ATR Signaling Uncouples the Role of RAD51 Paralogs in Homologous Recombination and Replication Stress Response

Graphical Abstract

Highlights

- ATR differentially regulates RAD51 paralog complexes through XRCC2 and XRCC3
- XRCC2 S247 phosphorylation limits DNA damage during perturbed replication
- XRCC2 phospho mutant cells exhibit early XRCC3 activation during replication stress
- XRCC3-mediated repair by HR promotes survival of XRCC2 phospho mutant cells

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In Brief

Mammalian RAD51 paralogs are conserved proteins that function in multiple aspects of genome maintenance. Here, Saxena et al. identify an ATR signaling governed pathway, wherein distinct RAD51 paralogs complexes cooperatively participate to facilitate genome integrity and cell survival during replication stress.
ATR Signaling Uncouples the Role of RAD51 Paralogs in Homologous Recombination and Replication Stress Response

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https://doi.org/10.1016/j.celrep.2019.09.008

SUMMARY

ATR kinase-mediated replication checkpoint is vital for genome maintenance following replication stress. Previously, we showed that XRCC2-RAD51D (DX2) sub-complex of RAD51 paralogs restrains active DNA synthesis during dNTP alterations, in a manner dependent on ATR-mediated phosphorylation of XRCC2. Here, we find that unrestrained fork progression in XRCC2 deficiency and phosphorylation defect causes replication-associated errors, subsequently resulting in genome-wide double-strand breaks (DSBs) and early activation of ATM signaling. Cells defective in XRCC2 phosphorylation exhibit ATM/ATR-mediated early activation of XRCC3 during perturbed replication, which facilitates recombination-mediated repair of the post-replicative DNA damage and thereby promotes cell viability. Collectively, our findings identify collaborative roles of RAD51 paralog complexes during replication stress and reveal their differential regulation by ATR signaling to promote cell survival and genome integrity.

INTRODUCTION

During DNA replication, various types of impediments cause slowing or stalling of the replication forks, leading to replication stress, which is a major driver of tumor progression (Técher et al., 2017; Zeman and Cimprich, 2014). Cells have hence evolved with multiple layers of regulation to manage replication stress, thereby protecting their genome against mutations and tumorigenesis (Saxena et al., 2018; Somyajit et al., 2017; Toledo et al., 2017). One of the primary functions of replication stress response is the protection of newly synthesized DNA against its nucleolytic erosion at stalled replication forks (Rickman and Smogorzewska, 2019). Fork protection involves BRCA1/2 and numerous other homologous recombination (HR) factors including RAD51, Fanconi anemia proteins, and RAD51 paralogs (Quinet et al., 2017b; Schlacher et al., 2011, 2012; Somyajit et al., 2015), in the absence of which replication forks are extensively remodeled or converted into DNA double-strand breaks (DSBs), resulting in genome instability (Liao et al., 2018).

Mammalian RAD51 paralogs are a family of conserved proteins (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) that are involved in HR (Somyajit et al., 2010; Suwaki et al., 2011), DNA damage signaling (Badie et al., 2009; Somyajit et al., 2012, 2013), protection and restart of stalled replication forks (Somyajit et al., 2015), and mitochondrial genome stability (Mishra et al., 2018). RAD51 paralogs exist in two functionally distinct complexes (BCDX2 and CX3) (Masson et al., 2001; Wiese et al., 2002). Moreover, a RAD51 paralog sub-complex (DX2) was recently found to be involved in aligning the rate of fork progression with cellular dNTP pool alterations. Notably, this function was regulated through ATR-mediated phosphorylation of XRCC2, thus implicating the paralogs as key components of the replication stress response (Saxena et al., 2018). Interestingly, another RAD51 paralog, XRCC3, was also identified as a target of ATR kinase, but specifically in response to DSBs (Somyajit et al., 2013). Although the activation and cellular functions of these phosphorylation events have been deciphered in the context of individual complexes, whether these two signaling pathways functionally interact remains elusive.

Here, we show that ATR signaling differentially regulates two RAD51 paralog complexes (DX2 and CX3) through the phosphorylation of XRCC2 and XRCC3. We find that XRCC2 phosphorylation is specifically induced by replication stress and is crucial to prevent the generation of post-replicative gaps and genome-wide DSBs. Notably, cells defective in XRCC2 phosphorylation undergo early activation of XRCC3 during replication stress, which allows HR-mediated repair of the accumulated DNA damage and cell survival. Taken together, our findings reveal an ATR signaling governed functional interplay between RAD51 paralogs, where distinct paralog complexes appear to collaboratively facilitate genome integrity during replication stress.

RESULTS

Unrestrained DNA Replication in XRCC2 Phospho-Defective Cells Induces Post-Replicative ssDNA Gaps
XRCC2-RAD51D (DX2) complex of RAD51 paralogs was recently shown to be involved in restraining active fork
Progression during nucleotide pool alterations. Cells deficient in XRCC2 exhibit permissive DNA replication upon dNTP perturbations, which is associated with an increase in chromosomal instability (Saxena et al., 2018). However, the mechanisms underlying this genome instability remain obscure. Recent evidence suggests that unrestrained replication during stressful conditions is associated with the formation of single-stranded DNA (ssDNA) gaps in the post-replicative genome (Lossaint et al., 2013; Peng et al., 2018). To test this possibility in XRCC2 deficient cells, we directly evaluated the presence of ssDNA gaps in ongoing forks, using a modified DNA fiber protocol (Quinet et al., 2016, 2017a). We subjected U2OS cells to 5-chloro-2′-deoxyuridine (CldU) labeling followed by a pulse of 5-iodo-2′-deoxyuridine (IdU) in the presence of the dNTP-depleting agent hydroxyurea (HU). Before spreading onto the glass slide, we permeabilized the cells and treated the nuclei with the ssDNA-specific enzyme S1 nuclease (Figure 1A). In the presence of gaps, S1 nuclease nicks the ssDNA opposite to the gap, thus converting the ssDNA gap into a DSB and resulting in shorter IdU tracts. Consistent with our previous study (Saxena et al., 2018), in the absence of S1 nuclease, XRCC2-depleted cells exhibited HU-resistant DNA synthesis, as assessed by a higher IdU/CldU ratio than control cells (Figure 1A). However, upon treatment with S1 nuclease, XRCC2-depleted cells, but not control cells, presented a significant decline in the IdU/CldU ratio (Figure 1A), suggesting the presence of ssDNA gaps in these cells. Notably, the formation of these gaps could be fully rescued by complementation with short hairpin RNA (shRNA)-resistant wild-type (WT) XRCC2, but not with the phospho-defective mutant (S247A XRCC2) (Figure 1A), significantly elevated in XRCC2 S247A cells compared with cells expressing WT or phospho-mimetic (S247D) XRCC2 (Figure S1A). Because ssDNA is rapidly coated by the RPA heterotrimer (Maréchal and Zou, 2015), we next used RPA staining as a marker to determine the global levels of ssDNA present in cells following HU exposure. Our immunofluorescence studies showed that while HU caused a modest increase in the RPA70-positive population in WT or S247D XRCC2 cells, S247A mutants exhibited a dramatic increase in the percentage of RPA70-positive cells (Figures 1B and 1C). This was not an indirect consequence of cell cycle perturbations, as no significant difference was observed in the cell cycle profiles of these cells (Figure S1B). Together, these results indicate that XRCC2 plays a vital role in preventing ssDNA gap generation at stressed replication forks, in a manner dependent on its phosphorylation by ATR kinase.

**XRCC2 Phosphorylation Prevents Replication-Born Lesions and Maintains Genome Stability following Replication Stress**

ssDNA gaps within replicated DNA molecules can serve as entry points for nucleases that degrade nascent DNA during prolonged stress (Hashimoto et al., 2010). To determine whether unrestrained fork progression and subsequent ssDNA gap generation in XRCC2 deficiency results in fork degradation, we measured the ratio of IdU to CldU tract lengths following prolonged HU treatment. Indeed, XRCC2-depleted cells and cells expressing S247A XRCC2 mutant showed enhanced fork degradation compared with control cells (Figure 2A). Immunofluorescence staining also showed an increase in the number of pRPA32 S4/8 foci in XRCC2 S247A cells (Figure S1C), which
serves as a marker for DNA resection. Moreover, the intensity of γH2AX in XRCC2 S247A cells increased immensely upon HU treatment, indicating high levels of replication stress (Figure S1D). Cells defective in fork protection pathway exhibit an increase in under-replicated DNA, which can ultimately manifest as chromosome breakage and micronuclei (Naim and Rosselli, 2009). We observed that XRCC2 S247A cells showed significantly more micronucleation than WT cells (Figure 2B), further confirming that XRCC2 phosphorylation is crucial for resolving replication stress. Notably, XRCC2 phospho-defective cells were proficient for HU-induced CHK1 S345 phosphorylation (Figure S2A) and regulation of origin firing (Figure S2B), indicating that activation of the ATR-CHK1 axis remains unaffected by the loss of XRCC2 phosphorylation. Collectively, these results indicate that ATR-mediated phosphorylation of XRCC2 is critical to protect nascent DNA and limit replication-associated damage during replication stress.

Unrepaired ssDNA gaps behind replication forks are likely to undergo breakage and can give rise to pathological DNA structures, including DSBs (Abe et al., 2018; Toledo et al., 2013). To test whether fork degradation in XRCC2 phospho-defective cells is associated with generation of DNA breaks, we studied 53BP1 foci formation as a marker for DSBs in U2OS cells upon HU treatment. Interestingly, XRCC2 S247A cells exhibited a severe increase in 53BP1 foci at merely 2 h of HU treatment (Figure 2C). This was confirmed by neutral comet assay, which showed significant DSB generation in XRCC2 S247A cells upon HU treatment (Figure S2C). Generation of DSBs at stalled replication forks leads to a switch in the DNA damage response from ATR to ATM kinase (Dungrawala et al., 2015; Sirbu et al., 2013; Toledo et al., 2013). ATM coordinates a signaling pathway through the RNF8 and RNF168 ubiquitin ligases, leading to ubiquitin conjugates at DSB sites (Altmeyer and Lukas, 2013a, 2013b), which can be scored using FK2 antibody. XRCC2 S247A cells displayed significantly higher intensity of FK2 than WT cells (Figures S2D and S2E) after a short pulse of HU, further supporting the notion that cells defective in XRCC2 phosphorylation undergo premature induction of DSBs during mild replication stress. Similar results were obtained in XRCC2-depleted MCF7 cells complemented with WT, S247A, or S247D XRCC2 (Figures S3A–S3D). Furthermore, U2OS cells depleted with XRCC2 or expressing the S247A mutant showed increased chromosomal aberrations following treatment with HU (Figures 2D and S2F). Thus,
XRCC2 phosphorylation is crucial for maintaining genome stability in response to replication stress.

Next, we asked whether the increased fork breakage and chromosome instability in XRCC2 S247A cells culminates in cell death. Hence, we studied cell survival in U2OS cells after exposure to different durations of HU treatment: 4 h, which induces replication stress, and 18 h, which causes global fork collapse and DSBs (Petermann et al., 2010). As expected, XRCC2-depleted cells exhibited sensitivity to prolonged HU treatment (18 h), given the role of XRCC2 in HR-mediated DSB repair (Johnson et al., 1999; Nagaraju et al., 2009). However, we failed to observe a survival defect in XRCC2-depleted cells upon short HU treatment (4 h) (Figure 2E). This observation is consistent with our previous results in Chinese hamster cells, in which RAD51C−/− and XRCC3−/− but not XRCC2−/− cells showed hypersensitivity to a short pulse of HU (Somayajit et al., 2015). Moreover, XRCC2 S247A mutants showed no survival defect at 4 or 18 h HU treatment (Figure 2E), the latter observation being consistent with our earlier report that XRCC2 phosphorylation is dispensable for HR (Saxena et al., 2018). Together, these results affirm the role of XRCC2 phosphorylation in limiting replication-associated DNA damage and genome instability during replication stress. However, loss of XRCC2 phosphorylation is competent with cell survival.

ATR Signaling Differentially Regulates XRCC2 and XRCC3

ATR kinase is the master regulator of cellular response to DNA replication stress (Clmprich and Cortez, 2008; Flynn and Zou, 2011). This is orchestrated through coordinated signaling events, involving phosphorylation of hundreds of substrates, which regulate origin firing (Toledo et al., 2013), maintenance of stalled forks (Branzei and Foiani, 2010) and various other aspects of DNA replication and repair (Cortez, 2015; Flynn and Zou, 2011). Moreover, there is now emerging evidence for the involvement of ATR in modulating replication fork progression upon stress (Lossaint et al., 2013; Mutreja et al., 2018; Saxena et al., 2018). XRCC2 is phosphorylated by ATR in response to dNTP pool alterations, and this event is crucial for restraining fork progression (Saxena et al., 2018). However, whether this activation of XRCC2 is specific only to replication stress remains obscure. To test this, we analyzed XRCC2 phosphorylation following HU-induced nucleotide pool depletion and camptothecin (CPT)-induced DSBs. Strikingly, we found that HU but not CPT led to the phosphorylation of XRCC2 on serine residues (Figure S3E), suggesting replication stress-responsive activation of XRCC2. Consistent with this, treatment with the ionizing radiation (IR)-mitotic drug zeocin failed to induce XRCC2 S247 phosphorylation (Figure 3A). Treatment with an ATM inhibitor (KU55933) led to a drastic loss of zeocin-induced pCHK2 T68 and pKAP1 S824 but caused no significant change in HU-induced XRCC2 phosphorylation (Figure 3A), suggesting that ATM is not involved in the activation of XRCC2. Collectively, these results show that XRCC2 is phosphorylated in an ATR-mediated pathway specifically in response to replication stress.

We have previously shown that XRCC3 is phosphorylated by ATR kinase specifically in response to DSBs, and its activation is crucial for DNA damage signaling and repair (Somayajit et al., 2013). Consistent with this, we observed significant XRCC3 S225 phosphorylation upon exposure to DSB-inducing drug, etoposide (Figure 3B). However, in agreement with our results from CPT and zeocin, no detectable XRCC2 phosphorylation was observed under these conditions (Figure 3B). To further validate this differential activation of XRCC2 and XRCC3, we used HU as a model agent to study the transition from fork stalling to collapse. HU-stalled replication forks remain stable in the early hours of treatment but progressively collapse into DSBs between 12 and 24 h (Petermann and Helledge, 2010; Petermann et al., 2010; Somayajit et al., 2015). Indeed, while XRCC2 phosphorylation reached a peak at 4 h of HU treatment, XRCC3 phosphorylation was evident only after 24 h of treatment (Figures 3C and 3D). Collectively, these results suggest that ATR signaling differentially activates individual RAD51 paralogs in response to distinct types of genotoxic insults.

XRCC3 phosphorylation is crucial for RAD51 loading at the sites of DSBs and subsequent HR-mediated repair (Somayajit et al., 2013). Consistent with these observations, XRCC3-depleted U2OS cells complemented with XRCC3 S225A mutant (Figure S3F) showed a significant reduction in IR-induced RAD51 focus formation compared with WT XRCC3-expressing cells (Figure S3G). No significant changes were observed in the cell cycle profiles upon expression of XRCC3 variants (Figure S3H), indicating that the difference in RAD51 foci formation was not a result of cell cycle alterations. In contrast, XRCC2 S247A mutant was found to be completely proficient in RAD51 foci formation (Figures 3E and 3F). To further explore the differential roles of XRCC2 and XRCC3 phosphorylation in genome stability, we studied replication tracts in these cells using DNA fiber assays. Replication rates were comparable in unchallenged cells (Figure S4A). However, unlike XRCC2 S247A mutants, cells defective in XRCC3 phosphorylation were found to be proficient in fork slowdown (Figure S4B) and nascent DNA protection during replication stress (Figure S4C). Together, these results show that disparate activation of the two RAD51 paralog complexes by ATR signaling ensures efficient DNA repair and genome stability upon different kinds of genotoxic stress.

XRCC3 Activation Promotes Cell Survival and Repair of Replication-Born Lesions in Cells Defective for XRCC2 Phosphorylation

It was intriguing that despite accumulation of replication-associated lesions, XRCC2 phospho-defective cells showed no survival defect upon short HU exposure. This was suggestive of the activation of a backup pathway involved in the timely repair of accumulated damage. HR has been implicated in the post-replicative repair of ssDNA gaps and collapsed forks generated during discontinuous replication (Branzei and Szakal, 2016; Petermann et al., 2010). Given that XRCC2 S247A cells are proficient in HR, we speculated that the accumulated lesions might be subsequently repaired in a recombination-dependent manner, thus allowing cell survival. Notably,
ATM/ATR-dependent phosphorylation of XRCC3 is essential for the HR-mediated repair of collapsed replication forks induced by long HU exposure (Somyajit et al., 2013). Because XRCC2 phospho-defective cells exhibited DSB generation and activation of ATM signaling during replication stress, we reasoned that this might result in early XRCC3 activation. Indeed, XRCC2 S247A cells displayed increased XRCC3 S225 phosphorylation, beginning as early as at 2 h of HU treatment, as evident by immunofluorescence studies (Figures 4A and 4B) and immunoblotting (Figure S4D).

Next, we sought to directly investigate the role of pXRCC3-mediated HR in the repair of replication-associated DNA damage accumulated in XRCC2 S247A cells. To this end, we complemented XRCC2 and XRCC3-depleted cells with shRNA-resistant WT/S247A XRCC2 and WT/S225A XRCC3 at levels comparable with endogenous protein levels (Figures S3F and S4E). We then exposed the cells to a short HU block (4 h) and studied persisting γH2AX foci 18 h after release from HU. As expected, XRCC2 S247A cells showed an increased percentage of γH2AX-positive population, compared with WT XRCC2-expressing cells (Figure 4C). Interestingly, co-expression of S247A XRCC2 and S225A XRCC3 (double-phospho-negative cells) led to a further increase in γH2AX-positive cells upon recovery (Figure 4C), suggesting impaired repair and persistence of DNA damage. Moreover, there was a significant increase in micronucleation in the double-phospho-negative cells (Figure S4F), supporting the notion that in the absence of XRCC2 phosphorylation, ATR mediates early activation of XRCC3 to promote HR-mediated repair of accumulated DNA damage.

Next, we tested whether abrogation of HR by inhibition of XRCC3 phosphorylation leads to cell death in XRCC2 phospho-defective cells during replication stress. As expected, expression of XRCC3 S225A mutant caused WT XRCC2 cells to become hypersensitive to long HU exposure, with no prominent effect on cell survival in response to short HU exposure (Figure 4D). This further confirms that although XRCC3 phosphorylation is crucial for DSB repair, it is per se functionally independent of the replication stress response. Strikingly, combined defect of XRCC2 and XRCC3 phosphorylation effectively sensitized cells to a short HU pulse (Figure 4D). Furthermore, ATR inhibition also sensitized XRCC2 S247A cells to short HU exposure (Figure S4G). Together, these observations indicate that cells defective in XRCC2 phosphorylation cause early activation of XRCC3 during replication stress in an ATM/ATR-mediated
When phosphorylated, XRCC3 facilitates recombination-mediated repair of the accumulated DNA lesions, thus promoting cell survival and genome integrity.

**DISCUSSION**

Here, we have identified an ATR governed pathway wherein distinct RAD51 paralogs cooperatively participate to maintain genome integrity and cell survival during replication stress. We find that cells defective in ATR-mediated phosphorylation of XRCC2 form post-replicative gaps during replication stress, resulting in increased fork processing and breakage. Consequently, these cells skew to HR-mediated DNA repair for survival, which is in turn dependent on ATM/ATR-mediated activation of XRCC3. These findings have important implications for understanding the complex interplay within this family of conserved proteins, in which each RAD51 paralog has a distinct and yet collaborative role in genome maintenance, depending on the type and extent of DNA damage.

An important implication of our study is that ATR functions at multiple stages to ensure genome stability and cell survival during replication stress, depending on the extent of damage incurred by the forks. First, ATR promotes fork slowdown through XRCC2 phosphorylation. This is crucial for the “prevention” of DNA damage by limiting aberrant DNA replication and formation of ssDNA gaps in the post-replicative genome (Figure 4E). However, in the absence of this first line of defense, as in cells harboring mutations in XRCC2, forks undergo breakage, resulting in the formation of DSBs. This poises ATR for the second stage of “repair,” wherein it activates XRCC3 and promotes recombination-mediated repair of the accumulated DNA damage (Figure 4E). This model is consistent with previous studies suggesting that prevention of fork collapse represents an early response of ATR to perturbed DNA replication (Chanoux et al., 2009; Couch et al., 2013). However, our results further extend these observations to propose a layered checkpoint response within the ATR pathway to limit DNA damage upon replication stress. In a similar scenario, a recent study showed that BRCA1-deficient cancer cells sequentially bypass their HR and fork protection functions during the acquisition of PARPi resistance. Notably, this rewiring is mediated by ATR, thus rendering these cells increasingly dependent on ATR for...
translocation events (Ochs et al., 2016; Willis et al., 2017). Of HR might further skew these cells to alternative, potentially reparative in the phosphorylation of both XRCC2 and XRCC3, suggesting the involvement of RAD51 paralogs in a layered DNA damage response pathway governed by ATR signaling. Together, these observations not only expand the current model for genome maintenance by RAD51 paralogs but also inspire their assessment as potential targets for cancer therapy from a translational perspective.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.09.008.

ACKNOWLEDGMENTS

We thank IISc confocal microscopy and fluorescence-activated cell sorting (FACS) facility for their help. We greatly acknowledge funding from the Department of Science and Technology (EMR/2015/001720); the Department of Biotechnology (DBT) (BT/PR23498/BRB/10/1590/2017); a DBT-National Bioscience Award (BT/HRD/NBA/37/01/2015); the IISc-DBT partnership program (DBT/PR/1NS/IISc-2011–12 and BT/PR27952/INF/22/212/2018), India; and infrastructure support provided by funding from DST and UGC, India. S.S. and S.D. are supported by fellowships from the IISc and CSIR, respectively. K.S. was supported by a fellowship from the CSIR, India; a Bristol-Myers Squibb fellowship, UK, and the Ranbaxy Science Foundation, India.

AUTHOR CONTRIBUTIONS

S.S., K.S., and G.N. conceived the project and designed the experiments. S.S. and K.S. performed the experiments. S.D. contributed to the generation of reagents. S.S., K.S., and G.N. analyzed the data. S.S. and G.N. wrote the manuscript.
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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rat anti-BrdU antibody | Abcam  | Cat# ab6326; RRID: AB_305426 |
| Donkey Anti-Rat Alexa Fluor® 594 | Abcam | Cat# ab150156 |
| Purified Mouse Anti-BrdU | BD Biosciences | Cat# 347580; RRID: AB_400326 |
| Rabbit Anti-Mouse IgG H&L (Alexa Fluor® 488) | Abcam | Cat# ab150125 |
| Anti-XRCC2          | Santa Cruz | Cat# sc-365854; RRID: AB_10846464 |
| Anti-XRCC3          | Santa Cruz | Cat# sc-271714; RRID: AB_10708416 |
| Anti-RRAD60         | Abcam  | Cat# ab9398; RRID: AB_1603759 |
| Anti-BrdU           | BD Biosciences | Cat# 555627; RRID: AB_10015222 |
| Anti-HA tag         | Roche  | Cat# 10952100 |
| Anti-pKAP1 S824     | Abcam  | Cat# ab70369; RRID: AB_1209417 |
| Anti-pCHK1 S345     | Abcam  | Cat# ab58567; RRID: AB_1056825 |
| Anti-pCHK1 S345     | Cell Signaling Technology | Cat# 2348S; RRID: AB_331212 |
| Anti-CHK1           | Santa Cruz | Cat# sc-377231 |
| Anti-pCHK2 T68      | Abcam  | Cat# ab85743; RRID: AB_1085765 |
| Anti-pCHK2 T68      | Cell Signaling Technology | Cat# 2197S; RRID: AB_2080501 |
| Anti-MCM3           | Santa Cruz | Cat# sc-365616; RRID: AB_10846721 |
| Anti-RAD51          | Santa Cruz | Cat# sc-8349; RRID: AB_2253533 |
| Anti-HSP90          | Santa Cruz | Cat# sc-69703; RRID: AB_2121191 |
| Anti-53BP1          | Millipore | Cat# 05-725; RRID: AB_309939 |
| Anti-Ubiquitylated proteins, clone FK2 antibody | Millipore | Cat# 04-263; RRID: AB_612093 |
| Anti-H2AX (pS139)   | BD Biosciences | Cat# 560443; RRID: AB_1645592 |
| Anti-Phospho RPA32 (S4/S8) | Bethyl | Cat# A300-245A; RRID: AB_210547 |
| Anti-Phospho Serine | Thermo Fisher Scientific | Cat# 61-8100; RRID: AB_2533940 |
| Anti-PARP1          | Santa Cruz | Cat# sc-365315; RRID: AB_10842296 |
| Anti-pXRCC3 S225    | Somyajit et al., 2013 | N/A |
| Anti-pXRCC2 S247    | Saxena et al., 2016 | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| 5-Chloro-2'-deoxyuridine | Sigma-Aldrich | Cat# C6891 |
| 5-Iodo-2'-deoxyuridine | Sigma-Aldrich | Cat# I7125 |
| 5-Bromo-2'-deoxyuridine | Sigma-Aldrich | Cat# B5002 |
| Hydroxyurea         | Sigma-Aldrich | Cat# H8627 |
| DAPI                | Sigma-Aldrich | Cat# D8417 |
| Camptothecin        | Sigma-Aldrich | Cat# C9911 |
| S1 nuclease         | Invitrogen | Cat# 18001016 |
| Propidium iodide    | Sigma-Aldrich | Cat# P4170 |
| cOmplete, Mini Protease Inhibitor Cocktail | Roche | Cat# 11836153001 |
| PhosSTOP            | Roche | Cat# 4906837001 |
| Thiazolyl Blue Tetrazolium Bromide (MTT reagent) | Sigma-Aldrich | Cat# M2128 |
| VE821 (ATR inhibitor) | Selleckchem | Cat# 8007 |
| KaryoMAX Colcemid Solution | Thermo Fisher Scientific | Cat# 15212012 |
| G 418 Sulfate       | Calbiochem | Cat# 108321-42-2 |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Prof. Ganesh Nagaraju (nganesh@iisc.ac.in). All plasmids and unique/stable reagents generated in this study are available on request without restrictions.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
Human cell lines U2OS, MCF7 and HeLa were obtained from ATCC. Source and identifier (if applicable) of cell lines used in this study are also listed in the key resources table.

METHOD DETAILS

Cell culture
Human cell lines U2OS, MCF7 and HeLa were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin (Sigma-Aldrich) at 37°C in humidified air containing 5% CO2. For generation of stable cell lines, cells were subjected to Neomycin selection 48 h after transfection (G418 sulfate; 1.6 mg/ml). The colonies were later pooled and propagated under constant neomycin selection (0.4 mg/ml).

DNA constructs and transfections
Human XRCC2 WT, phospho-defective and phospho-mimetic constructs, shRNA resistant constructs (Saxena et al., 2018) and human XRCC3 WT, phospho-defective (Somyajit et al., 2013) and shRNA resistant constructs were generated using PCR-based
mutagenesis and cloned into pcDNA3β vector using *E. coli* DH5α. Sequences of primers used for generation of the constructs are listed in Table S1. XRCC2 and XRCC3 shRNA constructs were generated using reported siRNA sequences and cloned into pRS shRNA vector.

XRCC2#1: 5′-TGCAACGACAACTATAAA-3′ (Saxena et al., 2018; Zheng et al., 2012)
XRCC3#1: 5′-GAATTATGCTGCAATTAA-3′ (Saxena et al., 2018; Somyajit et al., 2013)

All plasmid transfections for stable and transient expression were performed using a Bio-Rad gene pulsar X cell (250 V and 950 μF). 6-8 h after transfection, fresh media was added to the cells. Cells were harvested/proceeded for indicated treatments (18-24 h after the transfection for XRCC2 and 30-36 h after the transfection for XRCC3).

DNA fiber spreads
Approximately 5 × 10⁶ cells were plated in each well of a six-well plate. Cells were pulse-labeled with 25 μM CldU and 250 μM IdU as indicated in the sketches. Later, cells were harvested and re-suspended in 50 μL of chilled PBS. Cell suspensions (3 μL) were placed on glass slides (Thermo ScientificSuperfrost) and briefly allowed to air-dry. The cell suspension was then mixed with 8 μL of lysis buffer (0.5% sodium dodecyl sulfate, 200 mM Tris-HCl [pH 7.4], 50 mM EDTA) and incubated for 2 min. Slides were inclined at 45° to spread the suspension. Once dried, DNA spreads were fixed by incubation in a 3:1 solution of methanol-acetic acid for 10 min, followed by denaturation with 2.5 N HCl for 80 min. After several rinses in PBS, the slides were incubated with blocking buffer (2% BSA and 0.01% Tween-20 in PBS) for 15 min. This was followed by incubation with rat anti-BrdU antibody (1:100 in blocking buffer; Abcam; ab150156). After incubation for 1 h at RT, slides were washed twice with 0.1% Tween-20 and incubated overnight with mouse Anti-IdU antibody (1:25 in blocking buffer; BD Biosciences; 594-conjugated Anti-Rat secondary antibody (1:100 in blocking buffer; Abcam; ab150125). After 2 washes with 0.1% Tween-20, slides were mounted with coverslips using Dabco mounting media (Sigma-Aldrich). Fibers were imaged using an Olympus IX71 microscope. Between 100 and 250 fibers were measured using ImageJ software from 2-3 independent experiments and P values were calculated using Prism software.

Effect of drug treatment on fork progression is represented as the ratio of IdU (labeled in the presence of the drug) to CldU tract lengths. For calculation of replication rate (kb/min) in unchallenged cells, length of IdU tracts (in kb) was divided by the time of IdU labeling (in minutes).

S1 nuclease sensitivity assay
The S1 nuclease assay for detection of ssDNA gaps was performed as described previously (Quinet et al., 2016, 2017a). Briefly, after the IdU pulse, cells were treated with CSK100 buffer (100 mM NaCl, 10 mM MOPS, 3 mM MgCl2 [pH 7.2], 300 mM sucrose, and 0.5% Triton X-100) for 10 min at room temperature, then incubated with S1 nuclease buffer (30 mM sodium acetate [pH 4.6], 1 mM zinc acetate, 5% glycerol, and 50 mM NaCl) with or without 20 U/mL S1 nuclease (Invitrogen, 18001-016) for 30 min at 37°C. The cells were then scraped in PBS + 0.1% BSA and centrifuged at 7,000 rpm for 5 min at 4°C. Cell pellets were resuspended in chilled PBS and proceeded for DNA fiber spreading as described before.

Western blotting
Cells were harvested and lysed in RIPA buffer (without SDS) supplemented with Complete and PhosSTOP tablets (Roche). Protein concentrations were estimated by standard Bradford assay. Proteins were resolved on a 10% SDS-PAGE gel and transferred onto PVDF membranes (Millipore). The membranes were blocked using 5% dry milk (w/v) in TBST (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20). For analysis of phosphorylated proteins, 3% BSA (w/v) in TBST was used for blocking. The membranes were then incubated with primary antibody overnight at 4°C. The membranes were washed with TBST and incubated with respective HRP-conjugated secondary antibodies (1:8000; Santa Cruz) for 1 h at 4°C. After TBST washes, membranes were developed with chemiluminescent HRP substrate (Millipore) and imaged using Chemidoc (GE healthcare LAS 4000).

Immunoprecipitation
After harvesting, cells were washed in PBS and lysed in IP lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1% NP-40, 0.5% sodium deoxy cholate, 0.1% SDS) supplemented with Complete and PhosSTOP tablets (Roche). After 1 h incubation on ice, lysates were cleared by centrifugation. Where appropriate, antibodies were added to lysate and incubated for 12-16 h at 4°C. Lysates were then incubated with 25 μl of Protein A/G beads (GE Healthcare) for 2 h at 4°C. Ig-antigen complexes were washed extensively and eluted in 2x Laemml sample buffer at 90°C for 30 min before SDS-PAGE.

Cell cycle analysis
Cells were harvested and single-cell suspensions were fixed overnight with 70% ethanol in PBS at −20°C. After centrifugation, the cells were incubated with RNaseA (0.1 mg/ml) in PBS at 42°C for 4 h and then incubated for 10 min with PI (50 μg/ml) in dark. A total of
1x10⁴ cells were analyzed by Verse flow cytometer (Becton Dickinson). Aggregates were gated out and percentage of cells with 2N and 4N DNA content was calculated using FACSDiva Version 6.1.1 software (Becton Dickinson).

**Cell survival assay**
Cells (5000/well) were seeded in a 24-well plate. After treatment (or mock treatment) with HU as indicated, cells were allowed to grow for 7-10 days. Cell survival was monitored by MTT (0.1 mg/ml; Sigma-Aldrich) assay using microplate reader (VersaMax ROM version 3.13). Percent cell survival was calculated as treated cells/untreated cells × 100.

**Metaphase spreads**
Cells were treated with HU for 4 h, recovered for 24 h in fresh media and incubated with Demecolcine (1 μg/ml) for the last 4 h of recovery. Cells were then harvested and treated with hypotonic solution (75 mM KCl) for 12 min, washed three times with chilled fixative (methanol/acetic acid 1:1), and left overnight at 4°C. Cells were later dropped onto a chilled glass slide, air-dried and visualized using Olympus CKX41 inverted microscope. For each case, at least 100 metaphase plates were scored.

**Immunofluorescence**
Exponentially growing cells were seeded onto coverslips, then treated (or mock-treated) with HU as indicated. After treatment, the cells were washed with PBS and fixed in 3.7% formaldehyde for 10 min at RT. If indicated, pre-extraction was performed with 0.2% Triton X-100 on ice for 90 s before fixation. Later, cells were blocked in blocking buffer (0.5% BSA and 0.5% Triton X-100 in PBS) for 30 min. For native BrdU staining, cells were incubated with 10 μM BrdU for 24 h, washed and treated with 500 μM HU for 2 h. For staining, cells were pre-extracted on ice for 1 min, followed by blocking in BrdU blocking buffer (10% FBS and 0.01% sodium azide in DMEM). The coverslips were incubated with the indicated primary antibodies for 2 h at RT. After a wash with blocking buffer, the coverslips were incubated with respective FITC/TRITC/AlexaFluor-conjugated secondary antibodies for 1 h at RT, and then stained with DAPI (1 μg/ml; Sigma-Aldrich) for 10 min before mounting onto slides. Cells were acquired using a confocal microscope (LSM510; Carl Zeiss) and images were processed using Zeiss LSM image browser software.

**Neutral comet assay**
24 h before the experiment, Silane-Prep Slides from Sigma (S4651-72EA) were pre-coated in 0.8% agarose in PBS and stored at R.T. Cells were treated as required, harvested in PBS, centrifuged and resuspended in 200 μl of 0.8% low melting point (LMP) agarose. 75 μl of cell-agarose suspension was spread on precoated slides, covered with coverslip and placed on icepack for 10 min for solidification. The coverslip was removed and a layer of LMP agarose was spread on the top. This was again covered with a coverslip and placed on icepack for 10 min. The coverslip was removed, and the slides were placed in chilled lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris base (pH 10), 1% N-laurylsarcosine, 0.5% Triton X-100) at 4°C for 2 h. The slides were washed three times in the electrophoresis buffer (300 mM sodium acetate and 100 mM Tris-HCl (pH 8.3)), followed by electrophoresis at 15 V (0.5 V/cm) for 1 h at R.T. The slides were washed three times in PBS, fixed in absolute ethanol and air-dried. The slides were stained with 50 μL ethidium bromide (2 μg/ml in water), covered with coverslip and imaged using an Olympus IX71 microscope. Between 70-100 comets were measured using ImageJ software with OpenComet plugin from 2 independent experiments.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Statistical differences in RAD51 foci experiments, chromosomal aberrations assays, cell cycle analysis were determined in terms of p value from two-tailed Student’s t test. Statistics for DNA fiber and immunofluorescence data were calculated using GraphPad Prism software (Version 6.0). N (in DNA fiber data) denotes the number of fibers analyzed per condition. A minimum of 100 DNA fibers were analyzed for each condition. N (in immunofluorescence data) denotes the number of cells analyzed per condition. A minimum of 100 cells were analyzed for each condition. Values of N and P are provided in the respective figure legends.

**DATA AND CODE AVAILABILITY**
This study did not generate/analyze datasets/code.