BRCA2 associates with MCM10 to suppress PRIMPOL-mediated repriming and single-stranded gap formation after DNA damage

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The BRCA2 tumor suppressor protects genome integrity by promoting homologous recombination-based repair of DNA breaks, stability of stalled DNA replication forks and DNA damage-induced cell cycle checkpoints. BRCA2 deficient cells display the radio-resistant DNA synthesis (RDS) phenotype, however the mechanism has remained elusive. Here we show that cells without BRCA2 are unable to sufficiently restrain DNA replication fork progression after DNA damage, and the unrestrained fork progression is due primarily to Primase-Polymerase (PRIMPOL)-mediated repriming of DNA synthesis downstream of lesions, leaving behind single-stranded DNA gaps. Moreover, we find that BRCA2 associates with the essential DNA replication factor MCM10 and this association suppresses PRIMPOL-mediated repriming and ssDNA gap formation, while having no impact on the stability of stalled replication forks. Our findings establish an important function for BRCA2, provide insights into replication fork control during the DNA damage response, and may have implications in tumor suppression and therapy response.
**BRCA2** is a major tumor suppressor with a critical role in genome integrity maintenance. Germline, monoallelic mutations in **BRCA2** predispose to breast, ovarian and other cancers, whereas biallelic mutations in the gene cause a severe form of Fanconi anemia (FA-D1) characterized by development of solid tumors during the early childhood. Early studies established a critical role of **BRCA2** in the homologous recombination (HR)-based repair of DNA double strand breaks (DSBs). Then, **BRCA2** mutant cells were found to display the radio-resistant DNA synthesis (RDS) phenotype, indicative of a possible intra-S-phase checkpoint defect. Later, it was shown that **BRCA2** is required for the stability of stalled replication forks, and this has been a subject of intense studies in recent years. Additionally, **BRCA2** was also shown to facilitate fork restart after stalling. However, whether **BRCA2** regulates replication fork progression after DNA damage or replication stress remains unknown.

In mammals, DNA replication starts from tens of thousands of replication origins scattered throughout the genome. To ensure that DNA is replicated once and only once per cell cycle, the initiation of replication is a tightly controlled process that involves sequential and concerted actions of numerous factors. Among these factors, the MCM2-7 helicase unwinds DNA, and MCM10, a unique member of the MCM family, interacts with and promotes the final activation of MCM2-7 helicase, leading to full DNA unwinding and origin firing. In addition to origin activation, MCM10 has been reported to recruit DNA polymerase α (polα), which catalyzes initial DNA synthesis on both leading and lagging strands at origins and the synthesis of Okazaki fragments on the lagging strands. Once DNA replication commences, MCM10 “travels” with the replication fork to facilitate replication elongation. More recently, MCM10 was also found to possess potent strand-annealing activity and to inhibit fork reversal.

In the present study, we investigated the mechanism of the RDS phenotype of **BRCA2**-deficient cells by using the DNA fiber assay to analyze replication kinetics in cells with and without **BRCA2**. We found that **BRCA2**-deficient cells failed to sufficiently retrain replication fork progression after DNA damage, which likely underlies the RDS phenotype of the cells. Moreover, using affinity purification, we identified an association of **BRCA2** with MCM10. In the data presented below, we focus on the regulation of replication fork progression by **BRCA2** and the role of its association with MCM10 in the process.

**Results**

**BRCA2** represses replication fork progression after DNA damage. To explore the role of **BRCA2** in DNA replication fork progression, we examined the rate of fork elongation in U2OS cells depleted of **BRCA2** (Fig. 1a) using the DNA fiber assay, in which replicating DNA was labeled with thymidine analogs dCdU and IdU over a standard labeling period of 20 min and then visualized by staining with respective antibodies (Fig. 1b). Compared with cells treated with control siRNAs, **BRCA2**-depleted cells showed normal fork progression in unperturbed replication (Fig. 1c). At 6 h after 10 Gy of ionizing radiation (IR), control cells showed a substantial drop in replication tract length (Fig. 1c); while tract length was also reduced in **BRCA2**-depleted cells, it was significantly longer than that in control cells (Fig. 1c). We then conducted a time course experiment to assess the kinetics of fork progression in cells with and without **BRCA2** after either 10 or 2 Gy of IR, and **BRCA2**-depleted cells showed significantly longer tract lengths at all the time points (1, 3, and 6 h) after IR (Fig. 1d, e).

Next, we investigated the impact of **BRCA2** loss on fork progression after DNA damage induced by bleomycin (BLEO), a radiomimetic therapeutic DNA-damaging agent. Control and **BRCA2**-depleted U2OS cells were subjected to either a short (1 h) pulse with a high-concentration (10 μM) or a prolonged (6 h) and continuous treatment with a low concentration (1 μM) of the drug. Underrestrained fork progression was again seen in **BRCA2**-depleted cells under both conditions (Fig. 1f, g). Additionally, we also tested fork progression after continuous treatment with methylmethane sulfonate (MMS), an alkylating agent that causes base damage, camptothecin (CPT), a topoisomerase I inhibitor that produces covalent DNA-protein conjugates, and hydroxyurea (HU), which depletes cellular nucleotide pool leading to replication stress and ssDNA at the replication fork. Again, while all three drugs led to major reductions in fork speed in both control and **BRCA2**-depleted cells, the latter were found to have longer replication tracts after treatment with all three drugs (Fig. 1g). These findings demonstrate that the function of **BRCA2** to suppress fork progression after DNA damage and/or replication stress is general and not specific to IR and radiomimetic agents.

To further confirm the role of **BRCA2** in restraining fork progression after DNA damage, we analyzed fork progression in **VC8** cells, a Chinese hamster ovary (CHO) cell line with biallelic **BRCA2** mutations and the same cells reconstituted with a human **BRCA2** cDNA (Fig. 1h). While re-expression of **BRCA2** in **VC8** cells did not produce any effect on fork velocity during unperturbed replication, it significantly reduced replication tract length after either IR or BLEO treatment (Fig. 1i, j). Taken together, these results demonstrate that **BRCA2** restrains replication fork progression after DNA damage.

**BRCA2** suppresses primase-polymerase (PRIMPOL)-mediated repriming after DNA damage. Underrestrained fork progression after DNA damage may result from several distinct mechanisms. First, we considered fork reversal, as higher overall fork speed can result from defective fork reversal. Although **BRCA2** protects (stalled and) reversed forks, it is not required for fork reversal per se. Several fork remodeling factors, including HLT2, SMARCAL1, and ZRANB3 are required for the formation of reversed forks. Therefore, we used siRNAs to downregulate these fork remodeling factors (Fig. 2a) and measured the impact on fork progression in the presence and absence of **BRCA2**. While loss of none of these factors showed any effect on fork progression in unirradiated cells (Fig. 2b), depletion of each of them led to higher overall fork velocity in irradiated cells (Fig. 2c), indicating that fork reversal indeed contributed to fork slowdown upon DNA damage. Co-depletion of each of these factors with **BRCA2** caused further increased fork velocity, showing mostly an additive effect (Fig. 2d). These results confirm that fork reversal operates normally in the absence of **BRCA2** and, more importantly, they indicate that the increased fork progression in **BRCA2**-depleted cells results from a different mechanism.

Recent studies have established primase-polymerase (PRIMPOL), which possesses an activity to reprime DNA synthesis downstream of lesions, as a key regulator of replication fork progression after DNA damage. A recent study also demonstrates that PRIMPOL is induced after cisplatin treatment and promotes lesion skipping and prevents fork reversal and degradation in **BRCA1**-deficient cells. Therefore, we asked if PRIMPOL is responsible for the underrestrained fork progression observed in **BRCA2**-deficient cells after IR, as repriming and lesion skipping would prevent fork stalling, thereby increasing fork progression. siRNA-mediated depletion of PRIMPOL alone...
shown little to no effect on fork velocity either before or after IR (Fig. 2e, f); however, when PRIMPOL was co-depleted with BRCA2, the increased fork progression elicited by BRCA2 loss was completely reversed (Fig. 2f). Transient re-expression of wild-type (WT) PRIMPOL in the co-depleted cells restored the increased fork progression after IR, whereas the CH (primase dead) and AxA (catalytic dead) mutants both failed to do so (Supplementary Fig. 1), implying that both primase and polymerase activities of PRIMPOL are required for the underrestrained replication fork progression in BRCA2-depleted cells after DNA damage.

Repriming of DNA synthesis by PRIMPOL generates ssDNA gaps on replicated DNA as a result of lesion bypass. We therefore carried out a modified DNA fiber assay, in which cells were treated with the ssDNA-specific S1 endonuclease after being labeled with the thymidine analogs. In this assay, any shortening of labeled DNA tracts after S1 treatment would be evidence of the presence of ssDNA gaps. Indeed, significant shortening of
These results clearly demonstrate ssDNA gap formation in BRCA2-depleted cells after IR and further indicate that PRIMPOL-mediated repriming underlies the underrestrained fork progression in BRCA2-deficient cells after DNA damage.

Finally, we tested whether PRIMPOL-mediated repriming was operative in cells depleted of HLTF, SMARCAL1, and ZRANB3 after IR. We found that co-depletion of PRIMPOL with each of replication tracts was seen in irradiated BRCA2-depleted cells after S1 nuclease treatment, whereas in cells transfected with control siRNAs, S1 treatment did not cause any change (Fig. 2g). Moreover, in cells depleted of both BRCA2 and PRIMPOL, S1 nuclease treatment also did not produce any difference (Fig. 2g). These results clearly demonstrate ssDNA gap formation in
the three factors significantly reversed the increased fork progression after IR (Fig. 2h). This suggests that repriming by PRIMPOL contributes to increased fork progression after DNA damage in the absence of fork reversal; in other words, fork reversal precludes PRIMPOL from being recruited and/or acting at damaged forks.

Impact of PRIMPOL loss on genome stability and cell viability after DNA damage in the presence and absence of BRCA2. To determine the role of PRIMPOL and repriming in genome stability in BRCA2-deficient cells, we depleted the two proteins, alone and in combination (Fig. 3a), in U2OS cells and analyzed mitotic spreads (Fig. 3b) for genome instability. In the absence of external DNA damage, cells depleted of either PRIMPOL or BRCA2 both showed increased genome instability, primarily in the form of chromatid breaks (CTBs), as compared with cells treated with a control siRNA (Fig. 3c). A small number of chromosomal breaks (CSBs) were also detected in PRIMPOL- and BRCA2-depleted cells, whereas radial chromosomes were practically not observed. Following IR (2 Gy, 6 h), overall genome instability was elevated in all cells, with PRIMPOL- and BRCA2-depleted cells still showing higher levels of genome instability than control cells (Fig. 3d). Interestingly, PRIMPOL-depleted cells harbored more CTBs than did BRCA2-depleted cells prior to IR but less after IR. This suggests that in unperturbed cells, ssDNA gaps generated by PRIMPOL leads to more DSBs than does the inability of BRCA2-depleted cells to repair DSBs due to their HR deficiency, whereas after radiation-induced DNA damage, loss of HR-mediated DSB repair has a greater impact on the amount of unrepaired DNA breaks in the cell. Moreover, combined loss of PRIMPOL and BRCA2 led to further increased CTBs compared with individual loss of either protein before radiation, but reduced CTBs compared with BRCA2 loss after radiation. This suggests that the effects of loss of the two proteins may be additive in unperturbed cells, whereas after radiation, loss of PRIMPOL and the resulting loss of repriming and ssDNA formation may prevent DNA breakage (at regions of ssDNA), thereby reducing CTBs. In irradiated cells, the levels of CSBs were still much lower than those of CTBs but appeared to follow the same trend as CTBs. Radiation also led to the formation of a small number of radial chromosomes, with BRCA2 loss causing an increase in this form of chromosomal abnormality while PRIMPOL depletion having practically no effect (Fig. 3d).

Finally, we analyzed the sensitivity of the above cells to IR, BLEO, MMS, and CPT (Fig. 3d–g). As expected, loss of BRCA2 led to increased sensitivity to all four DNA-damaging agents, while depletion of PRIMPOL showed no effect, consistent with previous reports. Notably, co-depletion of PRIMPOL with BRCA2 led to a partial rescue of the hypersensitivity of BRCA2-depleted cells to radiation and bleomycin but not MMS and CPT, suggesting that PRIMPOL or ssDNA gaps resulting from its repriming activity contributes to cellular sensitivity after certain types of DNA damage, which remains to be fully defined.

Identification of MCM10 as a BRCA2 binding protein. We have previously used tandem affinity purification (TAP) coupled with mass spectrometry (MS) to identify new association partners of PALB2, a major BRCA2 binding protein that is required for its chromatin association, DNA damage-induced foci formation, and HR activity. The efforts led to the identification of BRCA1 and KEAP1 as PALB2 binding proteins. However, in these previous experiments, individual bands or gel sections were analyzed...
by MS, leading to incomplete identification of components of the complex. To further identify new components of the PALB2 complex, we reconducted the purification and subjected the entire content of the complex to MS analysis. As shown in Fig. 4a, in addition to known interactors such as BRCA2, BRCA1 (and its close partner BARD1), KEAP1, RAD51, MRG15, and MRGX34, our new TAP-MS analyses identified HNRNPM, LMNA, and MCM10 as candidate new components of the PALB2 complex. We decided to focus on MCM10, as it is an essential DNA replication factor.

To confirm the association between MCM10 and PALB2, we cloned the human MCM10 cDNA into a FLAG-HA double tagging vector, transiently expressed and immunoprecipitated (IPed) the protein, and tested the presence of endogenous PALB2 and BRCA2 in the precipitates. Indeed, both PALB2 and BRCA2 were readily detectable (Fig. 4b). We then IPed endogenous MCM10 and again detected both PALB2 and BRCA2 in the precipitates (Fig. 4c). Therefore, MCM10 is a bona fide component of the PALB2/BRCA2 complex. HU treatment did not produce any significant effect on the association between MCM10 and PALB2/BRCA2, although it triggered PALB2 phosphorylation.

Given the known role of BRCA2 in DNA replication and the relatively large amount of BRCA2 associated with PALB2, we asked if MCM10 in fact interacts with BRCA2. To this end, we tested a panel of patient-derived BRCA2 variants in its PALB2-binding motif31 for their ability to associate with MCM10. As shown in Fig. 4e, MCM10 was found to co-IP with not only PALB2 but also defective variants (G25R, W31R, and W31C), indicating that it indeed can associate with BRCA2 independently of PALB2. To rule out the possibility that the BRCA2–MCM10 association may be mediated by DNA in the cell lysate, we treated the IPed material on anti-FLAG beads with DNase I after the IP was completed, and the association remained intact (Fig. 4e). To determine the impact of DNA damage on the MCM10–BRCA2 association during DNA replication, we synchronized cells in the S phase, exposed them to different genotoxic agents, including IR, BLEO, CPT and cisplatin (a crosslinker), and then analyzed the association by BRCA2 IP. The results showed that the association remained largely unchanged after DNA damage induced by these agents (Fig. 4f).

The N-terminal coiled-coil motif of MCM10 is required for its BRCA2 association. To determine the structural element of MCM10 that is responsible for its association with BRCA2, we first generated a series of six deletions spanning the entire MCM10 coding sequence in a myc-MCM10-GFP vector (Supplementary Fig. 2a) and performed transient overexpression and IP-western. Surprisingly, BRCA2 was found to co-IP with all six deletion proteins under the conditions used (Supplementary Fig. 2b). This was likely an artifact as the proteins were expressed at very high levels. As we had been studying the BRCA1–PALB2 association mediated by their respective coiled-coil (CC) motifs35, we then took a candidate approach and focused on the N-terminal, which contains a conserved CC motif (Fig. 5a). Deletion of this motif abrogated the co-IP of BRCA2 with MCM10, indicating that it is required for their association (Fig. 5b). It has been reported that the CC motif of Xenopus MCM10 mediates its dimer/oligomerization, and mutations of conserved hydrophobic residues in the motif abrogate this self-association36. We therefore generated the corresponding mutations in the human MCM10 and tested their effect on BRCA2 association. Indeed, the 2D (L114D/L118D) and 4A (L114A/L118A/M125A/L128A)
showed significantly reduced ability to associate with BRCA2, whereas the 2A mutant MCM10-2A (Fig. 5c). In this setting, MCM10–BRCA2 association was largely abolished in the absence of DNA damage. Assay scheme is shown on the top, and IdU/CldU tract length ratio after HU treatment is shown below. Data in (c) mean ± s.d., with the number of dots shown below each column. 

MCM10 (Fig. 5b). Interestingly, this mutation also abrogated the BRCA2 association with MCM10 (Fig. 5b). Thus, it was concluded that interactions between MCM10 and BRCA2 are disrupted in the presence of DNA damage. 

MCM10 association restrains replication fork progression after DNA damage. To determine the role of the BRCA2–MCM10 association in DNA replication, we constructed stable U2OS cell lines expressing MCM10-WT, MCM10-ACC, or MCM10-2A (Fig. 5c). In this setting, MCM10-ACC remained largely unable to associate with BRCA2, whereas the 2A mutant showed significant residual association. We then depleted the endogenous MCM10 in the stable cell lines using a pool of two siRNAs against the 3’-UTR of its mRNA (Supplementary Fig. 3) and measured replication fork progression supported by the exogenous proteins. No significant difference was detected among the cells during unperturbed replication (Fig. 5d); after DNA damage elicited by either IR or BLEO, however, cells selectively expressing either mutant MCM10 protein showed under-restrained fork progression (Fig. 5d–f).

To confirm the above findings, we conducted the DNA combing analysis, in which DNA molecules would be stretched to their maximum length. Source data are provided as a Source Data file.

Mutations both led to loss of BRCA2 binding (Fig. 5b). Thus, it appears that the same residues are involved in both MCM10–MCM10 and BRCA2–MCM10 associations. Additionally, we also tested a 2A (L114A/L118A) mutation, which was shown to have no impact on MCM10 self-association. Interestingly, this mutation also abrogated the BRCA2 association with MCM10 (Fig. 5b).

BRCA2–MCM10 association restrains replication fork progression after DNA damage. To determine the role of the BRCA2–MCM10 association in DNA replication, we constructed stable U2OS cell lines expressing MCM10-WT, MCM10-ACC, or MCM10-2A (Fig. 5c). In this setting, MCM10-ACC remained largely unable to associate with BRCA2, whereas the 2A mutant showed significant residual association. We then depleted the endogenous MCM10 in the stable cell lines using a pool of two siRNAs against the 3’-UTR of its mRNA (Supplementary Fig. 3) and measured replication fork progression supported by the exogenous proteins. No significant difference was detected among the cells during unperturbed replication (Fig. 5d); after DNA damage elicited by either IR or BLEO, however, cells selectively expressing either mutant MCM10 protein showed under-restrained fork progression (Fig. 5d–f).

To confirm the above findings, we conducted the DNA combing analysis, in which DNA molecules would be stretched to their maximum length. Source data are provided as a Source Data file.
profund. Moreover, while replication tracts in cells expressing MCM10-2A were evidently longer than those in cells expressing the WT protein, they were significantly shorter than those in cells expressing the ΔCC mutant, a phenotype consistent with its partial loss of BRCA2 binding capacity. Collectively, the above results suggest that BRCA2 inhibits fork progression after DNA damage through its association with MCM10.

To rule out the possibility that the underrestrained fork progression in cells expressing the mutant MCM10 proteins was due to compromised MCM10 dimer/oligomerization or other functions of the CC motif, we also co-depleted BRCA2 with the endogenous MCM10 in the above stable cells and analyzed fork progression using DNA fiber assay. Under this condition, the differences between cells expressing WT and the mutant MCM10 proteins were no longer observed (Fig. 5d). Moreover, depletion of endogenous BRCA2 did not further accelerate fork progression in cells stably expressing the mutant MCM10 proteins. Thus, it is the association of BRCA2 with the CC motif of MCM10 that restrains fork progression after DNA damage.

As BRCA2 is required for the stability of stalled replication forks, we next asked whether its association with MCM10 contributes to its fork stabilizing function. We labeled the above cells selectively expressing exogenous WT and mutant MCM10 proteins, treated them with HU, and then determined the lengths of CdU- and IdU-labeled tracts (Fig. 5b). Blank U2OS cells depleted of BRCA2 were used as positive control. Loss of BRCA2 led to substantial shortening of newly synthesized DNA tracts after HU treatment, indicative of a degradation of stalled forks, whereas loss of BRCA2–MCM10 association had no impact (Fig. 5b). As such, the association between BRCA2 and MCM10 is required for the cell to restrain replication fork progression after DNA damage but is not involved in fork stabilization upon stalling.

**BRCA2–MCM10 association prevents PRIMPOL-mediated repriming after DNA damage.** Finally, we asked if the function of BRCA2 to suppress PRIMPOL-mediated repriming depends on its association with MCM10. We depleted PRIMPOL together with the endogenous MCM10 in the above stable cell lines expressing WT or MCM10 CC mutants (Supplementary Fig. 3) and measured the lengths of replication tracts. Depletion of PRIMPOL alone had no effect on fork progression in unperturbed cells but evidently reversed the increase in cells expressing MCM10 mutants after IR (Fig. 6a). Moreover, S1 nuclease treatment also reduced tract lengths in cells expressing the mutant MCM10 proteins, without any impact on cells expressing the WT protein (Fig. 6b). Taken together, these results indicate that BRCA2 functions through its association with MCM10 to restrain fork progression after DNA damage by suppressing PRIMPOL-mediated repriming and lesion skipping.

**Discussion**

In this study, we found that BRCA2-deficient cells underwent underrestrained replication fork progression after being treated with diverse types of DNA-damaging agents. Subsequently, we found that the increased fork progression in BRCA2-deficient cells was due to PRIMPOL-mediated replication repriming, leaving behind ssDNA gaps in the newly synthesized strand. Furthermore, we identified an association of BRCA2 with MCM10 mediated by the N-terminal CC motif of the latter. Loss of this association led to underrestrained fork progression after DNA damage, as cells in which the endogenous MCM10 was replaced by exogenous MCM10 lacking the CC motif behaved in the same fashion as did cells depleted of BRCA2, and depletion of BRCA2 eliminated the difference between cells expressing WT and the mutant MCM10 without causing further increase in fork progression in cells expressing the mutant proteins. Additionally, underrestrained fork progression in cells selectively expressing the mutant MCM10 proteins was also found to be due to PRIMPOL-mediated repriming. Based on these findings, we propose a model wherein BRCA2 is recruited to replication forks by MCM10 and, upon stalling of DNA polymerase ε at DNA lesions, inhibits PRIMPOL-mediated repriming, thereby restraining fork progression and preventing ssDNA gap formation (Fig. 6c).

How does BRCA2 inhibit repriming by PRIMPOL? One possible scenario is that it may physically and directly block PRIMPOL recruitment after its own recruitment by MCM10, given its very large size (≈384 KD). As it has been shown that PRIMPOL is recruited by replication protein A (RPA) to ssDNA and that BRCA2/DSS1 can displace RPA from ssDNA, it is also possible that BRCA2 may indirectly prevent PRIMPOL recruitment to damaged forks by removing RPA from the ssDNA arising from the uncoupling of DNA polymerase ε and the MCM2-7 helicase complex upon encounter of DNA lesions. In supporting these two possible scenarios, the amount of chromatin-associated PRIMPOL was larger in cells selectively expressing the MCM10 mutants than in cells expressing the WT protein after DNA damage (Supplementary Fig. 4), although this remains to be substantiated by more precise methodologies such as iPOND. Besides, the possibility that BRCA2 or the BRCA2/MCM10 complex may directly or indirectly inhibit PRIMPOL’s enzymatic activity cannot be ruled out.

The exact lesions that are bypassed by PRIMPOL in the absence of BRCA2 or BRCA2–MCM10 association also remain to be further defined. IR can cause a variety of DNA damage including base damage, single strand breaks (SSBs), DSBs, and DNA–DNA or DNA–protein crosslinks, and bleomycin, much like IR, can generate abasic site, SSBs, and DSBs. While it is unlikely for PRIMPOL to reprim across DSBs, any form of DNA damage that leads to ssDNA regions behind the replication fork could potentially trigger RPA-mediated PRIMPOL recruitment and repriming. Indeed, we found that BRCA2-depleted cells also showed underrestrained fork progression after treatment with MMS, CPT, and HU (Fig. 1g). These findings indicate that PRIMPOL can catalyze repriming across a variety of DNA lesions as a general mechanism for BRCA2-deficient cells to bypass DNA lesions other than DSBs. Careful analyses of repriming efficiency following treatment of BRCA2-deficient cells with different DNA agents at different doses are required to further define the preferred lesions for PRIMPOL action and the relative efficiency of repriming across different types of lesions.

Another key question is whether this newly discovered BRCA2 function is shared by RAD51 and BRCA1, its key partners in the HR pathway that have also been shown to play important roles in fork reversal and/or stability. In this regard, a previous study has shown that loss of RAD51 leads to PRIMPOL-dependent unrestrained replication progression and ssDNA gaps after UV irradiation. Therefore, we tested the impact of RAD51 loss on fork progression after IR. Indeed, RAD51 depletion led to longer replication tracts after IR; however, the underrestrained fork progression caused by RAD51 loss was barely reduced by co-depletion of PRIMPOL (Supplementary Fig. 5a, b). This indicates a limited involvement of PRIMPOL in the process and further suggests that the increased fork progression in RAD51-depleted cells may be mainly due to lack of fork reversal. A recent study has also shown PRIMPOL-mediated repriming in BRCA1-deficient cells, where PRIMPOL was found to be transcriptionally induced after cisplatin treatment and then reprim DNA synthesis. This study was focused on PRIMPOL-mediated repriming to preclude fork reversal and degradation of reversed forks, while in the current study we present a mechanism that is not only specific to BRCA2 but also independent of fork reversal. In fact, we found that depletion of BRCA1 in U2OS cells led to
PrimPol depletion on replication fork progression in stable U2OS cells selectively expressing exogenous WT vs NATURE COMMUNICATIONS | (2021) 12:5966 | https://doi.org/10.1038/s41467-021-26227-6 | www.nature.com/naturecommunications phase checkpoint defect of BRCA2-decidate the mechanism of the RDS phenotype and possible intra-S-independent of its HR function. of BRCA2 to inhibit PRIMPOL-mediated repriming is likely suggests that BRCA1, BRCA2, and RAD51 each has distinct roles (Supplementary Fig. 5c, d). Taken together, available evidence further reduced and remained slower than control after IR DNA damage and that fork speed in BRCA1-depleted cells was substantially reduced fork progression even without exogenous DNA damage and that fork speed in BRCA1-depleted cells was further reduced and remained slower than control after IR (Supplementary Fig. 5c, d). Taken together, available evidence suggests that BRCA1, BRCA2, and RAD51 each has distinct roles with respect to PRIMPOL-dependent lesion bypass, and the role of BRCA2 to inhibit PRIMPOL-mediated repriming is likely independent of its HR function.

Finally, as noted before, this study began as an effort to elucidate the mechanism of the RDS phenotype and possible intra-S-phase checkpoint defect of BRCA2-deficient cells. To address RDS, we initially depleted BRCA2 and used BrdU incorporation to semi-quantitatively measure DNA synthesis before and after IR. BRCA2-depleted cells showed similar levels DNA synthesis during normal growth but indeed incorporated more BrdU than control cells after IR (Supplementary Fig. 6a–c). Consistent with its being a critical replication factor acting in both replication initiation and elongation, depletion of MCM10 led to reduced DNA synthesis, as measured by BrdU incorporation (Supplementary Fig. 6a–c), and slower replication fork progression, as determined by the DNA fiber assay (Supplementary Fig. 6d), both before and after IR. Moreover, cells selectively expressing MCM10-ΔACC also showed increased BrdU incorporation than did cells expressing the WT protein (Supplementary Fig. 6e).

Separately, we conducted DNA fiber analysis using a modified labeling scheme, in which we first labeled BRCA2-depleted cells with CldU for 20 min and then with IdU for 40 min in the presence of bleomycin. Under this condition, BRCA2-depleted cells as expected showed PRIMPOL-dependent increase in fork progression and ssDNA gap formation (Supplementary Fig. 7a), while no increase in origin firing was observed during bleomycin treatment (Supplementary Fig. 7b). As such, unrestrained fork progression, rather than increased origin firing, is the primary contributor to the RDS phenotype of BRCA2-deficient cells.

**Methods**

**Cell lines and culture.** U2OS and HEK293T cells were purchased from ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin (Pen-Strep). VC8 and VC8 + BRCA2 cells were provided by Dr. Maria Jasin (Memorial Sloan Kettering Cancer Center) and grown in DMEM/F12 supplemented with 10% heat-inactivated FBS and Pen-Strep. HeLa S3 cells were obtained from Dr. Yoshihiro Nakatani’s lab (Dana-Farber Cancer Institute) and cultured in DMEM with 5% FBS and Pen-Strep. Cells were verified by morphology match and known growth properties. Cells used did not show signs of mycoplasma contamination and were not tested for mycoplasma. U2OS cells were periodically treated with Plasmocin (Invivogen) to prevent mycoplasma contamination.
**Plasmids and mutagenesis.** The MCM10 cDNA was amplified by RT-PCR from RNA prepared from HeLa S3 cells and cloned between XhoI and NotI sites of the pool of retroviral vector and a modified pM-MBCA2-GFP was previously described. Site-directed mutagenesis was conducted according to the Quick-Change protocol (Agilent Technologies) to generate the MCM10 mutants used. Details will be provided upon request. Primers used for cloning, mutagenesis, and sequencing are available from the authors upon reasonable request. Uncropped western blot images are provided in the Supplemental Table. Established tissue culture cell lines were Amersham ECL HRP-conjugated sheep anti-mouse IgG (GE Healthcare, NA931-1ML, 1:8000), donkey anti-rabbit IgG (GE Healthcare, NA934-1ML, 1:8000), and goat anti-rabbit antibody HRP conjugate (Sigma, AIP183P, 1:8000).

**DNA fiber assay.** The procedures were performed largely following published protocols. Briefly, active replication forks in cells were labeled by sequential 20 min pulses of two thymidine analogs, CldU (20 µM) followed by IdU (200 µM). Cells were then collected, washed, and resuspended in cold PBS at a density of 2 × 10^5 cells/µl. Two µl of the cell suspension were spotted on one to three glass slides. Slides were air-dried, fixed in 3:1 methanol/acetic acid at −20 °C for 15 min and stored at 4 °C overnight. Prior to staining, slides were washed with water, incubated in 2.5 M HCl for 80 min (to denature DNA), washed with PBS 3 times, and blocked with 0.5% BSA in PBS for 20 min. Slides were then incubated at 37 °C for 2 h with primary antibodies (CldU: 1:300, rat monoclonal anti-BrdU, Abcam 6326; IdU: 1:30, mouse monoclonal anti-BrdU, BD biosciences 347580) diluted in blocking buffer. After three washes with PBS, slides were incubated with secondary antibodies (CldU: goat anti-rat, Alexa Fluor 594 (Invitrogen, 610077); IdU: goat anti-mouse, Alexa Fluor 488 (Invitrogen, A-11029); 1:300, IdU: goat anti-mouse, Alexa Fluor 488 (Invitrogen, A-11029)); 1:300) in blocking buffer at 37 °C for 1 h. Slides were washed with PBS, air-dried, and mounted with liquid mountant (ProLong Gold Antifade Mountant, Thermofisher, P36930). Images were acquired with a Nikon TE2000 fluorescence microscope with NIS Elements 4.40 software. For each sample, at least 10 images were taken from the whole slide and at least 200 individual IdU-labeled tracts were measured using ImageJ 1.58i software.

**DNA combing assay.** DNA combing assay was carried out on the molecular combing system from Genomic Vision as described. Cells were pulse labeled sequentially with 50 µM CldU and 50 µM IdU (20 min each) and then incubated with 500 µM thymidine for 60 min. Cells were harvested and embedded in 0.75% agarose plugs. Agarose plugs were melted at 70 °C for 20 min in 0.5 M MES (2-(N-morpholino)ethanesulfonic acid) (pH 6.5). The melted agarose was cooled down to 42 °C and incubated in the presence of β Agarase I (NEB, M0392) overnight. Silanized coverslips were dipped in the DNA solution and incubated for 2 min at RT and then combed for 2 h at 60 °C to crosslink DNA to coverslips. Coverslips were then washed with 0.2 M NaOH for 20 min at RT, washed extensively with PBS and then stained using CldU and IdU antibodies, and single DNA molecules were stained by YOYO-1.

**Chromosomal abnormality.** U2OS cells were seeded at 150,000 cells per well in 6-well plates and transfected with control (AllStars), BRCA2 (1949), PRIMPOL (pool of 1138 and 1158), or BRCA2 + PRIMPOL (BRCA2-1949 + pool of 1138 and 1158). At 1 day after transfection, cells were subjected to 2 Gy of IR, and 6 h after IR, colcemid (0.2 µM) was added to enrich mitotic cells. Cells were harvested 90 min after colcemid addition, and chromosomal abnormality was measured by mitotic spread analysis as previously described. Further details are provided in Supplementary Methods.

**Radiation and drug sensitivity.** U2OS cells were transfected with siRNAs as above. At 30 h after transfection, cells were trypsinized and resuspended into 96-well plates at 2000 cells per well and allowed to attach overnight. Cells were then treated with IR or drugs at different doses. At 72 h after IR or drug addition, cell viability was determined using CellTiter-Glo® 2.0 Cell Viability Assay (G9241, Promega) according to the protocol provided by the manufacturer.

**Data availability**

The data supporting the findings of this study are available from the corresponding authors upon reasonable request. Uncropped western blot images are provided in the Source Data file.
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Author contributions

B.X. conceived the project and supervised the work. B.X., Z.K., M.I.A, Z.S., L.Z., A.-K.B., A.D. and M.S. performed additional interaction domain mapping, mutagenesis and IP analyses; R. B.X. and S.P. generated cDNA constructs, Dr. Maria Jasin (Memorial Sloan Kettering Cancer Institute of New Jersey Flow Cytometry and Cell Sorting Shared Resource, which is supported, in part, by NCI-CCSG P30CA072720-5921. Z.K. and T.R.F. were supported by postdoctoral fellowships from the New Jersey Commission on Cancer Research (NCCCR).

Competing interests

The authors declare no competing interests.
