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PIF1 helicase promotes break-induced replication in mammalian cells

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1st Editorial Decision 20th Feb 2020

Thank you for submitting your manuscript on PIF1 roles in break-induced replication and CFS protection to The EMBO Journal. It has now been assessed by three expert referees, whose comments are copied below for your information.

As you will see, the referees are somewhat divided in their opinion - while reviewer 3 is supportive of publication pending satisfactory revision of a number of specific issues, reviewer 2 (who is particular familiar with CFS expression/protection) does not consider key conclusions of the study sufficiently supported at the current stage. Finally, referee 1 expresses interest in principle, but lists a number of currently open questions that would need further investigation in order to turn the study into a more significant advance of our understanding.

This leads me to conclude that the study is at present not sufficiently conclusive and insightful to justify immediate, concrete further consideration for EMBO Journal publication, at least in its current form. However, I realize that the study may well become a more compelling candidate for an EMBO Journal article if extended along the lines suggested by all three referees, and after more careful re-investigation of many of the CFS analysis-related concerns, as well as the uncertainties around BIR vs. MiDAS involvement.

Given that it is difficult to predict the outcome of such required further work, which may well confound the original interpretations and result in decreased overall significance, I am at this stage not able to make strong commitments regarding eventual acceptance; but would nevertheless like to give you an opportunity to respond to the referees' comments by way of a substantially revised manuscript. Alternatively, I would also be open to discussing the manuscript and its reviews with the editors of our open-access partner journal Life Science Alliance, in case you should be interested in exploring options for rapid publication of a less-extensively revised version.

Should you decide to attempt revision for The EMBO Journal, please be reminded that our policy to allow only a single round of major revision will make it important to comprehensively answer to all points raised at this stage.

Referee #1:

In this manuscript, Li and Wang et al. have studied the role of break induced replication (BIR) in mediating long track and short track gene conversion upon generation of double strand breaks in mammalian cells. They have generated robust reporters to assay LTGC (BIR)/STGC in mammalian cells and their work identifies distinct dependencies of replication independent and dependent DSB repair on BIR replisome components. This paper demonstrates a novel role for Pif1 in BIR and protecting common fragile sites. The authors further demonstrated that both the helicase null Pif1 mutant and the breast cancer associated Pif1 L319P mutation were defective in BIR.

1) The authors have shown that LTGC events is Rad51 and PolD3 dependent (Fig 1C and Fig 2E). This is in contrast to the previous studies which show that LTGC events in mammalian cells are Rad51 independent and suppressed by BRCA1, BRCA2, CtIP and Rad51 (Chandramouly et al. 2013 and Willis et al. 2014). Is there a temporal switch to explain the Rad51 dependent and independent processes? Also, experiments with BRCA1, BRCA2, CtIP KO or mutant cells might delineate the differences in these events.

2) The authors have shown that DNA synthesis at common fragile sites uses the BIR mechanism which is Rad51 dependent (Fig 2E, F and G). Is this process distinct from MiDas which is Rad51 independent (Minocheromji et al. 2015)? The authors have also shown a role for Pif-1 in MiDas (Fig. 7A) and demonstrate that Pif1 is enriched at CFS after treatment with APH (7B, 7C). Determining the cell cycle phases in which the EGFP+ events are observed using the EGFP-Flex1-BIR-5086 and EGFP-Flex1-STGC-1541 reporters and studying the dependency of this BIR mediated DNA synthesis on Mus81-EME1 may again help distinguish these events.

3) The authors have shown that LTGC events are favored over STGC events at collapsed replication fork generated by Cas9n using a dual reporter system (Fig.4C). This reporter is an elegant system to study both types of events and understand the DSB response that is preferentially used and also to study the whether there is any temporal switch between these events. This reporter line if combined with the Flex system can help understand which type of repair (STGC/LTGC) occurs predominantly at CFs. The experiments mentioned in the previous point (2) with this dual reporter at CFs will help in determining whether there is a temporal switch between the LTGC and STGC events at CFS.

4) Classically BIR entails long tracks of DNA synthesis. The authors have shown that STGC upon replication fork collapse uses BIR replisome components and also is Rad51 dependent. But whether this is actually BIR or a SDSA event requiring components of BIR machinery is not very clear. The dependency of this process on other classical SDSA components can help understand this better.

5) The authors have demonstrated the role of PIF-1 in LTGC and STGC events at CFS and in MiDas (Fig. 2E, 3C, 4 A-D). They have shown the sensitivity of PIF-1 KO cells to HU and ApH

(Fig.2A-B) and its role in in protection of CFs (Fig.7A-D). The sensitivity of PIF1-KO cells to HU or APH suggests the cells are more sensitive to replication stress. Considering its role in protection of CFS, are PIF-1 KO cells sensitive to ATR inhibition? Are the L319P and E307Q mutants of PIF-1 also defective in the STGC at CFS (EGFP-Flex1-STGC-1541)?

6) The authors have shown that replication independent DSBs use BIR mechanism for LTGC but not for STGC (Fig. 1C and D, 4D) But replication dependent DSBs (generated when replication fork encounters ssDNA nicks) use BIR mechanism for both LTGC and STGC requiring PIF-1 (Fig 2E, 3C 4D). It would imply that PIF-1 KO cells should be more sensitive to DNA damaging agents that generate ssDNA nicks. Experiments studying the sensitivity of PIF-1 KO cells to such agents like MMS or PARPi will further strengthen this data.
7) Thea authors demonstrate a role for FANCM in BIR, and a novel synthetic relationship between FANCM and POLD3, and FANCM and PIF1. The authors demonstrate that decrease in FANCM with shRNA increases the frequency of BIR at Flex 1 sites, and that this increase is dependent on POLD3, RAD51 and PIF1 by using shRNA depletion (6A). Additionally, the authors show that shRNA depletion of PIF1 or POLD3 in FANCM KO cells causes a decrease in cell viability (6D). The authors call this a novel synthetic lethal relationship, but POLD3 is essential in humans.
8) The authors have shown that PCNA and RFC1 are required for BIR. The authors used shRNA to silence expression of RFC1 or PCNA and demonstrated a reduction in HU induced STGC when RFC or PCNA were depleted. Additionally, they showed that STGC after I-SceI cleavage did not require PCNA or RFC1, further establishing that there are two distinct processes happening.

Minor points

1) Dependence on PCNA for Pif-1 recruitment can be shown at the endogenous FRA3B and FRA16D sites which would further strengthen their data.
2) PIF-1 is mentioned as FIP-1 in the discussion.
3) POLD3 is a stocihiometric component of Pol delta. It is not BIR specific and should not be oversimplified this way in the text.

Referee #2:

In the manuscript "PIF1 promotes break-induced replication to suppress common fragile site instability" Li et al presents new EGFP-based reporter systems to study long -track gene conversion (LTGC) and short-track gene conversion (STGC). They find that BIR is used for LTGC and also for STGC, when replication forks collapse. They neatly show that oncogenic stress induces PIF1 dependent BIR. Furthermore, they identify a synthetic lethal genetic interaction between PIF1 and FANCM.

Whilst the study presents some useful tools and interesting insight into mammalian BIR it also suffers from lack of coherence and clarity - mainly with respect to claims regarding CFSs. This is exemplified by the following: 1. HU is used for most experiments in the manuscript despite the fact that APH is the drug that induces CFSs. 2. The BIR that takes place at CFSs in mitosis (MiDAS) is independent of RAD51, thus it is different from the mechanism that is described throughout this

manuscript. 3. The flex1 sequence from FRA16D is probably not the reason for FRA16D fragility. This is clear from the fact that CFSs need to be transcribed to become fragile. Therefore AT-rich sequences such as flex1 clearly do no underlie fragility at CFSs per se.
To improve the manuscript the authors should tone down the link to CFSs. The current data does for instance not justify the title. Furthermore, it would strengthen the manuscript if the authors included analysis of the involvement of RAD52 in the different reporter assays.

Major comments
Figure 1.
The authors should provide evidence that the suggested mechanism is indeed responsible for restoration of the GFP reading frame. They should perform PCR, sequencing and/or southern blots from GFP positive cells to show that they have undergone the suggested genetic change at the cassette. This is a general shortcoming. The authors need to validate all the new reporter-cassette that they present in the paper by showing that they recombine as suggested on the schematics.

Figure 1F.
The authors should comment on the activity that remains after PIF shRNA and especially after PIF1 KO. Around 33% BIR activity remains after PIF1 knockout - is this a redundant pathway? They should analyse some of the GFP positive clones from PIF1 knockouts to see if they are different from the ones that arise in PIF1 positive cells

Figure 2.
How do the authors explain that according to their fiber analysis 2 mM HU stalls all replication forks and at the same time their model seems to suggest that upon HU replication fork stalling takes place specifically at the flex region. This seems unlikely. The authors should provide data showing whether cells perform any DNA synthesis during the 24 hours that they are treated with 2 mM HU. If no DNA synthesis is observed the authors should explain how complete HU-induced stalling for 24 hours induce recombination at their BIR cassette.

Figure 3

It simply seems that BIR is used at stalled replication forks explaining why BIR is used for STGC with the flex cassette. This would fit the idea that BIR is used at seDSBs. It is not clear to me how the authors can be sure that deDSBs are formed at stalled replication forks in the flex cassette. The authors should provide data that proves that deDSBs are formed as indicated in the hypothetical model shown in figure 3B.

Figure 4
The authors should show the cell cycle distribution of cells depleted for PCNA and RIF1. The authors should more explicitly discuss and investigate the possibility that replication fork will never encounter the reporter cassette upon PCNA and RIF1 depletion.

Minor comments
p. 24 line 5
The authors claim that "chromosomal breaks and gaps in CFSs are accumulated when PIF1 is deficient". The authors have not shown that this is the case. The authors should use FISH on

metaphase spreads to probe for breaks at a few CFSs if they want to claim that CFS breakage is elevated in the absence of PIF1.

Figure 2A
The triangle and square symbols are missing in the APH assay

Figure 7 E, F, G and H
Why have the authors not shown/included the PIF1 knockout in these assays?

Figure S2 A
The authors should also show efficiency of PIF1 depletion by western blot.

Figure S3 A. The authors should show efficiency of FANCM depletion by western blot.

Referee #3:

Understanding how DNA repair is regulated in eukaryotic cells is an exciting and important area of research. The new study from Dr. Wu's lab is focused on one important pathway of double-strand break (DSB) repair called break-induced replication (BIR) in human cells. BIR is a pathway that mainly repairs one-ended DSBs that can result from replication collapse or from telomere erosion. BIR has been intensively studied in yeast, and these studies implicated BIR as a source of genetic instabilities associated with cancer. However, the role of BIR in DNA repair in human cells and its genetic control remain poorly investigated. The results of this study fill an important gap in our knowledge by shedding light on the contribution of BIR in repair of DNA breaks resulting from different sources in human cells. Also, this study demonstrates that similarly to what was shown in yeast, PIF1 plays an important role in BIR in humans. Importantly, the authors demonstrate that DSBs initiated by endonuclease cuts proceed through BIR involving PIF1 when they are repaired by long-track gene conversion (LTGC), but not when they lead to short track gene conversion (STGC). Even more interestingly, the authors demonstrate that when DNA breaks are initiated by replication fork collapse, both STGC and LTGC proceed through BIR and require PIF1. In addition, the authors show that Pif1 is recruited to a common fragile site (CFS) and that the breast cancer-associated PIF1 mutant L319P is defective in BIR, suggesting a direct link of BIR to the oncogenic process. Together, the paper represents an extremely elegant and throrough study. The number of different experimental systems that have been developed for this study and the quality and quantity of results obtained by the authors is simply astonishing. Together the paper represents an important breakthrough and will be of great interest for the diverse readership of EMBO. However, the authors need first to address several important questions.
Specific questions/comments:
1. In many experiments shown in the manuscript the authors deplete products of genes encoding major replication factors by using shRNA. For example, they deplete POLD3, known to be a part of the Pol delta complex that might be important for the stability of the entire complex. Similarly, the authors deplete RFC, PCNA, and also PIF1 (the latter might participate in processing of normal Okazaki fragments). In all cases of these depletions the authors observe the reduction of repair in various constructs. This brings the question of how the authors could exclude that the observed effect results from either change in the efficiency of break formation (for example due to sickness of a fraction of cells resulting from weakened replication) or from a massive stalling of replication forks

leading to sequestration of some other factors that are needed for BIR.
2. It is very important that the authors explain why depletion of many replication factors (PIF1, RFC, POLD3, etc) results in a decrease of BIR by approximately half. Where is this remaining part of BIR (independent of these replication factors) coming from? After all, it is unlikely that it results from less than 100% depletion of replication factors since the amount of these proteins in the cell should be very high as compared the amount that should be needed for the repair of one break. So, if the depletion is inefficient one would expect not to see any effect at all. All in all, the authors should discuss the nature of the remaining BIR following depletion of POLD3, PIF1, RFC, etc.
3. Do the authors know how the observed BIR is terminated? Is the termination mediated by NHEJ, MMEJ, etc? Did they ever try to sequence the junctions at termination positions?
4. While discussing the effects of depleting FANCM on the repair at EGFP-Flex1-BIR-5086, the authors assume that the increased frequency of BIR is explained by the increased frequency of replication collapse at positions of secondary structures. However, another possibility is that increased BIR results from a decrease in the anti-BIR role that FANCM might play by unwinding D-loops that are initiating BIR (based on results obtained using mph1 mutants in yeast). It will be important that the authors address this possibility.
5. P. 17, line 9: It should be "PIF1" instead of FIP1.
6. P. 19, the line before the last one: "It may take some time for REC to decide that BIR is the choice..." It remains possible that in the presence of two broken ends created by an endonuclease, the rare usage of BIR results not from the "choice" that is guided by REC, but simply from the interruption of ongoing BIR-like synthesis by an invasion or annealing of the second (catching) end that leads to the interruption of synthesis. This end might simply be absent in the case of replication collapse, which leads to a prevalence of BIR because it is not stopped by another end.
7. P. 20, line 9: "may convert to replication forks using conventional BIR mechanism." The authors need to explain what this means.
8. P. 20., line 13-14. "BIR is used at collapsed forks and BIR-specific replisomes promote more processive replication than BIR-independent GC." What kind of processive replication are the authors talking about here? Do they mean that GC is associated with some particular type of replication which is not very processive?
9. P. 20, line 20: "PIF1 and POLD3-deficient human cells are sensitive to replication stress ...., suggesting that BIR is essential mechanism to repair broken forks..." Why do the authors think that the sensitivity is explained by BIR defect rather than by the importance of these proteins for S-phase replication? Similarly, on p. 24, the authors mention that inviability of pfh1-L430P in S. pombe and cancer predisposition of patients with PIF1 mutation results from the role of PIF1 in BIR. How can it be excluded that the problems stem from the role played by PIF1 in normal replication?
10. Fig. 1 (panels C, D, E, F): was there any effect of shPOLD3 on the cells' viability? The same information will be important for the cases where other replication factors were suppressed.
11. Fig. 7. When comparing the effect of PIF1 on Rad51-dependent BIR with its effect on MIDAS, the authors need to mention that MIDAS is RAD51-independent and may represent a different type of BIR or different type of DSB repair.

Reviewer #1:
In this manuscript, Li and Wang et al. have studied the role of break induced replication (BIR) in mediating long track and short track gene conversion upon generation of double strand breaks in mammalian cells. They have generated robust reporters to assay LTGC (BIR)/STGC in mammalian cells and their work identifies distinct dependencies of replication independent and dependent DSB repair on BIR replisome components. This paper demonstrates a novel role for Pif1 in BIR and protecting common fragile sites. The authors further demonstrated that both the helicase null Pif1 mutant and the breast cancer associated Pif1 L319P mutation were defective in BIR.

We thank the reviewer for the valuable comments and suggestions.

1) The authors have shown that LTGC events is Rad51 and PolD3 dependent (Fig 1C and Fig 2E). This is in contrast to the previous studies which show that LTGC events in mammalian cells are Rad51 independent and suppressed by BRCA1, BRCA2, CtIP and Rad51 (Chandramouly et al. 2013 and

Willis et al. 2014). Is there a temporal switch to explain the Rad51 dependent and independent processes? Also, experiments with BRCA1, BRCA2, CtIP KO or mutant cells might delineate the differences in these events.

Response: The previous study by Scully's lab showed that when BRCA1, CtIP, RAD51 or other RAD51 paralogs are deficient, STGC and LTGC are both reduced after I-SceI cleavage, with more significant reduction for STGC than LTGC, thereby leading to an increase of the ratio of LTGC/STGC and a bias towards LTGC (Fig.2C in Chandramouly et al. 2013[1]; Fig.2A-2C in Nagaraju et al. 2009[2], and Fig.3A-C in Nagaraju et al. 2006[3]). They provided evidence that such increased LTGC/STGC ratio is due to impaired function for second end capturing when BRCA1 or other relevant players are deficient[1]. When they deleted the second end homology (non-invading strand), then the "gene conversion" with "single end" does not show a bias towards LTGC in BRCA1/CtIP-deficient cells (Fig.7A in Chandramouly et al. 2013[1]). They stated that "in the context of the one ended reporter, although overall HR retained dependence on BRCA1 and CtIP, loss of BRCA1/CtIP no longer influenced the balance between STGC and LTGC" (see discussion in Chandramouly et al. 2013[1]).

We have analyzed the repair products from EGFP-BIR-5085 reporter after I-SceI or Cas9 cleavage by sequencing the repair junctions of single green clones. We found that only 3.3% of the events completed replicating 3.8 kb and finished BIR by LTGC via SDSA, and the rest used end joining to finish BIR (Fig.1C and Fig.S14). We performed new experiments and showed that BIR scored by EGFP-BIR-5085 reporter in U2OS cells after I-SceI cleavage is significantly reduced when BRCA1, BRCA2 or CtIP is depleted by shRNAs, similar to the effect after RAD51 depletion (Fig.1D and Fig. S2C, Cas9). This suggests that BIR even when being completed with end joining is dependent on RAD51, BRCA1, BRCA2 and CtIP, which is consistent with Scully's findings from using "single end" DSBs to study LTGC[1]. We also analyzed STGC (green) and LTGC (red) in our competition reporter EGFP/STGC-mCherry/LTGC-5034 after Cas9 cleavage. We found that both STGC and LTGC are decreased to a similar extent when BRCA1, BRCA2, CtIP of RAD51 is depleted (Fig. S9A, top), but we did not observe an increase of LTGC/STGC ratio as reported by the Scully's lab. In addition to U2OS cells, we also performed the same experiments in HCT116 (EGFP/STGC-mCherry/LTGC-5034) cells and obtained similar results after I-SceI cleavage (Fig.S9B). Scully's lab used mouse embryonic stem (ES) cells for the study of LTGC/STRC, where HR is the dominant DSB repair pathway, whereas in human somatic cells, non-homologous end joining is a preferred pathway. It is possible that BRCA1 pathway has a unique activity in suppression of LTGC when HR is elevated in ES cells. We have added discussions in the manuscript.

In another study, Scully's lab showed that when replication fork is stalled at the replication fork barrier (RFB, induced by Tus/Ter), the absolute LTGC frequency is increased in BRCA1- and BRCA2-deficient cells[4], which is different from the increase of relative ratio of LTGC/STGC at endonuclease-induced DSBs[1-3]. We thus used Cas9 nickase (Cas9n) to induce DSB formation on forks in the EGFP-BIR-5085 reporter and found that BIR track length is substantially increased at broken forks compared to that at endonuclease-induced DSBs (Fig.2E and Fig.S14). However, depletion of BRCA1, BRCA2 or RAD51 also results in decrease of BIR at broken forks (Fig.S2C, Cas9n). Similar results were obtained when we induced fork breakage at Flex1 site in EGFP-Flex1-BIR-5086 reporter upon HU or APH treatment (Fig.S2D). We further used STGC and LTGC competition reporter EGFP/STGC-mCherry/LTGC-5034 and induced fork breakage by Cas9n. We showed that both STGC and LTGC are decreased when BRCA1 or BRCA2 are depleted with almost unchanged ratio of LTGC and STGC (Fig.S9A, Cas9n). Thus, our results from using nicks or Flex1 to induce fork breakage are

different from Scully's study that BRCA1- and CtIP-deficiency causes an absolute increase of LTGC at Tus/Ter (RFB)[4].

Based on the study in yeast, HR is rapidly induced at RFBs, but DSBs are usually not formed at RFBs[6-8], and HR at RFBs often exhibits the characteristics different from that at DSBs. For instance, upon DSB formation, BIR has a delay to initiate, but at RFBs, BIR is immediately launched[9,10]. Loss of RAD51 causes a reduction of deletion formation at RFBs, but induces a substantial increase of deletions at DSBs on broken forks induced by site-specific single-strand breaks[8,9]. Scully's lab also found that the key cNHEJ players XRCC4 and Ku70 suppress I-SceI-induced HR, but not Tus/Ter (RFB)-induced HR[5]. Thus, it is possible that the role of RAD51, BRCA1 and CtIP in LTGC at RFB is different from that at DSBs on broken forks (induced by nicks or Flex1); while RAD51, BRCA1 and CtIP suppress LTGC at RFBs, these HR players are required for both STGC and LTGC at DSBs on broken forks.

2) The authors have shown that DNA synthesis at common fragile sites uses the BIR mechanism which is Rad51 dependent (Fig 2E, F and G). Is this process distinct from MiDas which is Rad51 independent (Minocheromji et al. 2015)? The authors have also shown a role for Pif-1 in MiDas (Fig. 7A) and demonstrate that Pif1 is enriched at CFS after treatment with APH (7B, 7C). Determining the cell cycle phases in which the EGFP+ events are observed using the EGFP-Flex1-BIR-5086 and EGFP-Flex1-STGC-1541 reporters and studying the dependency of this BIR mediated DNA synthesis on Mus81-EME1 may again help distinguish these events.

Response: Our finding that BIR is dependent on RAD51 in asynchronized human cells is consistent with the previous observation by Halazonetis' lab (Fig.3D in [11]). However, POLD3-dependent MiDAS is RAD51 independent but requires RAD52. To understand this difference, we examined BIR using our EGFP-BIR-5085 reporter in both asynchronized cells and mitotic cells. Interestingly, despite that our reporter contains 1.3 kb homology for strand invasion, BIR scored in mitotic cells by the EGFP-BIR-5085 reporter is RAD51-independent (Fig.1E), which is different from the results in asynchronized cells (Fig.1D), but agrees to the observation that MiDAS is RAD51-independent.

It has been shown that in mitotic cells, DSBs are still sensed and γH2AX, MRE11 and MDC1 foci are localized to mitotic DSBs[12-16], but extensive end resection, CHK1 activation and RAD51 filament formation are abrogated, resulting in suppression of HR[17-21]. This is largely due to impaired recruitment of RNF8, RNF168 and BRCA1 to DSBs in mitosis[13,14,22]. Thus, because RAD51 pathway is impaired in mitotic cells, BIR becomes RAD51 independent and RAD52 dependent in mitotic cells (Fig. 1E).

Cell cycle distributions were determined before and after treatment of HU (2 mM, for 24 hours) as well as 3 or 6 days after removal of HU (Fig.S11F). The HR assay was performed 4 days after HU removal. We also depleted MUS81 prior to HU treatment and found that HU-induced γH2AX accumulation and Flex1-induced BIR were reduced upon MUS81 depletion (Fig.S6). This is consistent with the notion that MUS81 is required for generating DNA breakage at stalled forks[23,24] to induce BIR. The dependence of MUS81 is also found for MiDAS[25].

3) The authors have shown that LTGC events are favored over STGC events at collapsed replication fork generated by Cas9n using a dual reporter system (Fig.4C). This reporter is an elegant system to study both types of events and understand the DSB response that is preferentially used and also to study the whether there is any temporal switch between these events. This reporter line if combined with the Flex system can help understand which type of repair (STGC/LTGC) occurs predominantly at

CFs. The experiments mentioned in the previous point (2) with this dual reporter at CFs will help in determining whether there is a temporal switch between the LTGC and STGC events at CFS.

Response: We introduced Flex1 to the EGFP/STGC-mCherry/LTGC-5034 reporter and generated a new reporter Flex1-EGFP/STGC-mCherry/LTGC-5304. We showed that upon HU treatment, Flex1-induced gene conversion favors LTGC (Fig.S10). This is similar to the observation that LTGC is used more frequently than STGC at nick-induced DSBs (Cas9n) on broken forks, but is opposite to that at Cas9-induced DSBs where STGC is used more frequently (Fig.5B).

4) Classically BIR entails long tracks of DNA synthesis. The authors have shown that STGC upon replication fork collapse uses BIR replisome components and also is Rad51 dependent. But whether this is actually BIR or a SDSA event requiring components of BIR machinery is not very clear. The dependency of this process on other classical SDSA components can help understand this better.

Response: In yeast, BIR replication can proceed for hundreds of kbs to the end of a chromosome[26,27]. However, within the first 10 kb DNA synthesis during BIR, frequent template switching was observed, revealing repeated cycles of strand invasion, DNA synthesis and strand dissociation[28]. During this strand invasion and dissociation cycle, if disassociated strand can find homology at the other end of the break, BIR is finished by SDSA, but if not, strands would reinvade and BIR continues[29]. In addition, gap repair of double-ended DSBs with the gap size of several kbs shows dependence on Pol32 and is also thought to be mediated by BIR via SDSA[10]. Thus, BIR can be completed by SDSA.

In mammalian cells, at endonuclease-generated DSBs, BIR track length is short (rarely exceeding 3.8 kb), and newly synthesized invading strands are frequently dissociated from the template and BIR is completed either by SDSA (BIR-SDSA) or end joining (BIR-EJ) (Fig.1A, Fig.1C). Similar results were also observed by the Halazonetis group[11]. Thus, BIR and SDSA are not regarded as independent events.

BIR has a unique dependence on the non-essential Polδ subunit Pol32 in yeast, and this is used as a criterium to distinguish BIR from gene conversion. In mammalian cells, POLD3-dependence is also a feature of BIR. Based on our study, we believe that recruitment of PIF1 to form BIR replisomes is an important event to activate BIR. We propose to use POLD3 dependence and inclusion of PIF1 as a component of BIR replisomes to judge the use of BIR mechanism. Identifying additional components of BIR replisomes will be a focus for the future study.

5) The authors have demonstrated the role of PIF-1 in LTGC and STGC events at CFS and in MiDas (Fig. 2E, 3C, 4 A-D). They have shown the sensitivity of PIF-1 KO cells to HU and ApH (Fig.2A-B) and its role in in protection of CFs (Fig.7A-D). The sensitivity of PIF1-KO cells to HU or APH suggests the cells are more sensitive to replication stress. Considering its role in protection of CFS, are PIF-1 KO cells sensitive to ATR inhibition? Are the L319P and E307Q mutants of PIF-1 also defective in the STGC at CFS (EGFP-Flex1-STGC-1541)?

Response: PIF1-KO cells showed moderate sensitivity to ATR inhibitor AZD6738 (Fig. S4A). We also showed that both PIF1 mutants L319P and E307Q mutants are defective in STGC at Flex1/CFS upon HU treatment as scored by the EGFP-Flex1-STGC-1541 reporter (Fig.5H).

6) The authors have shown that replication independent DSBs use BIR mechanism for LTGC but not for STGC (Fig. 1C and D, 4D) But replication dependent DSBs (generated when replication fork

encounters ssDNA nicks) use BIR mechanism for both LTGC and STGC requiring PIF-1 (Fig 2E, 3C 4D). It would imply that PIF-1 KO cells should be more sensitive to DNA damaging agents that generate ssDNA nicks. Experiments studying the sensitivity of PIF-1 KO cells to such agents like MMS or PARPi will further strengthen this data.

Response: We showed that PIF1-KO cells are sensitive to CPT (Fig.S4B), MMS (Fig.S4C) and PARP inhibitor Olaparib (Fig.S4D).

7) Thea authors demonstrate a role for FANCM in BIR, and a novel synthetic relationship between FANCM and POLD3, and FANCM and PIF1. The authors demonstrate that decrease in FANCM with shRNA increases the frequency of BIR at Flex 1 sites, and that this increase is dependent on POLD3, RAD51 and PIF1 by using shRNA depletion (6A). Additionally, the authors show that shRNA depletion of PIF1 or POLD3 in FANCM KO cells causes a decrease in cell viability (6D). The authors call this a novel synthetic lethal relationship, but POLD3 is essential in humans.

Response: Thank the reviewer to point this out. We have revised the relevant parts and only claimed synthetic lethality interactions of FANCM with PIF1 but not POLD3.

8) The authors have shown that PCNA and RFC1 are required for BIR. The authors used shRNA to silence expression of RFC1 or PCNA and demonstrated a reduction in HU induced STGC when RFC or PCNA were depleted. Additionally, they showed that STGC after I-SceI cleavage did not require PCNA or RFC1, further establishing that there are two distinct processes happening.

Response: Thank the reviewer for the comment.

Minor points
1) Dependence on PCNA for Pif-1 recruitment can be shown at the endogenous FRA3B and FRA16D sites which would further strengthen their data.
Response: We performed ChIP at FRA3B and showed that PIF1 recruitment is impaired when PCNA is depleted by shRNA (Fig.S13).

2) PIF-1 is mentioned as FIP-1 in the discussion.
Response: We have corrected this typo.

3) POLD3 is a stocihiometric component of Pol delta. It is not BIR specific and should not be oversimplified this way in the text.
Response: We have modified the relevant parts and stated that POLD3 is a component of replisomes, which is required for BIR but not for STGC in mammalian cells.

Referee #2:

In the manuscript "PIF1 promotes break-induced replication to suppress common fragile site instability" Li et al presents new EGFP-based reporter systems to study long -track gene conversion (LTGC) and short-track gene conversion (STGC). They find that BIR is used for LTGC and also for STGC, when replication forks collapse. They neatly show that oncogenic stress induces PIF1

dependent BIR. Furthermore, they identify a synthetic lethal genetic interaction between PIF1 and FANCM.
Whilst the study presents some useful tools and interesting insight into mammalian BIR it also suffers from lack of coherence and clarity - mainly with respect to claims regarding CFSs. This is exemplified by the following: 1. HU is used for most experiments in the manuscript despite the fact that APH is the drug that induces CFSs. 2. The BIR that takes place at CFSs in mitosis (MiDAS) is independent of RAD51, thus it is different from the mechanism that is described throughout this manuscript. 3. The flex1 sequence from FRA16D is probably not the reason for FRA16D fragility. This is clear from the fact that CFSs need to be transcribed to become fragile. Therefore AT-rich sequences such as flex1 clearly do no underlie fragility at CFSs per se.
To improve the manuscript the authors should tone down the link to CFSs. The current data does for instance not justify the title. Furthermore, it would strengthen the manuscript if the authors included analysis of the involvement of RAD52 in the different reporter assays.

We thank the reviewer for the valuable comments and suggestions.

Response: We have repeated the key experiments after APH treatment to determine BIR in the EGFP-Flex1-BIR-5086 reporter and examine PIF1 recruitment to CFS-ATs (Fig.S2D, Fig.S11D and Fig.S13), and obtained similar results as after HU treatment.

It has been shown that replication often stalls at AT-rich sequences in CFS[30-32]. However, we agree that multiple factors in addition to AT-rich sequences contribute to CFS instability, and transcription of large genes is one important factor causing CFS breakage[33-35]. We have added discussion to state that AT-rich sequences are only one of the many factors contributing to the CFS instability. We have changed the title to "PIF1 promotes break-induced replication in mammalian cells". Instead of focusing on CFSs, we have shifted our emphasis towards the role of PIF1 in protecting DNA secondary structures (such as Flex1) to prevent chromosomal breakage.

Regarding RAD51 dependence, we showed that BIR scored by the EGFP-BIR-5085 reporter is RAD51-dependent in asynchronized cells (Fig.1D), which is consistent with the previous observation in Halazonetis' lab (Fig.3D in [11]). However, using the same reporter, we found that BIR exhibits RAD51 independence in mitotic cells (Fig.1E), which is in agreement with MiDAS[36]. We believe that this difference in RAD51 dependence is largely due to the suppression of RAD51 pathway caused by impaired recruitment of RNF8, RNF168 and BRCA1 to DSBs in mitosis[13,14,22] (also see discussion in "Response to Referee #1, comment 2"). We have added discussion in the manuscript.

We have also conducted extensive study and carefully analyzed the involvement of RAD52 in BIR. We found that the requirement of RAD52 for BIR is conditional in mammalian cells. We first showed that when a long homology is present for BIR strand invasion, RAD52 is only required for BIR when HR activity is compromised. Using the EGFP-BIR-5085 reporter, which contains 1.3 kb homology, we do not see BIR dependence on RAD52 in asynchronized cells (See below Fig.SS1A and Fig. SS1D, left). However, in mitotic cells, BIR is dependent on RAD52 (Fig.1E), possibly because the RAD51 pathway is suppressed. To further test this idea, we inactivated HR by depleting BRCA1 and then tested the BIR dependence on RAD52. Interestingly, as scored by the EGFP-BIR/LTGC-5085 reporter (1.3 kb homology), BIR is much reduced when RAD52 shRNA was expressed in BRCA1-depleted cells compared to BRCA1 depletion alone, whereas RAD52 depletion does not reduce BIR in BRCA1-WT cells (see below Fig.SS1B). This suggests that BIR in the context of the EGFP-BIR/LTGC-5085 reporter becomes RAD52 dependent when BRCA1 is deficient in asynchronized cells.

Secondly, we showed that BIR becomes more reliant on RAD52 when homology at DSB ends to the donor is limited. In contract to our EGFP-BIR-5085 reporter carrying 1.3 kb homology, when we used the BIR reporter constructed by the Halazonetis' lab, which contains 0.3 kb homology[11], we observed RAD52 dependence in asynchronized cells (see below Fig.SS1C), consistent with their previous observation[11]. This raised the possibility that RAD52 becomes important for BIR when the homology is short. To further test this, we constructed a new reporter EGFP-BIR (0.1kb)-5378 by reducing the homology in EGFP-BIR-5085 to 0.1 kb. Reducing the size of homology in the same reporter backbone results in RAD52 dependence (see below Fig.SS1D).

**Figure for Referees not shown.

We have included in this manuscript the results of BIR dependence/independence on RAD52 and RAD51 in mitotic cells, using the EGFP-BIR-5085 reporter (Fig.1E). For the requirement of RAD52 based on the homology size, we hope to analyze further to clarify more details, such as the homology size threshold for RAD52 dependence by constructing more reporters with homology size between 0.1-1.3 kb. We will also determine the role of RAD52 in BIR when BRCA1 is deficient in the context of different homology size. In addition, careful analysis of the repair products from newly constructed reporters by sequencing the repair junctions of single repair clones will also be needed. These in-depth analyses will take substantial time and we hope the study related to homology size can be allowed to publish in the future manuscript, which will focus more on the role of RAD52 in BIR.

Major comments

Figure 1.

The authors should provide evidence that the suggested mechanism is indeed responsible for restoration of the GFP reading frame. They should perform PCR, sequencing and/or southern blots from GFP positive cells to show that they have undergone the suggested genetic change at the cassette. This is a general shortcoming. The authors need to validate all the new reporter-cassette that they present in the paper by showing that they recombine as suggested on the schematics.

Response:

We thank the reviewer for this suggestion. We have analyzed single clones of EGFP positive cells derived from different reporters including EGFP-BIR-5085 after I-SceI, Cas9 and Cas9n cleavage, EGFP-Flex1-BIR-5086 after APH treatment, competition reporter EGFP/STGC-mCherry/LTGC-5034 after Cas9 and Cas9n cleavage, EGFP-Flex1-STGC-1541 after APH and EGFP-STGC-1731 after Cas9n cleavage. These analyses significantly help our understanding of the BIR, STGC and LTGC mechanism in mammalian cells.

We analyzed the green single clones derived from the EGFP-BIR-5085 reporter after I-SceI cleavage by sequencing analysis. Interestingly, among 30 clones, only 1 clone (3.3%) completed DNA synthesis of 3.8 kb to reach the second end homology and finished the repair by SDSA, and the rest 29 clones (96.7%) aborted BIR replication by template disassociation and completed the repair by end joining (Fig.1C, left and Fig.S14). Southern blot analysis verified this observation (Fig.S2A). Halazonetis' lab also showed similar observation using their reporter after I-SceI cleavage that BIR events often terminate BIR replication early and finish the repair by end joining[11]. Collectively, these results suggest that in contrast to yeast, BIR replication at endonuclease-generated DSBs is not very processive in mammalian cells and usually cannot exceed 4 kb. Similar results were obtained when we used Cas9 to generate DSBs in the EGFP-BIR-5085 reporter (Fig.1C, right and Fig.14). However, when Cas9n was used to make nicks in the EGFP-BIR-5085 reporter (Fig.2E), 27 cloned out of 39 clones (69.3%) reached the second homology (3.8 kb away) and used SDSA, which is in sharp contrast to that after Cas9 cleavage (3.3% completed 3.8 kb replication). This is also consistent with our observation from the STGC and LTGC competition reporter EGFP/STGC-mCherry/LTGC-5034 that Cas9n cleavage significantly increases LTGC compared to Cas9 cleavage (Fig.5B), supporting the notion that BIR/LTGC is promoted at broken replication forks.

We also analyzed the STGC (green) and LTGC (red) events derived from the competition reporter EGFP/STGC-mCherry/LTGC-5034 (Fig.5B). Sequencing analysis showed that all green clones derived after either Cas9 or Cas9n cleavage contain the intact EGFP cassette as expected. For red clones, replication needs to proceed at least 1.1 kb to complete mCherry open reading frame, but for completing LTGC, replication needs to continue for 2.2 kb to reach the second FP homology (Fig.5A). Sequencing red events revealed that only 9.2% of the events (7 out of 76 clones) after Cas9 cleavage are terminated early and finished by end joining, while ~90% of the events reached the second FP homology (2.2 kb away) and used SDSA to complete LTGC (Fig.5C and Fig.S14). However, after Cas9n cleavage, all red cells (100%, 48 clones analyzed) completed 2.2 kb replication and used the FP homology for LTGC or BIR continues further (Fig. 5C and Fig.S14). Thus, this EGFP/STGC-mCherry/LTGC-5034 reporter is appropriate for scoring the usage of STGC versus LTGC.

We also analyzed 38 green clones derived from the EGFP-Flex1-BIR-5086 reporter (Fig.3A) after APH treatment, and all of them (100%) completed 3.8 kb DNA synthesis and no end joining events were found (Fig.S14), which is in sharp contrast to the analysis of EGFP-BIR-5085 after I-SceI

or Cas9 cleavage where BIR-EJ is used dominantly (Fig.1C, Fig.S14). This is consistent with the notion that BIR is activated for repairing DSBs at broken replication forks.

In addition, 20 green clones derived from EGFP-Flex1-STGC-1541 reporter after APH treatment and EGFP-STGC-1731 reporter after Cas9n cleavage have been analyzed and they all completed STGC as expected.

Figure 1F.
The authors should comment on the activity that remains after PIF shRNA and especially after PIF1 KO. Around 33% BIR activity remains after PIF1 knockout - is this a redundant pathway? They should analyze some of the GFP positive clones from PIF1 knockouts to see if they are different from the ones that arise in PIF1 positive cells.

Response: We have analyzed the remaining BIR events in *PIF1*-KO cells carrying the EGFP-BIR-5085 reporter after I-SceI and Cas9 cleavage by examining the single green clones. Interestingly, the remining BIR events in *PIF1*-KO cells show shorter repair replication track length (Average: 1.5 kb after I-SceI and 1.4 kb after Cas9) compared to WT cells (Average: 2.2 kb after I-SceI and 1.9 kb after Cas9) (Fig.1C). In *PIF1*-KO cells, events with track length longer than 2.5 kb (up to 3.8 kb in WT cells) are missing. These results suggest that at DSBs, PIF1 is important for promoting more processive replication, resulting in longer BIR track length. Besides PIF1, there are likely other unknown helicases that are also involved in promoting BIR.

Figure 2.
How do the authors explain that according to their fiber analysis 2 mM HU stalls all replication forks and at the same time their model seems to suggest that upon HU replication fork stalling takes place specifically at the flex region. This seems unlikely. The authors should provide data showing whether cells perform any DNA synthesis during the 24 hours that they are treated with 2 mM HU. If no DNA synthesis is observed the authors should explain how complete HU-induced stalling for 24 hours induce recombination at their BIR cassette.

Response: In our reporter assay, we treated cells with 2 mM HU for 24 hours and removed HU, followed by FACS analysis three days after HU removal (Fig.3B and Fig.4E). We showed that treatment of 2 mM HU for 24 hours abolished EdU incorporation, but DNA replication started to reassume one day after removal of HU and continuously increased at day 3 (Fig.S11E). It is plausible that BIR is used to promote replication restart at the sites such as Flex1 with DNA breakage during the recovery from HU treatment when replication reassumes.

Figure 3
It simply seems that BIR is used at stalled replication forks explaining why BIR is used for STGC with the flex cassette. This would fit the idea that BIR is used at seDSBs. It is not clear to me how the authors can be sure that deDSBs are formed at stalled replication forks in the flex cassette. The authors should provide data that proves that deDSBs are formed as indicated in the hypothetical model shown in figure 3B.

Response: In our reporter design of EGFP-STGC-1731 (Fig.4B) and EGFP-Flex-STGC-1541 (Fig. 4D), the donor (iEGFP, containing only G and F parts) does not contain the C-terminal part of EGFP (P part is missing), so the invading strand has to anneal back to the second end at the right side of

Flex1 to obtain the P part of EGFP to produce green cells. Since the second end has to be involved for producing green cells, our reporter only scores the recombination events using both DSB ends at broken forks. We recovered the green cells after APH treatment of the U2OS (EGFP-Flex1-STGC-1541) cells and after Cas9n cleavage in U2OS (EGFP-STGC-1731) cells, and showed that all repair products (20 clones analyzed in each case) contain the second end homology as expected shown in the model (Fig.4B and Fig.4D).

Figure 4
The authors should show the cell cycle distribution of cells depleted for PCNA and RIF1. The authors should more explicitly discuss and investigate the possibility that replication fork will never encounter the reporter cassette upon PCNA and RIF1 depletion.

Response: As revealed by qPCR, we depleted about 70-80% of PIF1, PCNA and RFC1 by shRNAs (Fig.S11A), and determined cell cycle profile at day 6 after shRNA expression (Fig.S11B). FACS analysis was also performed on day 6 following shRNA expression of these genes (endonuclease delivery and HU treatment were on day 2 after shRNA expression and FACS was performed after 4 additional days). We also monitored cell growth and observed minor reduction of cell growth on day 6 in shRNA expressing cells compared to the vector control (Fig. S11C). More obvious reduction of cell growth was observed 2-3 weeks after expressing shRNAs for these genes. Within the time frame that we performed the repair assays, replication still occurs and thus replication forks would encounter nicks or flex1 in the reporter cassettes even when PCNA, RFC1 or PIF1 was depleted.

Minor comments
p. 24 line 5
The authors claim that "chromosomal breaks and gaps in CFSs are accumulated when PIF1 is deficient". The authors have not shown that this is the case. The authors should use FISH on metaphase spreads to probe for breaks at a few CFSs if they want to claim that CFS breakage is elevated in the absence of PIF1.
Response: In the revision, we have put emphasis on the role of PIF1 in protecting structure-prone DNA sequences such as Flex1, and in preventing chromosomal breakage upon replication stress. We have revised relevant parts.

Figure 2A
The triangle and square symbols are missing in the APH assay
Response: We have made the correction.

Figure 7 E, F, G and H
Why have the authors not shown/included the PIF1 knockout in these assays?
Response: We have shown the data using *PIF1*-KO cells in Fig.7G, Fig.7H, Fig.S3D and Fig.S3E.

Figure S2 A
The authors should also show efficiency of PIF1 depletion by western blot.
Response: We have tested several commercial PIF1 antibodies (Santa Cruz, sc-48377; Abcam, ab192369; Sigma, SAB4301117; Genetex, GTX55973), and none of them truly recognize endogenous PIF1. Due to the antibody issue, we were not able to perform western blot analysis of PIF1.

Figure S3 A. The authors should show efficiency of FANCM depletion by western blot.
Response: We have shown FANCM Western blot in Fig.S12A and Fig.S12B.

Referee #3:
Understanding how DNA repair is regulated in eukaryotic cells is an exciting and important area of research. The new study from Dr. Wu's lab is focused on one important pathway of double-strand break (DSB) repair called break-induced replication (BIR) in human cells. BIR is a pathway that mainly repairs one-ended DSBs that can result from replication collapse or from telomere erosion. BIR has been intensively studied in yeast, and these studies implicated BIR as a source of genetic instabilities associated with cancer. However, the role of BIR in DNA repair in human cells and its genetic control remain poorly investigated. The results of this study fill an important gap in our knowledge by shedding light on the contribution of BIR in repair of DNA breaks resulting from different sources in human cells. Also, this study demonstrates that similarly to what was shown in yeast, PIF1 plays an important role in BIR in humans. Importantly, the authors demonstrate that DSBs initiated by endonuclease cuts proceed through BIR involving PIF1 when they are repaired by long-track gene conversion (LTGC), but not when they lead to short track gene conversion (STGC). Even more interestingly, the authors demonstrate that when DNA breaks are initiated by replication fork collapse, both STGC and LTGC proceed through BIR and require PIF1. In addition, the authors show that Pif1 is recruited to a common fragile site (CFS) and that the breast cancer-associated PIF1 mutant L319P is defective in BIR, suggesting a direct link of BIR to the oncogenic process. Together, the paper represents an extremely elegant and thorough study. The number of different experimental systems that have been developed for this study and the quality and quantity of results obtained by the authors is simply astonishing. Together the paper represents an important breakthrough and will be of great interest for the diverse readership of EMBO. However, the authors need first to address several important questions.

We thank the reviewer for the valuable comments and suggestions.

Specific questions/comments:
1. In many experiments shown in the manuscript the authors deplete products of genes encoding major replication factors by using shRNA. For example, they deplete POLD3, known to be a part of the Pol delta complex that might be important for the stability of the entire complex. Similarly, the authors deplete RFC, PCNA, and also PIF1 (the latter might participate in processing of normal Okazaki fragments). In all cases of these depletions the authors observe the reduction of repair in various constructs. This brings the question of how the authors could exclude that the observed effect results from either change in the efficiency of break formation (for example due to sickness of a fraction of cells resulting from weakened replication) or from a massive stalling of replication forks leading to sequestration of some other factors that are needed for BIR.

Response: We agree that an indirect effect of depletion of these proteins could possibly influence the outcome of BIR, but based on our study, it is more likely that these proteins are involved directly in BIR. We performed ChIP of γH2A at Flex1 after depleting PCNA, RFC1 or PIF1 by shRNAs, and showed increased DSB formation at Flex1 (Fig.S11G). Thus, reduced BIR in PCNA-, RFC1- or PIF1-deficient cells is not likely due to reduced DSB formation caused by impaired replication. Increased

DSB formation in these shRNA expressing cells likely reflects a defect in BIR to repair DSBs at Flex1 when replication forks are broken.

For PIF1, we showed that its helicase activity is required for BIR (Fig.1F). We also showed that the BIR track length is reduced in *PIF1*-KO cells (Fig.1C). Along with the biochemical data that yeast PIF1 promotes D-loop extension *in vitro*[37], it is reasonable to believe that PIF1 plays a direct role in BIR by supporting processivity of BIR replication. Unfortunately, we were not able to monitor the track length in PCNA- or RFC1-deficient cells since single clones for analyzing track length could not grow out when PCNA or RFC1 was depleted. However, we showed that PIF1 loading to Flex1 after HU or APH is dependent on PCNA (Fig.5G, Fig.S11D), suggesting that PCNA likely has a direct role in recruiting PIF1 to promote BIR. Yeast Pif1 directly interacts with PCNA[37,38]. We have also found that when overexpressing in 293T cells, human PIF1 interacts with PCNA. We are still in the process to analyze the interaction of endogenous human PIF1 and PCNA and hope to figure out the regulation of this interaction upon replication stress. In-depth characterization of this interaction will take time and we hope to publish the interaction data in the future manuscript. The interaction of PIF1 and PCNA further supports a direct involvement of PCNA and possibly also RFC1 in BIR.

2. It is very important that the authors explain why depletion of many replication factors (PIF1, RFC, POLD3, etc) results in a decrease of BIR by approximately half. Where is this remaining part of BIR (independent of these replication factors) coming from? After all, it is unlikely that it results from less than 100% depletion of replication factors since the amount of these proteins in the cell should be very high as compared the amount that should be needed for the repair of one break. So, if the depletion is inefficient one would expect not to see any effect at all. All in all, the authors should discuss the nature of the remaining BIR following depletion of POLD3, PIF1, RFC, etc.

Response: We believe that multiple sub-pathways are involved in promoting BIR. The track length analysis of the remaining BIR events in *PIF1*-KO cells showed that the average track length is shorter in *PIF1*-KO cells compared to WT cells (Fig.1C and Fig.S14), and events with track length longer than 2.5 kb (up to 3.8 kb in WT cells) are missing in *PIF1*-KO cells. It is very likely that other unknown helicases are also involved in promoting BIR in the absence of PIF1.

Besides PCNA and RFC1, other pathways may also be involved in activating BIR. Along this line, we found that inhibition of ATR leads to a reduction of BIR. However, since ATR is also needed for end resection for general HR, at this stage, we do not know whether its role in BIR is through activating BIR or by promoting end resection. More extensive work will be needed to clarify how ATR is involved in BIR and how ATR coordinates with PCNA and RFC1 to promote BIR. Regarding POLD3, we do not have evidence to show whether POLD3-independent BIR exists or not. Nevertheless, we believe that the remaining BIR observed after depleting our proposed BIR players is due to the existence of multiple BIR pathways.

3. Do the authors know how the observed BIR is terminated? Is the termination mediated by NHEJ, MMEJ, etc? Did they ever try to sequence the junctions at termination positions?

Response: Thank the reviewer for pointing this out. In this revision, we performed extensive analysis of the repair products derived from different reporters. We found that in majority BIR events from the EGFP-BIR/LTGC-5085 reporter after I-SceI or Cas9 cleavage, BIR replication cannot complete 3.8 kb to reach the second homology end, and BIR is terminated by end joining (Fig.1C, BIR-EJ). This is consistent with the previous findings by Halazonetis' lab that BIR track is relatively short in

mammalian cells[11]. About 60% of the end joining junctions show 1-5 bp microhomology and more than 10% of the events contain 1-8 bp insertions (Fig.S2B), exhibiting MMEJ features. However, at broken forks, BIR track length is much longer compared to that at endonuclease-generated DSBs. Majority BIR events after Cas9n cleavage (Fig.S14, EGFP-BIR-5085) and all BIR events when forks are broken at Flex1 after APH (Fig.S14, EGFP-Flex1-BIR-5086) have reached the second homology (3.8 kb away) and either complete BIR by SDSA or continue BIR further downstream.

4. While discussing the effects of depleting FANCM on the repair at EGFP-Flex1-BIR-5086, the authors assume that the increased frequency of BIR is explained by the increased frequency of replication collapse at positions of secondary structures. However, another possibility is that increased BIR results from a decrease in the anti-BIR role that FANCM might play by unwinding D-loops that are initiating BIR (based on results obtained using mph1 mutants in yeast). It will be important that the authors address this possibility.

Response: We performed ChIP analysis of γH2AX at Flex1 after HU treatment and showed that DSBs are accumulated at Flex1 upon FANCM depletion (Fig.S12C). This supports the idea that FANCM is involved in preventing DSB formation at Flex1, and accumulated DSBs at Flex1 would lead to increased BIR. Although FANCM is involved in dismantling D-loop *in vitro* like Mph1[39,40], we showed previously that the overall HR activity is not increased in FANCM-deficient cells after I-SceI cleavage[41], suggesting that FANCM may not have anti-recombination activity in mammalian cells. Furthermore, we showed that FANCM exhibits a synthetic lethal interaction with PIF1. Since PIF1 is required for BIR, if FANCM has an anti-recombination activity, loss of FANCM should rescue the defect in PIF1-deficient cells rather than causing synthetic lethality. Although we cannot totally exclude the possibility that FANCM has a second role in suppressing BIR, it is more likely that increased BIR at Flex1 in FANCM-deficient cells is due to accumulated DSBs at Flex1 caused by loss of the FANCM activity in removing DNA secondary structures activity.

5. P. 17, line 9: It should be "PIF1" instead of FIP1.
Response: We have corrected the typo.

6. P. 19, the line before the last one: "It may take some time for REC to decide that BIR is the choice..." It remains possible that in the presence of two broken ends created by an endonuclease, the rare usage of BIR results not from the "choice" that is guided by REC, but simply from the interruption of ongoing BIR-like synthesis by an invasion or annealing of the second (catching) end that leads to the interruption of synthesis. This end might simply be absent in the case of replication collapse, which leads to a prevalence of BIR because it is not stopped by another end.

Response: We agree with this alternative mechanism. However, at endonuclease-generated DSBs, DSB ends seem still need to be checked to activate BIR, whereas at fork-associated DSBs, no check is required for BIR activation. In this revision, we proposed two models for REC to activate BIR at endonuclease-generated DSBs. One is that REC directly senses the presence of two ends and acts to control of initiation of DNA repair synthesis of the invading strands to regulate BIR activation as proposed in yeast [10]. The other is that the repeated cycles of strand invasion and disassociation may be detected by REC to launch BIR. Upon REC detection, BIR replisomes are assembled by recruiting factors such as PIF1. However, at DSBs on broken forks, BIR replisomes are immediately assembled

to activate BIR without using REC. We have added discussions of this alternative pathway in the manuscript.

7. P. 20, line 9: "may convert to replication forks using conventional BIR mechanism." The authors need to explain what this means.

Response: BIR is used to repair DSBs at broken forks. After strand invasion, BIR proceeds by D-loop migration, but as proposed in yeast, BIR D-loops may be converted to replication forks after MUS81 cleavage[42]. We have revised the relevant parts in the manuscript.

8. P. 20., line 13-14. "BIR is used at collapsed forks and BIR-specific replisomes promote more processive replication than BIR-independent GC." What kind of processive replication are the authors talking about here? Do they mean that GC is associated with some particular type of replication which is not very processive?

Response: The gene conversion track length is usually very short with more than 80% of track length below 100 bp[43], and thus GC replisomes do not need to be very processive. At this stage, we speculate that the composition of GC replisomes may be different from that of replisomes for general replication and for BIR, and the difference could be that the accessory factors such as helicases for three different replisomes are different. For instance, recruitment of PIF1 and possibly other factors as well, may be a key step to assemble BIR replisomes, which could be more processive than GC replisomes. Limiting GC track length is important for reducing the mutagenic effect, such as mutation and loss of heterozygosity. Clarifying the components of replisomes used for GC or BIR, and figuring out the difference of the replisomes for DNA repair and for general replication will be an important research goal for us.

9. P. 20, line 20: "PIF1 and POLD3-deficient human cells are sensitive to replication stress ...., suggesting that BIR is essential mechanism to repair broken forks..." Why do the authors think that the sensitivity is explained by BIR defect rather than by the importance of these proteins for S-phase replication? Similarly, on p. 24, the authors mention that inviability of pfh1-L430P in S. pombe and cancer predisposition of patients with PIF1 mutation results from the role of PIF1 in BIR. How can it be excluded that the problems stem from the role played by PIF1 in normal replication?

Response: We agree that we cannot exclude the possibility that observed sensitivity could also be caused by other function of these proteins related to replication in addition to their functions in BIR. However, the correlation of loss of BIR activity, increased DSB formation and impaired replication restart upon replication stress supports the idea that BIR defect would contribute to the sensitivity to replication stress although it may not be the only cause. Similarly, PIF1-E307Q and PIF1-L319P mutants have a defect in BIR and this defect could contribute to but may not be the sole cause for the sensitivity to replication stress. We have revised the discussion.

10. Fig. 1 (panels C, D, E, F): was there any effect of shPOLD3 on the cells' viability? The same information will be important for the cases where other replication factors were suppressed.

Response: By qPCR, we showed that about 70% to 80% of PCNA, RFC1 and POLD3 were depleted by shRNAs. We examined cell growth and cell cycle distribution after depletion of these proteins and

found minor reduction of cell growth on day 6 when FACS analysis was performed to determine the repair efficiency (Fig.S11B and Fig.S11C). More obvious reduction of cell growth was observed 2-3 weeks after expressing shRNAs for these genes. We also showed that depleting POLD3 under our experimental condition has a minor effect on cell growth in Fig.6D, left.

11. Fig. 7. When comparing the effect of PIF1 on Rad51-dependent BIR with its effect on MIDAS, the authors need to mention that MIDAS is RAD51-independent and may represent a different type of BIR or different type of DSB repair.

Response: MiDAS is RAD51-independent, but BIR assayed by our reporters as well as by the reporter from Halazonetis' lab[11] is RAD51 dependent in asynchronized cells. To understand this difference, we used the same EGFP-BIR-5085 reporter that we used in asynchronized cells to assay BIR in mitotic cells. Interestingly, using the same reporter which contains substantial homology (1.3 kb) at the DSB to the donor, we found that BIR is RAD51-dependent in asynchronized cells (Fig.1D) but Rad51-independnet in mitotic cells (Fig.1E). This difference is likely due to impaired function of the RAD51 pathway in mitotic cells (see more detailed discussion in the Response to Referee #1, comment 2). We have discussed this in the revised manuscript.

References

1. Chandramouly, G. et al. BRCA1 and CtIP suppress long-tract gene conversion between sister chromatids. *Nat Commun* **4**, 2404 (2013).
2. Nagaraju, G., Hartlerode, A., Kwok, A., Chandramouly, G. & Scully, R. XRCC2 and XRCC3 regulate the balance between short- and long-tract gene conversions between sister chromatids. *Mol Cell Biol* **29**, 4283-94 (2009).
3. Nagaraju, G., Odate, S., Xie, A. & Scully, R. Differential regulation of short- and long-tract gene conversion between sister chromatids by Rad51C. *Mol Cell Biol* **26**, 8075-86 (2006).
4. Willis, N.A. et al. Mechanism of tandem duplication formation in BRCA1-mutant cells. *Nature* **551**, 590-595 (2017).
5. Willis, N.A., Panday, A., Duffey, E.E. & Scully, R. Rad51 recruitment and exclusion of non-homologous end joining during homologous recombination at a Tus/Ter mammalian replication fork barrier. *PLoS Genet* **14**, e1007486 (2018).
6. Lambert, S. et al. Homologous recombination restarts blocked replication forks at the expense of genome rearrangements by template exchange. *Mol Cell* **39**, 346-59 (2010).
7. Mizuno, K., Lambert, S., Baldacci, G., Murray, J.M. & Carr, A.M. Nearby inverted repeats fuse to generate acentric and dicentric palindromic chromosomes by a replication template exchange mechanism. *Genes Dev* **23**, 2876-86 (2009).
8. Ahn, J.S., Osman, F. & Whitby, M.C. Replication fork blockage by RTS1 at an ectopic site promotes recombination in fission yeast. *EMBO J* **24**, 2011-23 (2005).
9. Nguyen, M.O., Jalan, M., Morrow, C.A., Osman, F. & Whitby, M.C. Recombination occurs within minutes of replication blockage by RTS1 producing restarted forks that are prone to collapse. *Elife* **4**, e04539 (2015).

10. Jain, S. et al. A recombination execution checkpoint regulates the choice of homologous recombination pathway during DNA double-strand break repair. *Genes Dev* **23**, 291-303 (2009).
11. Costantino, L. et al. Break-induced replication repair of damaged forks induces genomic duplications in human cells. *Science* **343**, 88-91 (2014).
12. Kato, T.A., Okayasu, R. & Bedford, J.S. Comparison of the induction and disappearance of DNA double strand breaks and gamma-H2AX foci after irradiation of chromosomes in G1-phase or in condensed metaphase cells. *Mutat Res* **639**, 108-12 (2008).
13. Giunta, S., Belotserkovskaya, R. & Jackson, S.P. DNA damage signaling in response to double-strand breaks during mitosis. *J Cell Biol* **190**, 197-207 (2010).
14. van Vugt, M.A. et al. A mitotic phosphorylation feedback network connects Cdk1, Plk1, 53BP1, and Chk2 to inactivate the G(2)/M DNA damage checkpoint. *PLoS Biol* **8**, e1000287 (2010).
15. Gomez-Godinez, V. et al. Analysis of DNA double-strand break response and chromatin structure in mitosis using laser microirradiation. *Nucleic Acids Res* **38**, e202 (2010).
16. Britton, S., Coates, J. & Jackson, S.P. A new method for high-resolution imaging of Ku foci to decipher mechanisms of DNA double-strand break repair. *J Cell Biol* **202**, 579-95 (2013).
17. Peterson, S.E. et al. Cdk1 uncouples CtIP-dependent resection and Rad51 filament formation during M-phase double-strand break repair. *J Cell Biol* **194**, 705-20 (2011).
18. Ayoub, N. et al. The carboxyl terminus of Brca2 links the disassembly of Rad51 complexes to mitotic entry. *Curr Biol* **19**, 1075-85 (2009).
19. Freire, R., van Vugt, M.A., Mamely, I. & Medema, R.H. Claspin: timing the cell cycle arrest when the genome is damaged. *Cell Cycle* **5**, 2831-4 (2006).
20. Esashi, F. et al. CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair. *Nature* **434**, 598-604 (2005).
21. Krajewska, M. et al. Forced activation of Cdk1 via wee1 inhibition impairs homologous recombination. *Oncogene* **32**, 3001-8 (2013).
22. Nelson, G., Buhmann, M. & von Zglinicki, T. DNA damage foci in mitosis are devoid of 53BP1. *Cell Cycle* **8**, 3379-83 (2009).
23. Hanada, K. et al. The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks. *Nat Struct Mol Biol* **14**, 1096-104 (2007).
24. Li, S. et al. ERCC1/XPF Is Important for Repair of DNA Double-Strand Breaks Containing Secondary Structures. *iScience* **16**, 63-78 (2019).
25. Minocherhomji, S. et al. Replication stress activates DNA repair synthesis in mitosis. *Nature* **528**, 286-90 (2015).
26. Davis, A.P. & Symington, L.S. RAD51-dependent break-induced replication in yeast. *Mol Cell Biol* **24**, 2344-51 (2004).
27. Malkova, A., Naylor, M.L., Yamaguchi, M., Ira, G. & Haber, J.E. RAD51-dependent break-induced replication differs in kinetics and checkpoint responses from RAD51-mediated gene conversion. *Mol Cell Biol* **25**, 933-44 (2005).
28. Smith, C.E., Llorente, B. & Symington, L.S. Template switching during break-induced replication. *Nature* **447**, 102-5 (2007).
29. Llorente, B., Smith, C.E. & Symington, L.S. Break-induced replication: what is it and what is it for? *Cell Cycle* **7**, 859-64 (2008).
30. Kaushal, S. & Freudenreich, C.H. The role of fork stalling and DNA structures in causing chromosome fragility. *Genes Chromosomes Cancer* **58**, 270-283 (2019).

31. Walsh, E., Wang, X., Lee, M.Y. & Eckert, K.A. Mechanism of replicative DNA polymerase delta pausing and a potential role for DNA polymerase kappa in common fragile site replication. *J Mol Biol* **425**, 232-43 (2013).
32. Zhang, H. & Freudenreich, C.H. An AT-rich sequence in human common fragile site FRA16D causes fork stalling and chromosome breakage in S. cerevisiae. *Mol Cell* **27**, 367-79 (2007).
33. Helmrich, A., Ballarino, M. & Tora, L. Collisions between replication and transcription complexes cause common fragile site instability at the longest human genes. *Mol Cell* **44**, 966-77 (2011).
34. Brison, O. et al. Transcription-mediated organization of the replication initiation program across large genes sets common fragile sites genome-wide. *Nat Commun* **10**, 5693 (2019).
35. Blin, M. et al. Transcription-dependent regulation of replication dynamics modulates genome stability. *Nat Struct Mol Biol* **26**, 58-66 (2019).
36. Bhowmick, R., Minocherhomji, S. & Hickson, I.D. RAD52 Facilitates Mitotic DNA Synthesis Following Replication Stress. *Molecular Cell* **64**, 1117-1126 (2016).
37. Wilson, M.A. et al. Pif1 helicase and Poldelta promote recombination-coupled DNA synthesis via bubble migration. *Nature* **502**, 393-6 (2013).
38. Buzovetsky, O. et al. Role of the Pif1-PCNA Complex in Pol delta-Dependent Strand Displacement DNA Synthesis and Break-Induced Replication. *Cell Reports* **21**, 1707-1714 (2017).
39. Prakash, R. et al. Yeast Mph1 helicase dissociates Rad51-made D-loops: implications for crossover control in mitotic recombination. *Genes Dev* **23**, 67-79 (2009).
40. Gari, K., Decaillet, C., Delannoy, M., Wu, L. & Constantinou, A. Remodeling of DNA replication structures by the branch point translocase FANCM. *Proc Natl Acad Sci U S A* **105**, 16107-12 (2008).
41. Wang, H. et al. The concerted roles of FANCM and Rad52 in the protection of common fragile sites. *Nat Commun* **9**, 2791 (2018).
42. Mayle, R. et al. DNA REPAIR. Mus81 and converging forks limit the mutagenicity of replication fork breakage. *Science* **349**, 742-7 (2015).
43. Elliott, B., Richardson, C., Winderbaum, J., Nickoloff, J.A. & Jasin, M. Gene conversion tracts from double-strand break repair in mammalian cells. *Mol Cell Biol* **18**, 93-101 (1998).

2nd Editorial Decision 11th Nov 2020

Thank you for submitting your revised manuscript for our editorial consideration. All three original referees have now assessed it again, and found their original concerns satisfactorily addressed. We shall therefore be happy to proceed with acceptance and publication of the study in The EMBO Journal, following incorporation of a number of minor comments the referees still have (see reports below).

In this final round of modification, please also address the following editorial points.

REFEREE REPORTS

Referee #1:

The authors have addressed the majority of my concerns with an impressive amount of data. The study is significant for implicating Pif1 in mammalian BIR, while also defining lengths of BIR tracks and usage in one sided vs 2-ended DSBs. Improvements have also occurred by focusing the study on BIR rather and clarifying Pif1 function in both Rad51-dependent BIR in interphase and Rad52 dependent BIR in mitosis. The discussion is also quite thoughtful and informative albeit a bit long.

Based on these advances, I believe this will be an important contribution to the DNA repair literature and am in favor of publication.

Referee #2:

The authors have significantly improved the manuscript by addressing the issues raised by the reviewers.
In particular, analyses of the repair products have validated the tools and provided mechanistic insight. Thus, altogether this manuscript presents novel insight as well as a number of new tools with a clear focus on BIR.

Minor issues:
P30: "MiDAS often occurs at CFSs upon replication stress and its dependence on POLD3 suggests that BIR It was shown previously that when BRCA1, CtIP is involved (Bhowmick et al., 2016; Minocherhomji et al., 2015b)." This sentence is not clear, please reformulate.

Referee #3:

The authors went above and beyond in addressing all of the reviewer's comments. The revised version of the manuscript is significantly improved. I am convinced that it will be of great interest to the broad readership of EMBO.
Minor comments:

1. The "n" number of sequences analyzed needs to be included in the legends to Figures Fig. 2E and Fig. S2B.
2. p. 30, line 11. The extra line starting from "It was shown previously..." needs to be removed.

Point-to-point response to reviewers' questions:

Reviewer #1:

The authors have addressed the majority of my concerns with an impressive amount of data. The study is significant for implicating Pif1 in mammalian BIR, while also defining lengths of BIR tracks and usage in one sided vs 2-ended DSBs. Improvements have also occurred by focusing the study on BIR rather and clarifying Pif1 function in both Rad51-dependent BIR in interphase and Rad52 dependent BIR in mitosis. The discussion is also quite thoughtful and informative albeit a bit long.

Based on these advances, I believe this will be an important contribution to the DNA repair literature and am in favor of publication.

Response: Thank the reviewer for the comment.

Referee #2:

The authors have significantly improved the manuscript by addressing the issues raised by the reviewers.
In particular, analyses of the repair products have validated the tools and provided mechanistic insight. Thus, altogether this manuscript presents novel insight as well as a number of new tools with a clear focus on BIR.

Minor issues:
P30: "MiDAS often occurs at CFSs upon replication stress and its dependence on POLD3 suggests that BIR It was shown previously that when BRCA1, CtIP is involved (Bhowmick et al., 2016; Minocherhomji et al., 2015b)." This sentence is not clear, please reformulate.

Response: We have corrected the sentence.

Referee #3:

The authors went above and beyond in addressing all of the reviewer's comments. The revised version of the manuscript is significantly improved. I am convinced that it will be of great interest to the broad readership of EMBO.

Minor comments:
1. The "n" number of sequences analyzed needs to be included in the legends to Figures Fig. 2E and Fig. S2B.

Response: We have included the numbers of analyzed sequences in the legends to Fig 2E and S2B.

2. p. 30, line 11. The extra line starting from "It was shown previously..." needs to be removed.

Response: We have corrected the sentence.

Accepted **10th Dec 2020**

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

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Xiaohua Wu
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-104509

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was not pre-determined, and we did not have pre-specified effects sizes. All cell biology experiments were curated from at least three independent experiments
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We used transfection or infection to deliver shRNAs and endonucleases (e.g. I-SceI and Cas9). When the transfection or infection efficiency is low as judged by drug resistance (<50%), we excluded the experiments from further analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes. We randomly assigned cells to treatment and control groups
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The experiments were repeated by different investigators.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes. We used t-test to analyze the significance of the difference between samples
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Normality is assessed by graphical inspection and K-S test if necessary
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	BRCA1 (GTX70121, GeneTex), BRCA2 (sc-293185, Santa Cruz Biotechnology), CTIP (A300-487A, Bethyl), RAD51 (sc-398587, Santa Cruz Biotechnology), RAD52 (sc-365341, Santa Cruz Biotechnology), POLD3 (ab182564, Abcam), PCNA (NA03, Millipore), FLAG (F1804, Sigma-Aldrich), HA (MMS-101P, Covance), RFC1 (A300-320A, Bethyl), MUS81 (sc-376661, Santa Cruz Biotechnology), KU70 (sc-17789, Santa Cruz Biotechnology), rat anti-BrdU (ab6326, Novus), mouse anti-BrdU (347580, BD Biosciences), Alexa 594 anti-rat (A11007, Invitrogen), Alexa 488 anti-mouse (A11029, Invitrogen),Anti-FLAG antibody (F3165, Sigma-Aldrich), Anti-phospho-H2AX (Ser139) antibody (07-164, EMD Millipore).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	U2OS(ATCC),Lenti-X 293T (Clontech), HCT116 WT and FANCM-KO(from Dr. Lei Li (University of Texas, MD Anderson Cancer Center)).All cells were tested negative for mycoplasma contamination.

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8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
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22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No