f (left), the authors suggest, "We propose that 53BP1 deficiency disrupts the balance of end fill-in DNA synthesis on ssDNA overhangs by interfering with the coordination between Polα-primase-directed priming and subsequent DNA synthesis elongation (Fig. 7f, left)." Given that synthesis in Polα-primase-directed priming occurs using ssDNA overhangs as templates, whereas BIR-like synthesis utilizes ssDNA overhangs as primers in a contrary direction, it is challenging to reconcile these mechanisms.

Response: We thank the reviewer for raising this point. We include Fig. S13b to explain the model in more detail. Polα/primase-directed priming takes place on ssDNA overhangs prior to strand invasion, whereas BIR-like synthesis occurs after strand invasion, indicating that there are two separate DNA polymerase loading steps. We propose that in the first step, Polα/primase-mediated fill-in DNA synthesis is stalled, which causes PCNA ubiquitination and PIF1 binding to PCNA-Ub, resulting in the recruitment of PIF1 to DSB ends (on ssDNA overhangs). After RAD51-mediated strand invasion, BIR DNA synthesis is launched. In this step, the PIF1/PCNA-Ub complex, as it is already enriched around DSBs, could be loaded to support Polδ for BIR DNA synthesis and D-loop migration (Fig. S13b).

The authors might also explore an alternative scenario. It is established that 53BP1 is essential for maintaining the stability of reversed arms at stalled replication forks. Similarly, 53BP1 could be crucial for stabilizing the ends at double-strand break (DSB) sites. In the absence of 53BP1, the resulting instability at these deDSB sites—akin to that observed in the reversed arms of forks—may lead to significant gaps. These gaps could necessitate a greater reliance on PCNA-PIF1-dependent synthesis for repair. This is my personal speculation, and the authors are not obliged to accept this viewpoint.

Response: Thanks to the reviewer for considering an alternative model. Although the role of 53BP1 in protecting reversed forks at stalled replication forks has been tested in multiple labs, the findings have been inconsistent, with some showing extensive nascent strand degradation, which leads to gap formation[1,2], and others indicating no effects, when 53BP1 is inactivated[3,4]. Further analysis revealed that the 53BP1 fork protection function is cell type dependent[5]. In addition, in BRCA1-deficient cells, where DNA gaps are often accumulated, inactivation of 53BP1 actually reduces gap formation[6-8]. Based on these observations, loss of 53BP1 does not always lead to gap accumulation on replication forks.

At DSBs, loss of 53BP1 leads to hyper end resection [9]. In our study, we found that inactivation of 53BP1 activates the onset of BIR mechanism in both wildtype cells and BRCA1-deficient cells. As discussed in the manuscript, since end resection extent in BRCA1/53BP1-deficient cells is similar to that in WT cells [10], super end resection is not likely the underlying mechanism to activate BIR. Regarding the possibility of gap formation, 53BP1 loss decreases gap formation observed in BRCA1-deficient cells[6-8]. However, compromised 53BP1 function promotes the BIR mechanism and rescues HR and BIR defect in BRCA1-deficient cells (Fig. 3b). Collectively, the role of 53BP1 related to fork protection and gap formation may not serve as the general mechanism underlying BIR activation when 53BP1 is deficient.

2. In several Proximity Ligation Assays (PLAs), such as those shown in Figures 4b-g and 5a-g, it is claimed that recruitment of PCNA or PIF1 is augmented when the PLA signal (of PCNA/gH2AX or PIF1/gH2AX) increases. How can it be ascertained that the enhanced signal is not merely due to an increase in γH2AX levels?

Response: This is a very good point worth considering. 53BP1 plays an important role in promoting KU-dependent NHEJ, although its loss causes milder radiosensitivity than a defect in the NHEJ core factors such as KU70 and XRCC4 [11,12], and thus DSBs are expected to increase upon loss of 53BP1. To make sure that the enhanced PLA signals of PIF1 and PCNA with γH2AX observed in 53BP1-deficient cells are not simply due to an increase in γH2AX levels, we compared the PLA signals after expressing shRNAs for 53BP1, KU70 or XRCC4. We found that the PLA signals of PIF1 and PCNA with γH2AX are increased significantly only after depletion of 53BP1, but not KU70 or XRCC4 (Fig. S7a), confirming that the increased recruitment of PCNA and PIF1 to DSBs in 53BP1-deficienct cells is not simply due to DSB accumulation.

3. “With our reporter system, we observed local jumping/template switching in the BIR-EJ events in WT U2OS cells (14.6%), with an increase in 53BP1-KO cells (22.6%).” is this difference statistically significant?

Response: We previously performed two sets of experiments on the wildtype U2OS (EGFP-BIR/LTGC) cell line and one set of experiments on the *53BP1*-KO cell line to analyze the BIR repair products using green single clones. In this revision, we have conducted additional analysis on the *53BP1*-KO cell line. The combined data from the two sets of experiments are presented in Fig. 2, with the data for each set shown separately in Fig. S2. The increase of jumping/template switching from 14.6% in WT cells to 22.9% in *53BP1*-KO cells is not statistically significant (P value: 0.0942) (Fig. 2c). Nevertheless, the overall increase of BIR events in 53BP1-deficient cells would lead to an increase in overall chromosomal rearrangements due to BIR-mediated jumping/template switching. We have updated the main text and included more discussion on this topic.

4. The abstract states, "We demonstrated that loss of 53BP1 induces BIR-like hyperrecombination, which depends on Polα-primase-mediated end fill-in DNA synthesis on ssDNA overhangs at DSBs." There is no evidence provided that Polα-primase-mediated DNA synthesis specifically occurs on ssDNA overhangs as opposed to other structures, such as D-loops.

Response: To show that the activation of BIR is triggered by Polα-primase-mediated end fill-in

DNA synthesis on ssDNA overhangs, as opposed to other structures such as D-loops, we depleted RAD51 in *53BP1*-KO cells. We observed that RAD51 depletion does not reduce PCNA-Ub accumulation at DSBs, as revealed by PLA of PCNA-Ub with γH2AX in *53BP1*-KO cells (Fig. S7b), while inhibition of primase activity by the inhibitor CD437 strongly inhibits PCNA-Ub accumulation at DSBs. These data suggest that accumulation of PCNA-Ub at DSBs in a manner dependent on primase activity likely occurs prior to RAD51-mediated strand invasion. This supports our working model that induction of BIR in 53BP1-deficient cells is largely due to Polα-primase-mediated end fill-in DNA synthesis on ssDNA overhangs at DSBs.

5. On page 9, it is mentioned, "By performing PLA of stably expressed Flag-PIF1 with endogenous PCNA, we found that human PIF1 interacts with PCNA in U2OS cells without treatment, and this interaction is enhanced upon HU treatment (Fig. 5e left and middle)." The PLA experiments demonstrate proximity between PIF1 and PCNA but do not definitively show interaction between them.

Response: We performed co-immunoprecipitation of Flag-PIF expressing in U2OS cells with endogenous PCNA before and after HU treatment. We observed the interaction of PIF1 and PCNA before HU treatment and their interaction is significantly increased following HU treatment (Fig. S7c), supporting the conclusion that PIF1 and PCNA interact.

Reviewer #2:

Comments:

In this work titled “53BP1 deficiency leads to hyperrecombination using break-induced replication (BIR)” by Shah and colleagues, new insights are shown that clarify how 53BP1 is involved in choice of repair pathways following single- and double-ended DNA breaks. The importance of this pathway choice is mostly seen in BRCA-deficient cancers that rely on mutagenic pathways to restore complicated DNA lesions, induced by perturbed replication or chemotherapeutics. One of these mutagenic pathways is break-induced replication, which, similar to HR, uses the sister-chromatid as template for repair. Using a specialized reporter-system, the authors clearly show that loss of 53BP1 results in an increase in HR but also in BIR. Surprisingly, proteins essential for BIR, PIF1 and PolD3, seem to be required for this increase, both for HR and BIR, and independent of BRCA-status. The authors continue to explore this phenotype mostly for PIF1, demonstrating that a requirement of ubiquitinated-PCNA and SMARCAD1 to drive BIR in absence of 53BP1. Lastly, 53BP1-deficient cells seem to rely strongly on PIF1, as determined by an observed synthetic lethal growth inhibition. This suggests that in absence of 53BP1, the pathway choice is not dominantly HR, but might be driven by BIR or a pathway utilizing BIR-proteins.

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repair of this reporter could take place at the transition of G2/M when MiDAS is shown to be executed. As no mention is made in this study of RAD52, or MiDAS besides the introduction, this should be addressed, preferably experimentally, that BIR observed in the BIR/LTGC assay is different from MiDAS and what the effect of RAD52 loss is in these reporter assays.
If it is the case that 53BP1 normally limits MiDAS to G2/M transition, but in 53BP1-deficient cells is allowed during S-G2, this would be a very promising addition to the current hypothesis.

Response: We thank the reviewer for raising the point of the connection of our study with MiDAS and we acknowledge that this is an important aspect.

In our study, we demonstrated that 53BP1 plays a critical role in restricting BIR in cycling cells. In mitosis, however, it has been shown that 53BP1 recruitment to DSB ends is attenuated [13], suggesting 53BP1 is not engaged in regulating DSB repair in mitosis. The suppression of the binding of 53BP1 to DSB ends in mitosis is likely due to CDK1 and PLK1-mediated phosphorylation[14]. Along this line, inactivating 53BP1 does not cause a defect in MiDAS[15]. This is similar to the situation for RAD51 in mitosis, where its activity is attenuated[16-20], and consequently RAD51 is not required for MiDAS[21-23]. We speculate that the absence of 53BP1 at DSBs in mitosis could be an important mechanism to deactivate the 53BP1-mediated BIR suppression, thereby allowing active MiDAS to complete replication of under-replicated DNA regions in mitosis.

We have also performed additional experiments to examine the dependence of BIR and MiDAS on RAD51 and RAD52. Consistent with previous findings [22,23], MiDAS is independent of RAD51 but requires RAD52 (Fig. S14a). BIR assayed using the EGFP-BIR/LTGC reporter, shows dependence on RAD51 and BRCA1 in both WT cells [21] and *53BP1*-KO cells (Fig. 1d). However, depletion of RAD52 in U2OS (EGFP-BIR/LTGC) cells does not cause a defect in BIR in cycling cells (Fig. S14b top). In addition, depletion of 53BP1 in WT and *RAD52*-KO cells leads to a similar increase of BIR (Fig. S14b bottom), suggesting that increased BIR due to 53BP1 deficiency is also independent of RAD52. Previously, using the same BIR reporter, we demonstrated that in mitotic arrested cells, RAD51 is dispensable, whereas RAD52 is required for BIR[21]. The shift of BIR from RAD51 to RAD52 dependence in mitosis is likely due to the suppression of RAD51 activity in mitotic cells[17,19].

Together, the dependence on RAD51 and RAD52 for BIR in cycling cells (interphase) and for MiDAS is different. The BIR suppression activity by 53BP1, which we identified in interphase cells, is likely attenuated in mitosis, which may contribute to the activation of MiDAS. We have included more discussion in the manuscript on this topic.

2. - In line with this, Spies et al. in 2019 showed how 53BP1-nuclear bodies can determine repair of under-replicated DNA via Rad51 or Rad52 (https://www.nature.com/articles/s41556-019-0293-6). Can the authors comment how they see their model of 53BP1 limiting BIR in this context? It could be possible that 53BP1-loss shifts repair of ur-DNA from Rad51-dependent HR, to Rad52-dependent BIR, due to the loss of 53BP1-NB formation.

Response: It is an interesting finding that 53BP1-nuclear bodies (NBs) restrain replication of the embedded genomic loci until late S-phase, and then enable the RAD52-mediated repair of underreplicated DNA (UR-DNA) lesions. The major role of 53BP1 in this process is to recruit RIF1 to control the origin firing in NB-embedded DNA, ensuring replication occurs late in S-phase. This would couple RAD52-mediated repair of UR-DNA lesions appropriately with DNA replication. They proposed that by delaying DNA replication in NBs, before fork convergence to

UR-DNA lesions, RAD52 could fix UR-DNA lesions, possibly through RNA-assisted DSB repair (see discussion in [24]). Therefore, the mechanism by which 53BP1-NB promotes the repair of UR-DNA lesions is likely different from its role in controlling BIR as reported in this study.

3. - Regarding data quality, it is not clear from the plotted data, especially in figures 4, 5, and 6, whether these are from single biological replicates or multiple. At least, in the way currently shown, it seems all data points are treated as a single experiment and tested as such for significance. These should be properly tested across the medians/means of each experiment, and preferably the data points of each biological replicate should be shown individually, for clarification.

Response: We have repeated three sets of experiments for Figures 4b, 4c, 4d, 4e, 4f and 4g; Figures 5a, 5b, 5c, 5d, 5e, 5f, and 5g; and Figure 6a, 6b, 6c, 6d and 6e. In each experiment, ~100 nuclei were analyzed. The data from three experiments are combined together in the main figures (~300 nuclei), with the average and significance of the three experiments presented in the corresponding supplementary figures now. For example, Fig. 4b shows the combined data of three experiments, while Fig. S5c presents the average and significance of the three sets of experiments. Please see details in the main text and figure legends.

4. - Statements on BIR involvement on broken replication forks seem to be derived from a few experiments where IR is exchanged for HU. To claim that these are indeed broken forks it would strengthen the data to show this experimentally besides PLA-based assays, i.e. DNA fiber assays to show replication stress or stalling.

Response: We have performed DNA fiber analysis as suggested, and showed that HU treatment stalls DNA replication (See below Fig. R1a). We also showed by γH2AX Western blot analysis that short HU treatment (1 mM, 2h) only causes fork stalling but not fork breakage, whereas long HU treatment (1 mM, 24h) leads to fork breakage (See below Fig. R1b).

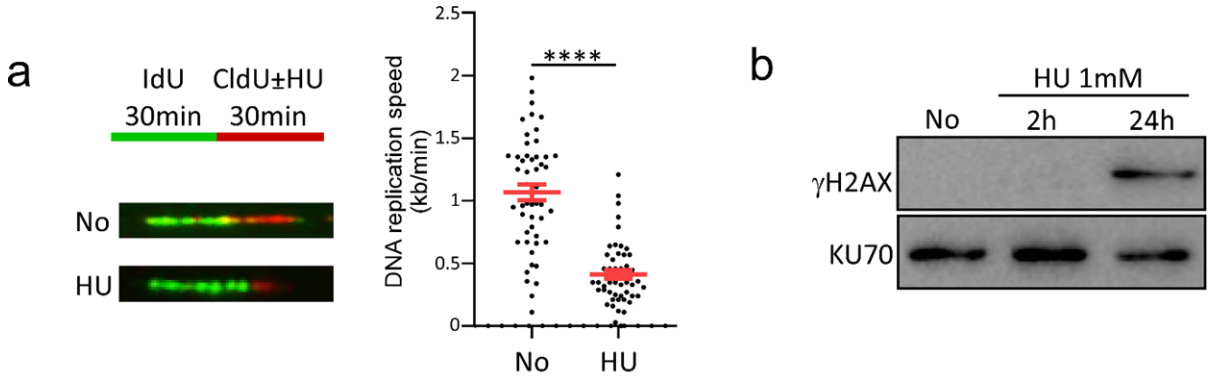


Fig. R1
Analysis of fork stalling and fork breakage upon HU treatment.
a, Schematic drawing of DNA fiber assay to measure replication speed upon HU treatment (left top). U2OS cells were first labeled with 200 μM IdU (green) for 30 min, washed with PBS, and then labeled with 40 μM CldU (red, with or without 1 mM HU, 30 minutes). Representative DNA fiber images (left bottom) and the quantification of CldU labeling length to indicate replication speed (right) by the scatter plot are shown.
b, γH2AX Western blot analysis was conducted before and after treating U2OS cells with HU (1 mM, 2h and 24h) with KU70 as a loading control.

5. - "BIR utilization in 53BP1-deficient cells results in a synthetic lethal interaction between the 53BP1 and BIR pathways, providing new opportunities for targeted cancer treatment."
While the synthetic lethality effects seem very clear, TP53BP1 mutation or loss, similar for TIRR amplification, is a low frequency event in cancers. The frequencies should be properly addressed in the discussion besides the current phrase “TIRR amplification is frequently observed in a wide range of cancers, with the highest frequencies present in breast, prostate and pancreatic cancers”.

Response: The statement regarding the high frequency of TIRR amplification in our manuscript was initially based on the literatures [25-27]. We have updated it in this revision by accessing the cancer genomics data sets through cBioPortal. We have confirmed that TIRR is frequently amplified across various cancer types (Fig. S12d), but the top three cancers (breast invasive carcinoma: 4.06%, cholangiocarcinoma: 2.78%, diffuse large B-cell lymphoma: 2.08%) with highest frequencies are not the same as those in our cited references. We have revised the relevant parts and added the percentage of TIRR amplification for the top three cancers with highest frequencies.

6.- The synthetic lethal effect observed in U2OS cells is very promising, is synthetic lethality similarly observed for shPOLD3? It would strengthen the synthetic lethality effect observed by repeating the 53BP1-KO +shPIF1 depletion in at least one other cell line model, possibly a model with inherent BRCA-deficiency to show the independence of this lethality to BRCA1/2.

Response: We have included data of cell viability in U2OS WT and *53BP1*-KO cells with and without depleting POLD3 (Fig. S10b). We also examined cell viability with and without depleting PIF1 or POLD3 in RPE1 WT and *53BP1*-KO cells (Fig. S10d and Fig. S10e).

In addition, we used BRCA1-deficient ovarian cancer cell line UWB1 and showed that depleting 53BP1 causes BRCA1-deficient UWB1 cells resistant to PARP inhibitor Olaparib. Significantly, inactivation of PIF1 sensitizes the resistant UWB1+53BP1sh cells to Olaparib (Fig. S11b).

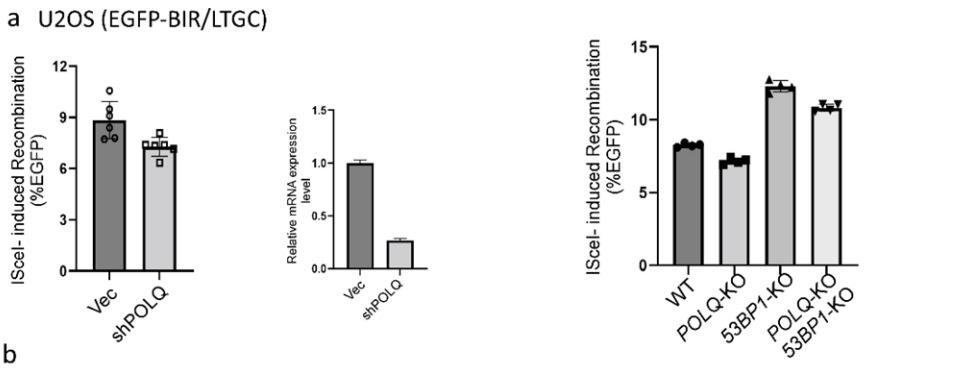
7. - Fig2: "With our reporter system, we observed local jumping/template switching in the BIR-EJ events in WT U2OS cells (14.6%), with an increase in 53BP1-KO cells (22.6%). Most jumping/template switching events contain MH sequences at the jumping sites (WT: 72.0%; 53BP1-KO: 77.8%) (Fig2c)"
Is this data significant over multiple experiments? Seems a small increase and possibly due to variance in repair outcomes, and will probably level out after sufficient clones are analyzed. Otherwise, I suggest rephrasing the conclusions drawn on these data.

Response: We have conducted another set of experiments analyzing BIR repair products in U2OS (EGFP-BIR/LTGC) *53BP1*-KO cells (Fig. 2 and Fig. S2). We detected a notable but not statistically significant increase in jumping/template switching events when comparing WT to *53BP1*-KO cells (P value: 0.0942). Also see response to Reviewer 1, point 3. We have modified the text.

8. - Fig2a: "suggesting that MMEJ is the major mechanism for end joining to complete BIR-EJ (Fig. 2a)"
Do the authors have evidence of SSA as a method to complete BIR with this reporter? Or is that

outcome not possible due to lack of an appropriate homology domain for SSA? If so, it would be fair to state that BIR-EJ works via MMEJ as can be judged by this reporter specifically. Furthermore, larger deletions due to extensive end resection are generally repaired via SSA. PolQ-KO or depletion should be done to show it is really MMEJ that drives BIR-EJ events.

Response: In WT cells, analysis of the repair junctions reveals that ~73% of the BIR-EJ events contain 1-6 bp (Ave: 2.5 bp) microhomology (MH) at the breakpoints, suggesting that MMEJ is involved (Fig. 2a). As SSA requires at least 20 bp homology, our reporter is not suitable for analyzing SSA involvement in mediating BIR-EJ. We have modified the statement as suggested.



b

	WT	*POLQ*-KO-22
Total green clones	30	28
BIR-EJ (>0.9 kb, <3.8 kb)	23 (23/30:76.7%)	24 (24/28: 85.7%)
BIR-SDSA (3.8 kb)	7 (7/30:23.3%)	4 (4/28: 14.3%)
Average tract length	2214.3 bp	2101 bp
BIR-EJ with MH at EJ sites	18 (18/23: 78.3%)	9 (9/24: 37.5%)
Size of MH	1-6 bp (Ave: 2.6 bp)	1-6 bp (Ave: 2.0 bp)

R2.
The Role of POLQ in BIR-EJ.
a, U2OS (EGFP-BIR/LTGC) cells, infected with lentiviruses encoding POLQ shRNA with a vector control (left) or harboring either *POLQ*-KO, *53BP1*-KO or *POLQ*-KO-*53BP1*-KO (right), were assayed for BIR by determining the percentage of EGFP positive cells with FACS analysis 5 days post-infection of I-SceI lentiviruses following puromycin selection.
b, A symmary displaying the features of BIR repair products from single green clones derived from WT and *POLQ*-KO (EGFP-BIR/LTGC) reporter cell lines after I-SceI cleavage. Genomic DNA extracted from single green clones was characterized by Sanger sequencing following PCR to determine the repair junctions and repair products.

Regarding POLQ, we are currently engaged in another project, specifically focusing on the study of the end joining mechanism underlying BIR-EJ. We depleted POLQ and also generated *POLQ*-KO in U2OS (EGFP-BIR/LTGC) cell line. We found that loss of POLQ activity only slightly reduces BIR-EJ frequency (Fig. R2a left). However, analysis of repair junctions of the BIR-EJ repair products derived from single green clones reveals a significant reduction of the BIR-EJ events using MH for end joining (78.3% in WT and 37.5% in *POLQ*-KO cells) (Fig. R2b). These data suggest that POLQ is involved in BIR-EJ, but in its absence, NHEJ and/or another alternative MMEJ pathway, which uses less homology, are likely used instead to mediate EJ. We are conducting more experiments to investigate the potential involvement of NHEJ in BIR-EJ, and search for factors required for BIR-EJ in POLQ-deficient cells, aiming to define the alternative MMEJ pathway(s). We have also knocked out *POLQ* in *53BP1*-KO cells, and observed a small reduction of BIR in *POLQ*-KO/*53BP1*-KO cells compared to *53BP1*-KO cells, similar to the effect of *POLQ* KO in WT cells (Fig. R2a right), suggesting that alternative end joining pathways to POLQ are also used for BIR-EJ when 53BP1 is deficient. We anticipate completing this study in the near future.

9. - Fig3a: 53BP1-KO cells show an increase in HR/STGC events of ~100%. Knockdown of PIF1 or POLD3 reduces this to WT levels again, suggesting the increase in HR is solely due to

BIR repair events. The authors claim this is due to seDNA caused by nicks. Can the authors explain how this is possible with I-Sce1 induced breaks?

Response: At seDSBs after fork breakage, BIR is activated to promote repair of broken forks (Fig. S4a left). This is largely due to PCNA ubiquitination and PIF1 recruitment to broken forks. However, at deDSBs, BIR is suppressed in wildtype (WT) cells and HR (STGC) is used preferentially (Fig. S4a right top, WT). In this study, we provided evidence that 53BP1 pathway plays a critical role in suppression of BIR at deDSBs. When 53BP1 is deficient, Polα-primase-mediated end fill-in DNA synthesis on ssDNA overhangs at deDSBs is stalled, leading to PCNA ubiquitination (Fig. 5a, IR) and PIF1 accumulation at deDSBs (Fig. 4b, IR). The enrichment of PCNA-Ub and PIF1 at deDSBs in *53BP1*-KO cells is reminiscent of broken forks with seDSBs, where PCNA-Ub and PIF1 are enriched after HU treatment in wildtype cells (HU/WT, Fig. 5a and Fig. 6c right). We propose that upon the loss of the 53BP1 control, BIR mechanism is established at deDSBs due to assembly of BIR replisomes facilitated by PCNA-Ub and PIF1 enrichment (Fig. S4a, right bottom, 53BP1-deficient), a situation similar to the onset of BIR at seDSBs in WT cells (Fig. S4a left). Therefore, when BIR replisomes are assembled at seDSBs on broken forks in WT cells and at deDSBs in 53BP1-deficient cells, BIR mechanism is used for both HR (STGC) and BIR (LTGC). As a result, increased HR in 53BP1-deficient cells at deDSBs after I-SceI cleavage is promoted by the BIR mechanism.

10. - In Fig4 and to some extent fig5 and 6, PLA is used to monitor localization of Flag-PIF1 to yH2AX or PCNA after 4Gy irradiation.
I have a number of concerns for these experiments. First of all, Flag-PIF1 is stably expressed, is this on top of endogenous Pif1 and how strong is the expression of Flag-Pif1 or EGFP-Pif1?

Response: Flag-PIF1 is expressed in WT cells in the presence of endogenous PIF1. We performed qPCR to determine Flag-PIF1 expression and showed that the expression of Flag-PIF1 is ~1.8 fold over endogenous PIF1 (Fig. S5b).

Second, with EGFP or Flag-tagged PIF1, I would expect to see standard immunofluorescence of localization to yH2AX. Why was PLA used for PIF1 staining?

Response: We used Flag-PIF1 and performed immunostaining of Flag-PIF1 and γH2AX, and showed that colocalization of Flag-PIF1 and γH2AX is significantly increased in *53BP1*-KO cells compared to WT cells (Fig. S6a).

Third, why was PLA used for PCNA and yH2AX localization in 4e, and 4f? A number of the PLA stainings could be done with conventional immunofluorescence, especially when analyzing DNA lesions induced by 4Gy irradiation.

Response: We performed immunostaining of PCNA and γH2AX, and showed that colocalization of PCNA and γH2AX is significantly increased in *53BP1*-KO cells compared to WT cells (Fig. S6b).

Fourth, typically, loss of 53BP1 results in an increase in HR following irradiation at 4Gy or HU treatment, and measured by increased RAD51 foci at yH2AX. Is such an increase observed in

these cells, and is that similarly overlapping with PCNA and PIF1? If not, this would indeed show there is a BRCA1/RAD51 independent pathway that utilizes BIR in absence of 53BP1.

Response: We examined colocalization of RAD51 foci with γH2AX, PCNA and Flag-PIF1 after IR (4 Gy). We observed a moderate increase of RAD51 colocalized with γH2AX, but a substantial increase of colocalization of RAD51 with PCNA and Flag-PIF1 in *53BP1*-KO cells compared to WT cells after IR (4 Gy) (Fig. S15).

Lastly, although as stated ~100 nuclei were measured for each sample, the experiments with PLA in figure 4 and 5 seem to be derived from one biological replicate in each panel. If this is not the case, it would be appropriate to show the medians of each biological replicate, and perform statistical testing on the repeated medians instead of taking all data points together. If these data do come from single biological replicates, ilt would be prudent to repeat the crucial panels at least with multiple experiments.

Response: We have repeated three sets of experiments for all PLA experiments and ~100 nuclei were analyzed in each experiment. See more detailed description in the Response above for Comment Point 3.

11. -Fig5 b,c,d These experiments use irradiation to produce deDSBs. Why are replication forks mentioned here (page9 "these data... ssdna overhangs."? If this related to the HU treatment used in Fig5a, then please show the effect of shPRIM or CD437 treatment on HU-treated cells, in both WT cells and 53bp1-deficient cells.

Response: We apologize for the ambiguity in this sentence on page 9. Our intention was to describe the stalling of Polα-primase-mediated end fill-in DNA synthesis on ssDNA overhangs at DSBs, rather than general fork stalling. We have revised that sentence in the main text.

12. -Fig 5i: %EGFP positive cells in FIG5i is around 14% in BIR/LGTC for WT cells, this is much higher compared to previous assays in Fig1 and Fig3 where it's ~5% for WT cells, and this high percentage is seen only in 53BP1-KO cells. Can the authors explain this?

Response: In this experiment, we used a new batch of I-SceI lentiviruses before determining the virus titer, and consequently a higher titer of I-SceI lentiviruses was used compared to other experiments. We have repeated this experiment using the standard titer of I-SceI lentiviruses (Fig. 5i).

13. -Fig6a-e, Were these experiments not conducted in untreated cells? Furthermore, similar as in Fig4 and Fig5, it seems these are single experiments performed. Where the effect sizes in Fig4 and 5 are substantial, for panels such as PIF1-yH2AX PLA for example in fig6 the effect sizes are small and warrent repeated measurements.

Response: We have included no treatment controls and repeated the experiments three times. See revised Figures 4b, 4c, 4d, 4e, 4f and 4g; Figures 5a, 5b, 5c, 5d, 5e, 5f, and 5g; and Figure 6a, 6b, 6c, 6d and 6e. Also see Response to Comment point 3 described above.

14. Fig7. The synthetic lethality observed for 53BP1-KO cells with PIF1 depletion, where it is clear these cells no longer proliferate or die within two or three days of plating up until 5days. In the STGC or LTGC reporter assays, a 5 day incubation period is also present before measurement of %EGFP cells. Is a similar loss of viability or proliferation observed in those cells? As in these reporter assay experiments PIF1 depletion is similarly performed in 53BP1-KO cells, viability loss could seriously affect the %EGFP outcomes.

Response: For the synthetic lethal interaction assay, the general protocol is described below. We infect cells with lentiviruses encoding shRNA, and 24 hours later, viruses are removed (Day 0 post viral infection: post-D0), followed by changing to medium with drug (e.g. puro) for 2-day selection of cells expressing shRNAs (post-D2). Cells are recovered for three days without drugs and then are plated for cell growth analysis (post-D5). The Day 1 of the growth curves presented in Fig. 7a, 7b and 7e is post-D5 after shRNA lentiviral infection (virus removal).

For the reporter assays involving the depletion of genes that are synthetic lethal with each other, to minimize cell death, we purified shRNA lentiviruses to achieve high titers and used sufficient amounts of viruses to reach 100% infection efficiency without requiring drug selection. After infection (post-D0), cells are recovered for a day in medium without drug (post-D1), followed by I-SceI (or Cas9) lentiviral infection for one day (post-D2) and FACS was performed 5 days after I-SceI infection (or Cas9) (post-D7). The date we performed FACS analysis corresponds to Day 2 on the growth curves in Fig. 7a, 7b and 7e, when the difference in cell growth is not so significant with expression of different shRNAs.

To more carefully determine BIR in reference to cell growth, we performed new experiments. For cell viability, we infected cells with shRNA lentiviruses for 24 hours (post-D0) (see below Fig. R3a) and after one day recovery (post-D1), cells were plated. We designated the cell plating date as Day 1 for the growth curve in Fig. R3b. Slower growth in *53BP1*-KO cells with PIF1 depletion is detected on Day 6 (post-D6) and becomes more evident on Day 7 (post-D7). The date we performed FACS analysis (shown in R3c) corresponds to Day 6 on this growth curve (Fig. R3b).

In order to assay for BIR efficiency at the time point when cells do not show growth defect. We adopted tet-on inducible I-SceI system to U2OS (EGFP-BIR/LTGC) cells, and induced I-SceI expression with doxycycline (DOX) for 48 hours post shRNA lentiviral infection (Fig. R3a). We performed FACS analysis 1 day after removal of DOX (post-D3), when there was no detectable growth defect of *53BP1*-KO cells with PIF1 depletion (Day3 in growth curve, Fig. R3b). We observed significant reduction of BIR when PIF1 is depleted in cells expressing 53BP1 shRNA (Fig. R3c), similar to the results after I-SceI lentiviral infection shown in Fig. 1d.

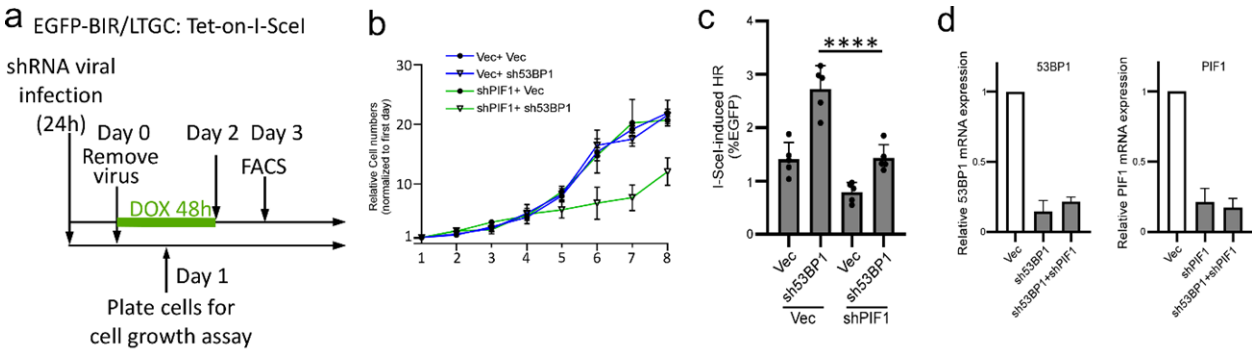


Fig. R3
EGFP-BIR/LTGC assay using Tet-on I-SceI system.
a, The timeline of the key steps for the BIR assay using the Tet-on-I-SceI system and the cell growth assay.
b, The growth curves of U2OS (EGFP-BIR/LTGC-tet-on I-SceI) cells were plotted after infection with lentiviruses expressing shRNAs targeting 53BP1 and PIF1, with a vector control (Vec).
c, U2OS (EGFP-BIR/LTGC-tet-on I-SceI) cells were infected with lentiviruses encoding 53BP1 and PIF1 shRNAs with a vector control, followed by inducing I-SceI expression with doxycycline (DOX, 2 μg/ml) at Day 0 (see **a**) for 48 hours. FACS analysis was performed 1 days after removal of DOX (Day 3).
d, qPCR analysis was performed to determine the shRNA knockdown efficiency of 53BP1 and PIF1.

Minor points:
- Can the authors state the aim of this study in the introduction, before summarizing the results?

Response: We have now included a description of the aim of this study in the introduction.

- "However, considering that SSA is mediated by a deletion mechanism between repetitive sequences it may be too specialized to salvage a general HR defect."
Which is the case for BIR as well, as the authors mention the 100x-1000x increases mutation rate for BIR. Therefore, I dont see the relevance of this statement on SSA. It would seem both SSA and BIR are not refered repair pathways unless specifically needed.

Response: We have removed this sentence.

- "Notably, long tract gene conversion (LTGC) generally employs the BIR mechanism" The mention of LTGC on page3 first paragraph seems out of the blue. It is unclear if LTGC is a repair mechanism on its own or if its part of BIR. Can the authors clarify what this sentence refers to?

Response: We have removed this sentence.

- Throughout the text the authors make use of BIR-mode, BIR mode, BIR mechanism, BIR pathway. Please use a consistent term for BIR.

Response: We have changed BIR mode to BIR mechanism.

- Multiple mentions in results of "green cells", please change to "EGFP+cells" which is what is measured.

Response: We have changed “green cells” to EGFP-positive cells throughout the text.

- "and we determined the role of 53BP1 for BIR in BRCA1-deficient cells. "Unclear statement, please rephrase.

Response: We have rephased this sentence.

- In this study, we showed that deficiency in 53BP1 leads to uncontrolled assembly of BIR-like replisomes"
If with "in this study" the authors mean their previous study (ref13), then this should be more clearly stated.

Response: Sorry for the ambiguity of this sentence. We do refer to our current 53BP1 study. We did not perform BIR analysis in 53BP1-deficient cells in the previous study[21]. We have modified this sentence to “In our current study---".

- "this data suggests that while generating ssDNA overhangs is necessary, their length is not the key determinant in triggering the overloading of PCNA and PIF1 to IR-induced DSBs in the absence of 53BP1."
This should be confined to the discussion section, or experimentally address why the ssDNA overhang length is relevant here.

Response: We have performed the end resection experiments and confirmed that the end resection extent in BRCA1-ΔBRCT/*53BP1*-KO cells is comparable to that in WT cells (Fig. S5j). However, the recruitment of PCNA and PIF1 to IR-induced DSBs in *53BP1*-KO/BRCA1-ΔBRCT cells is significantly more compared to WT cells (Fig. 4g and S5i), suggesting ssDNA overhang length is not the key determinant for PCNA and PIF1 recruitment.

- Fig4a: Please show quantification of the experiment where these images came from.

Response: We have performed quantitation analysis of GFP-PIF1 recruitment shown in Fig. 4a and the results are presented in Fig. S5a(n=5).

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors addressed all my questions.

Reviewer #2 (Remarks to the Author):

The authors have adequatly addressed my comments on the first manuscript and I see no further need for additional experiments.

The description of RAD52 involvement is now described fully in the discussion section, which I suggest to move partly to the results section in Fig1 or Fig3, while keeping the speculation to the discussion.

Furthermore, I would suggest to incorporate some of the data shown to reviewers (R1b, R3b+c) in the final manuscript for transparency.

Response to the Second Reviewer 2:

- The description of RAD52 involvement is now described fully in the discussion section, which I suggest to move partly to the results section in Fig1 or Fig3, while keeping the speculation to the discussion.
  Response: RAD52 data has been moved from Fig. S14b to Fig. 1e.

- Furthermore, I would suggest to incorporate some of the data shown to reviewers (R1b, R3b+c) in the final manuscript for transparency.

  Response: R1b has been placed to S9g, and data in R3 are now in Fig. S16.