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# Differential Roles of XRCC2 in Homologous Recombinational Repair of Stalled Replication Forks

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**Abstract** Homologous recombination is an important mechanism in DNA replication to ensure faithful DNA synthesis and genomic stability. In this study, we investigated the role of XRCC2, a member of the RAD51 paralog family, in cellular recovery from replication arrest via homologous recombination. The protein expression of XRCC2, as well as its binding partner RAD51D, is dramatically increased in S- and $G_2$-phases, suggesting that these proteins function during and after DNA synthesis. *XRCC2* mutant irs1 cells exhibit hypersensitivity to hydroxyurea (HU) and are defective in the induction of RAD51 foci after HU treatment. In addition, the HU-induced chromatin association of RAD51 is deficient in irs1 mutant. Interestingly, irs1 cells are only slightly sensitive to thymidine and able to form intact RAD51 foci in S-phase cells arrested with thymidine. Irs1 cells showed increased level of chromatin-bound RAD51 as well as the wild type cells after thymidine treatment. Both HU and thymidine induce γ-H2AX foci in arrested S-phase nuclei. These results suggest that XRCC2 is involved in repair of HU-induced damage, but not thymidine-induced damage, at the stalled replication forks. Our data suggest that there are at least two sub-pathways in homologous recombination, XRCC2-dependent and -independent, for repair of stalled replication forks and assembly of RAD51 foci following replication arrest in S-phase. J. Cell. Biochem. 95: 942–954, 2005. © 2005 Wiley-Liss, Inc.

**Key words:** XRCC2; RAD51; RAD51 paralogs; homologous recombinational repair; stalled replication forks; replication inhibitor

Homologous recombinational repair (HRR) is an essential cellular process that is highly conserved from bacteria to humans. HRR serves as an important mechanism for eliminating DNA double-strand breaks (DSBs) from chromatin in an error-free manner, thereby maintaining genomic integrity and stability. A critical process in HRR is the polymerization of RAD51 onto single-stranded DNA (ssDNA) ends of a DSB, forming nucleoprotein filaments, which facilitate the homologous searching, pairing, and strand exchange [reviewed in Sung et al., 2003]. The assembly of the RAD51-ssDNA nucleoprotein complex is a rate-limiting step, as RAD51 needs to replace replication protein A (RPA), which has much higher affinity to ssDNA. In eukaryotic cells, a number of proteins, including RAD52, RAD54, BRCA1, BRCA2, and the five RAD51 paralogs (XRCC2, XRCC3, RAD51B, RAD51C, and RAD51D), are required to mediate or promote the process of RAD51 nucleation [reviewed in Thompson and Schild, 2001, 2002; Sung et al., 2003; West, 2003]. In cells exposed to ionizing radiation or other DNA damaging agents, RAD51 accumulates in multiple discrete foci, which are likely the sites where HRR takes place [Haaf et al., 1995; Raderschall et al., 1999]. A common characteristic of the RAD51 mediators is that their functions are essential for the formation of DNA damage-induced RAD51 foci. Hamster and chicken RAD51 paralog mutants are defective in formation of RAD51 foci after exposure to ionizing radiation and other DNA damaging agents [Bishop et al., 1998; O'Regan et al., 2001;

Abbreviations used: dCTP, deoxycytidine triphosphate; DSB, DNA double-strand breaks; HRR, homologous recombinational repair; HU, hydroxyurea; MMC, mitomycin C; NHEJ, non-homologous end joining; RPA, replication protein A; ssDNA, single-stranded DNA; TR, thymidine.

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Takata et al., 2001; Godthelp et al., 2002; Liu, 2002]. These data suggest that RAD51 paralogs are involved in loading RAD51 onto ssDNA at a DSB site in the early stage of HRR.

Recent studies have suggested that RAD51 paralogs play roles in S-phase of the cell cycle for repair of DSBs at stalled replication forks. In an in vivo assay using the hypoxanthine phosphoribosyltransferase locus as a reporter gene, it was demonstrated that XRCC3 is essential for the repair of camptothecin-induced DSBs following replication fork arrest [Arnaudeau et al., 2001]. In addition, it was found that *xrcc3* deficient cells (irs1SF) showed an increased sensitivity to DNA replication elongation inhibitors hydroxyurea (HU) and thymidine, both of which induce replication arrest-associated homologous recombination in vivo [Lundin et al., 2002]. Recently, Henry-Mowatt et al. reported that XRCC3 is involved in the mechanism that controls the progression of replication forks after DNA damage. They found that the rate of replication fork progression was reduced in normal vertebrate cells by treatment with UV or cross-linking agent cisplatin, but the reduced fork progression was less severe in irs1SF and *xrcc3−/−* chicken DT40 cells treated with the same agents [Henry-Mowatt et al., 2003]. The defects in the slowing of replication forks of *xrcc3* mutants can be corrected by introduction of purified human RAD51C-XRCC3 complex or RAD51 protein [Henry-Mowatt et al., 2003]. These data suggest that XRCC3 and RAD51 cooperatively modulate the progression of replication forks on damaged chromosomes. In addition, XRCC3 directly interacts and co-immunoprecipitates in human cell extracts with RPA [Yoshihara et al., 2004], which plays an essential role in DNA synthesis [reviewed in Wold, 1997]. Moreover, RAD51D physically and functionally interacts with the product of *BLM*, the causal gene for Bloom's Syndrome [Braybrooke et al., 2003]. BLM interacts and colocalizes with RPA and RAD51 [Brosh et al., 1999; Wu et al., 2001], and plays a role in recovery from S-phase replication arrest [Davies et al., 2004].

In this study, we investigated the role of XRCC2 in RAD51-mediated homologous recombination following DNA replication fork arrest. Our data showed that the function of XRCC2 is required for HU-induced RAD51 focus formation, but not essential for the foci induced by thymidine. The disability of forming HU-induced RAD51 foci in *xrcc2*-deficient mutant is related to the defect in loading of RAD51 onto chromatin. Although both HU and thymidine induce $\gamma$-H2AX foci, irs1 cells were hypersensitive to HU, but much less sensitive to thymidine. These results suggest that the XRCC2 is involved in repair of HU-induced damage, but is less important for repair of thymidine-induced damage. Our data also suggest that there are at least two sub-pathways in homologous recombination, XRCC2-dependent and -independent, for repair of stalled replication forks and assembly of RAD51 foci following replication arrest in S-phase.

## MATERIALS AND METHODS

### Cell Lines

Hamster cell lines V79 (wild type), irs1 (*xrcc2* mutant), and irs1/XRCC2 (*XRCC2* complemented irs1 cell line) were cultured at 37°C in an atmosphere of 5% $CO_2$ in $\alpha$-MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics as described [Liu, 2002]. Irs1/XRCC2 cells were obtained by stable transfection of irs1 cells with the XRCC2 expression vector pGFP-XRCC2, which corrects the hypersensitivity of irs1 to mitomycin C [Liu et al., 2002]. The normal human diploid somatic fibroblast cell line (MJ90), a gift from Dr. Miguel Rubio (Lawrence Berkeley National Laboratory, Berkeley, CA), was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics.

### Cell Survival

Cells were plated in 100 mm dishes in 10 ml medium at different cell concentrations depending upon the treatment. After incubation at 37°C for 16 h, HU or thymidine was added to the culture at various concentrations. Cells were grown in the drug-containing medium until visible colonies are formed. Cells were then fixed with 95% ethanol and stained with crystal violet. Colonies that contain >50 cells were counted.

### Cell Synchronization

For the normal human MJ90 cells, we used serum starvation and aphidicolin blocking/ release method as described previously [Dulic et al., 1998]. To obtain $G_0$ phase cells, cells were grown in the medium containing 0.2% serum for 72 h. $G_1$ cells were obtained by stimulating $G_0$

phase of cells with the medium containing 15% serum for 12 h. To prepare S and $G_2$/M cells, $G_0$ cells were stimulated with the medium containing 15% serum for 12 h and then incubated with aphidicolin (2 μg/ml) for 24 h. After extensive washing of cells with phosphate buffered saline (PBS), the medium containing 15% serum was then added and cells were incubated for 2 or 6 h for collecting S- and $G_2$/M-phase cells, respectively. To synchronize hamster cells in S-phase, cells were first incubated with 2 mM thymidine for 16 h, and then released in regular medium supplemented with 10% serum for 8 h, followed by incubation with 2 mM HU or thymidine for another 16 h. Cells were washed with PBS extensively and released in regular medium for 1.5 h before harvest.

### Analysis of Cell Cycle Distribution by FACScan

To monitor the quality of cell synchrony, cell cycle profiles were analyzed by flow cytometry. Cells were trypsinized and fixed in 70% ethanol in PBS at 4°C overnight, followed by treatment with RNase A and staining with propidium iodide. In some experiments, the S-phase cells were pulse-labeled with bromodeoxyuridine (BrdU) by incubation with 10 μM BrdU at 37°C for 15 min before harvest. Cells were then fixed with ethanol and stained with Fluorescein-conjugated anti-BrdU antibody following manufacturer's instruction (Becton-Dickinson, San Jose, CA). Data were acquired on a FACScan and analyzed with Cell Quest$^{TM}$ software (Becton-Dickinson).

### Ionizing Irradiation

MJ90 cells were irradiated with X-ray (10 Gy) using a Pantak® X-ray generator operating at 320 kV/12 mA. Cells were incubated further at 37°C for 1 h and then harvested for cell extraction and immunoblotting.

### Immunoblotting

Whole cell extracts were prepared in cell extract buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, and 0.5% NP-40. A total of 50 μg of cell extracts was resolved onto 4–15% pre-cast SDS–PAGE gels and immunoblotted with the designated antibodies. Rabbit anti-human XRCC2 and rabbit anti-human RAD51 antibodies were described previously [Liu et al., 2002]. Rabbit anti-human RAD51D antibody was purchased from Novus Biologicals (Littleton, CO). Anti-tubulin and anti-Histone H3 antibodies were kindly provided by Dr. Matthew Coleman (Lawrence Livermore National Laboratory). Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibodies (Bio-Rad, Hercules, CA) were used as secondary antibodies. The proteins were detected by using ECL plus chemiluminescent detection system (Amersham Biosciences, Piscataway, NJ) followed by autoradiography.

### Immunostaining

Similar procedures were followed as described previously [Liu, 2002] with some modification. Cells grown on monolayer were trypsinized and resuspended at $1 \times 10^5$ cells/ml in medium, and 300 μl of cells were spun onto a glass slide by centrifugation at 2000 rpm for 5 min on a Cytospin centrifuge (Thermo Shandon, San Jose, CA). The cells on the slides were fixed with 4% paraformadehyde in PBS for 15 min at room temperature. Cells were permeabilized with 0.5% triton X 100 in PBS at room temperature for 10 min or in ice-cold acetone-methanol mixture (1:1 v/v) for 5 min, blocked with 1× PBS containing 1% bovine serum albumin (BSA) for 1 h followed by incubation with rabbit anti-human RAD51 antibody in PBS with 1% BSA for 1 h at room temperature or overnight at 4°C. After washing with PBS three times for 10 min with gentle agitation, cells were incubated with Alexa-fluor 546-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR) for 1 h followed by washing with PBS three times. Immunostaining of γ-H2AX foci was done using mouse anti-human γ-H2AX antibody (Upstate, Inc., Charlottesville, VA) followed by staining with Texas Red-conjugated anti-mouse IgG (Molecular Probes). Slides were then mounted with Vectashield mounting medium (Vector laboratories, Burlingame, CA) containing 4′,6′-diamidino-2-phenylindole (DAPI) at 0.1 mg/ml. Immunostained slides were examined under a Zeiss fluorescent microscope, and fluorescent images were captured and recorded using software Pathvysion (Applied Imaging, San Jose, CA). At least 200 nuclei were scored. Cells containing more than five foci were recorded as positive.

### Cell Compartment Fractionation

Subcellular fractionation was carried out using the methods described by Tarsounas et al. [2003] with some modifications. Briefly,

exponentially growing hamster V79 and irs1 cells were harvested by trypsinization. Approximately $5 \times 10^6$ cells were pelleted and resuspended in hypotonic Buffer A (10 mM HEPES-KOH pH 7.1, 50 mM NaCl, 0.3M sucrose, 0.1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, and protease inhibitors) and incubated on ice for 15 min. Nuclei were pelleted by centrifugation at $1500 \times g$ for 5 min. Supernatants (cytoplasmic fraction) were transferred into fresh tubes. Nuclear pellets were washed once with Buffer B (10 mM HEPES-NaOH pH 7.1, 0.1 mM EDTA, 1 mM DTT, and protease inhibitors), resuspended in Buffer C (10 mM HEPES-NaOH pH 7.1, 0.5M NaCl, 0.1 mM EDTA, 0.5% NP40, 1 mM DTT, and protease inhibitors), and incubated on ice for 15 min with occasional vertexing. The extracts were centrifuged at $16{,}000 \times g$ at 4°C for 10 min. The supernatant (nuclear fraction) was collected in fresh tubes. After washing with Buffer C once, the pellet (chromatin fraction) was then resuspended in 20 μl SDS–PAGE loading buffer, sonicated to shear chromatin DNA, and boiled for 5 min. Proteins were separated by SDS–PAGE and RAD51 was analyzed by Western blotting with rabbit anti-human RAD51 antibody [Liu, 2002]. The membranes were re-blotted with tubulin, actin, or H3 antibodies, which were used as loading controls for cytoplasmic, nuclear, and chromatin fractions, respectively. To quantify the proteins, signals from Western blots for RAD51 were scanned and analyzed using KODAK 1D Imaging Systems (New Haven, CT). The levels of RAD51 in chromatin fractions were normalized with H3, and the ratios of RAD51 induction relative to the asynchronous cells were calculated.

## RESULTS

### Protein Levels of XRCC2 and RAD51D are Increased in S- and $G_2$/M-Phases

To elucidate the function of RAD51 paralogs in HRR and DNA replication, we first examined the cell cycle regulation of XRCC2 protein expression in human normal fibroblast MJ90 cells. As shown in Figure 1, the XRCC2 protein level is very low and almost undetectable in cells synchronized at $G_0$- or $G_1$-phases, but is dramatically increased in S- through $G_2$/M-phases. This expression pattern is consistent with that of RAD51D, the protein directly interacts with XRCC2 [Braybrooke et al., 2000], and RAD51, both of which also peak at S- and $G_2$/M-phases (Fig. 1). In addition, we observed that X-ray irradiation does not induce the expression of XRCC2 and RAD51D in any phase of the cell cycle (Fig. 1), which is consistent with the observations in asynchronous cells [Liu, 2002]. These results suggest that the expression of XRCC2 and RAD51D are regulated during the cell cycle and are probably coordinated with that of RAD51. The increased protein levels in S- and $G_2$-phases suggest that these proteins are primarily functioning during or after DNA replication. Notably, we could not detect the cell cycle-dependent expression of XRCC2, RAD51D, and RAD51 in HeLa S3 cells synchronized with thymidine/aphidicolin double blocking and release, as the levels of these proteins showed no change throughout the cell cycle (data not shown).

### Synchronization and Recovery of Hamster Cell From S-Phase Arrest

To obtain high population of cells arrested in S-phase, we first used thymidine block/release to synchronize cells. Then the cells were incubated with either HU or thymidine at 2 mM for 16 h, followed by releasing cells in drug-free medium for 1.5 h to allow cells to progress into S-phase. As shown in Figure 2, we achieved >90% S-phase cells using this approach as determined by FACS analysis of cell cycle profiles. The cell cycle recovery of V79 and irs1

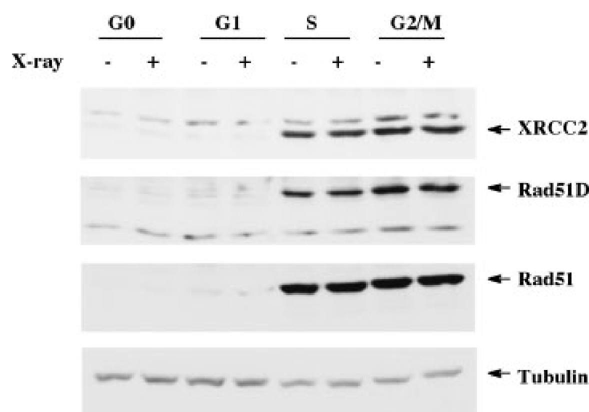


**Fig. 1.** Protein expression of XRCC2, RAD51D, and RAD51 in different phases of cell cycle. The normal human diploid somatic fibroblasts were synchronized as described in "Materials and Methods," and irradiated or unirradiated with 10 Gy X-ray. A total of 50 μg of whole cell extracts was dissolved by SDS–PAGE and analyzed by Western blotting with the antibodies for respective proteins. Anti-tubulin antibody was used as a loading control for each whole cell extract. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cells from the replication arrest was examined in cells that were released in the drug-free medium for 1.5, 6, 9, and 24 h. The results show that V79 or irs1 cells treated with HU are accumulated in $G_2$-phase even after 24 h (Fig. 2A). In contrast, both V79 and irs1 cells treated with thymidine progressed through $G_2$-phase effectively (Fig. 2B). No obvious differences were observed between V79 and irs1 after HU or thymidine treatment (Fig. 2). These results suggest that although both HU and thymidine effectively arrest cells in S-phase, the stalled replication forks induced by HU are apparently not recovered efficiently in the first 24 h and continue to prevent cell division. The stalled replication forks induced by thymidine, however, does not alter the cell cycle progression of either V79 or irs1 cells.

### Survival Sensitivity of irs1 Cells to HU and Thymidine

We examined the survival sensitivity of V79, irs1, and the *XRCC2* gene transfectant to HU or thymidine. The cells were grown in the medium containing the drugs at different concentrations until the visible colonies were formed. Figure 3 shows that irs1 cells display increased sensitivity to HU compared to V79 cells, whereas transfection of *XRCC2* cDNA can partially complement the hypersensitivity. In contrast, irs1 cells showed only a slightly increased sensitivity to thymidine compared to V79 and the *XRCC2* complemented cells (Fig. 3). These results with thymidine are rather unexpected because it was reported that *xrcc3* mutant showed increased survival sensitivity

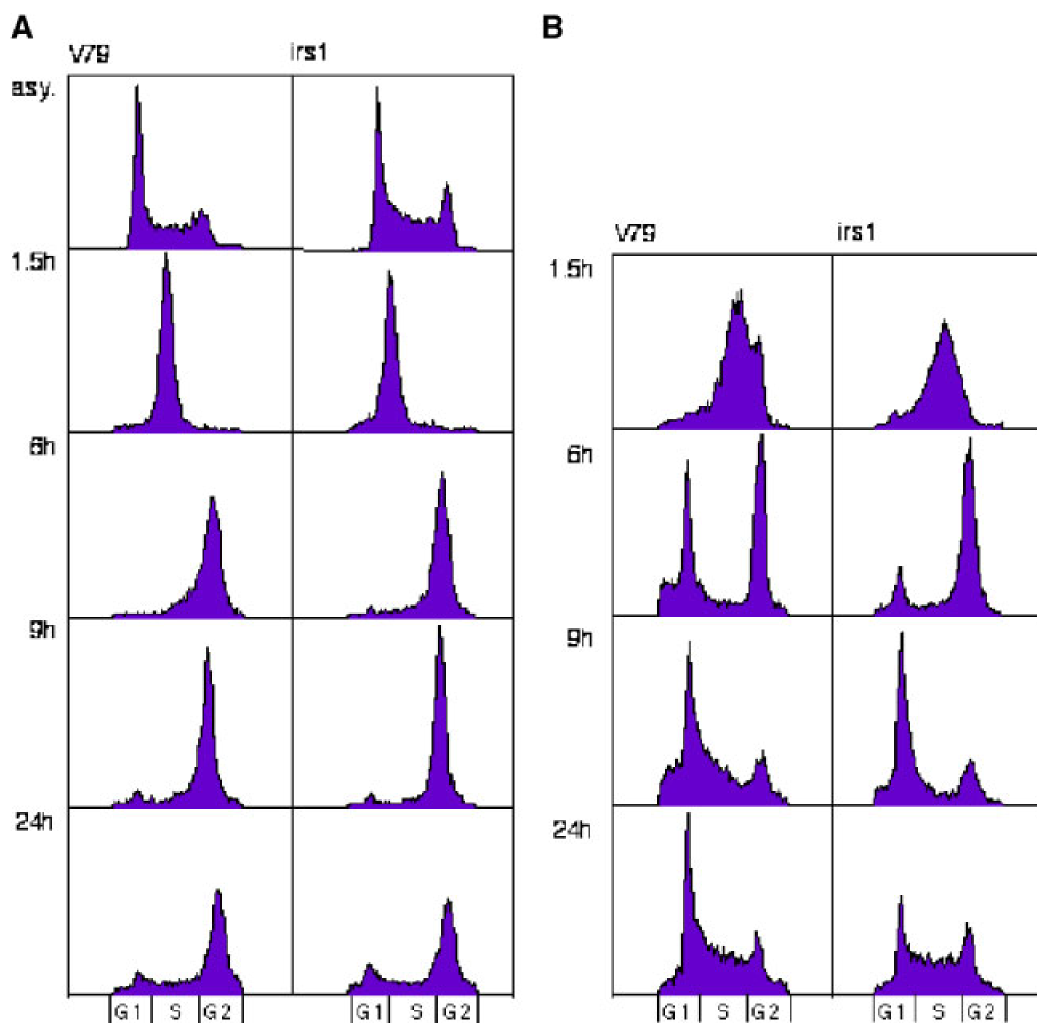


**Fig. 2.** FACS analysis of V79 and irs1 cells following release from treatment with HU (**A**) or thymidine (**B**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

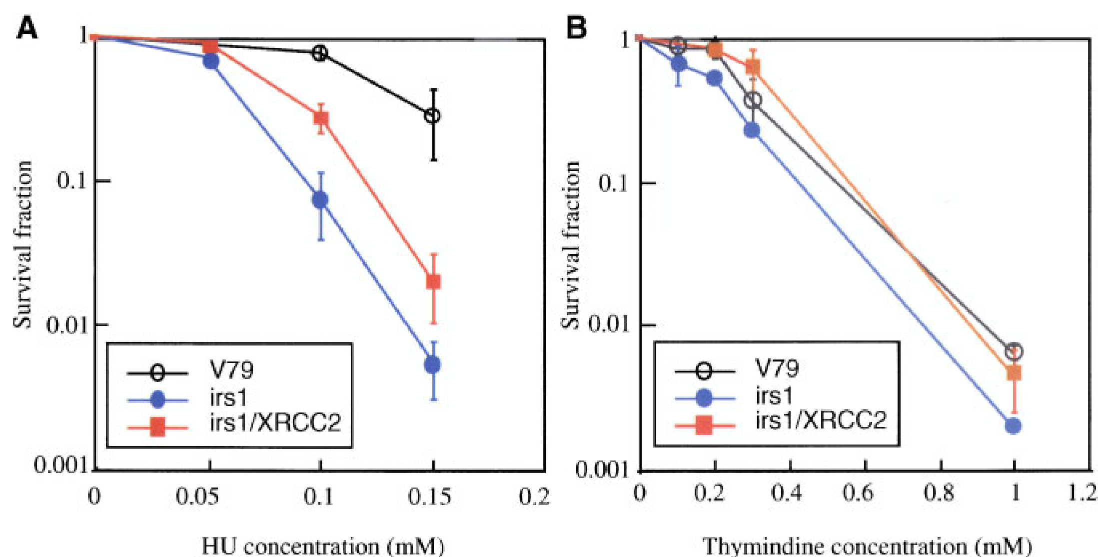


**Fig. 3.** Survival sensitivity of V79 and irs1 cells to HU (**A**) and thymidine (**B**). Data are shown as averages and standard errors from two to three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to thymidine [Lundin et al., 2002]. But our finding that irs1 cells are not sensitive to thymidine is in agreement with the results in Figure 2 that the first cell cycle progression immediately after thymidine treatment was not altered in the mutant.

### XRCC2 is Required for RAD51 Focus Formation Induced by HU

We next examined RAD51 focus formation in irs1 and V79 cells arrested in S-phase after HU treatment. As shown in Figure 4A, HU-induced RAD51 foci are readily detected in V79 nuclei. In untreated asynchronous V79 cells, only <3% of cells contained RAD51 foci, while in HU-treated cells, 86% of cells became RAD51 foci positive (Fig. 4B). Most of these RAD51 focus-positive V79 cells contain >20 foci per nucleus (Fig. 4C). In contrast, irs1 cells lacked this response and showed no induction of RAD51 foci by HU (Fig. 4). The defect in irs1 cells can be complemented by expression of a functional *XRCC2* gene (Fig. 4). Both the percentage of RAD51 focus positive cells and the distribution of the foci per cell are corrected in *XRCC2* complemented irs1 cells (Fig. 4B,C). These results suggest that XRCC2 is required for the formation of RAD51 foci at the stalled replication forks induced by HU.

We also observed that very few cells (2%–3%) displayed RAD51 foci in asynchronous V79 and irs1 cells, although S-phase cells comprised 40%–50% in asynchronous cells. These results are consistent with that reported before [Liu, 2002], and suggest that RAD51 foci are not formed in S-phase cells that are undergoing normal cell cycle progression but rather appear in cells with arrested or slowed replication.

### XRCC2 is not Required for the Formation of RAD51 Foci Induced by Thymidine

Thymidine treatment induces RAD51 foci in V79 cells as shown in Figure 5. We noticed that thymidine (2 mM) induced fewer RAD51 foci per cell in V79 cells compared to HU (2 mM), as most of the thymidine-treated V79 cells contain less than 20 foci per cell (Fig. 5C). Surprisingly, RAD51 foci are also induced in irs1 cells treated with thymidine (Fig. 5A,B). The percentage of RAD51 focus positive cells are 45% and 54% for V79 and irs1 cells, respectively, indicating that there is no significant difference between V79 and irs1 for induction of RAD51 foci by thymidine. We then scored the number of foci per cell and found that there is still no significant difference between V79 and irs1 cells (Fig. 5C). These results indicate that XRCC2 is not required for the assembly of RAD51 foci in S-phase nuclei arrested by thymidine.

Since RAD51 foci are induced by thymidine treatment, we questioned whether the RAD51 foci in HU-treated V79 cells (Fig. 4) resulted from the incubation with thymidine in the double blocking/release procedure to synchronize cells at S-phase (see "Materials and Methods"). Because the RAD51 foci are not

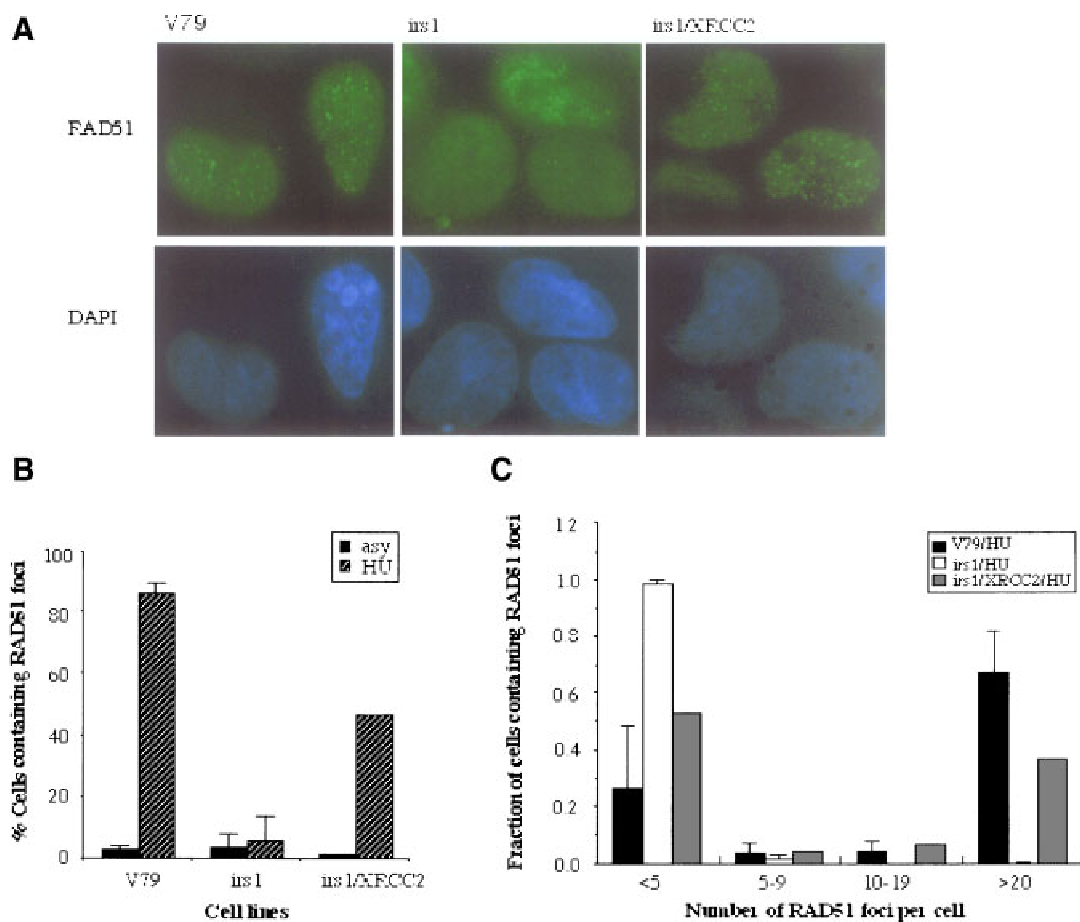


**Fig. 4.** RAD51 focus formation induced by HU. **A**: HU-induced RAD51 foci in V79, irs1, and the *XRCC2* complemented irs1 cells (irs1/XRCC2). **B**: Percentage of cells containing RAD51 foci (>5 foci per cell) in HU-treated cells compared with asynchronous cells. **C**: Distribution of RAD51 foci per cell in HU-treated V79, irs1, and irs1/XRCC2 cells. At least 200 nuclei were scored for each sample. Error bars represent standard errors from the mean of two to three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

formed in thymidine/HU treated irs1 cells (Fig. 4), the foci induced by the first thymidine block seem to have disappeared after 24 h incubation in thymidine-free medium. This indicates that the RAD51 foci seen in HU-treated V79 cells are solely induced by HU, and are not a result of the thymidine treatment.

### Induction of γ-H2AX Foci by HU and Thymidine

The different responses of irs1 cells to HU and thymidine prompted us to examine whether these differences are due to the production of double strand breaks (DSBs) at the stalled replications forks. Several labs have previously reported that HU, but not thymidine, induces DSBs as measured by pulse-field gel electrophoresis (PFGE) [Saintigny et al., 2001; Lundin et al., 2002, 2003; Mohindra et al., 2004]. We examine the formation of γ-H2AX nuclear foci, which are thought to relate to DSBs in chromatin [Rothkamm et al., 2003; Rothkamm and Lobrich, 2003], after HU or thymidine treatment. As shown in Figure 6, the γ-H2AX foci are detected in cells treated with either HU or thymidine. The γ-H2AX foci co-localize with RAD51 foci, which are also induced by HU and thymidine in V79 cells (Fig. 6). Similarly, the γ-H2AX foci are observed in irs1 cells (data not shown). These results suggest that both HU and thymidine result in damages or structural alternations at stalled replication forks that induce H2AX phosphorylation.

### HU-Induced Chromatin-Association of RAD51 is Reduced in irs1 Cells

It was reported that the level of RAD51 bound to chromatin is increased in S-phase cells

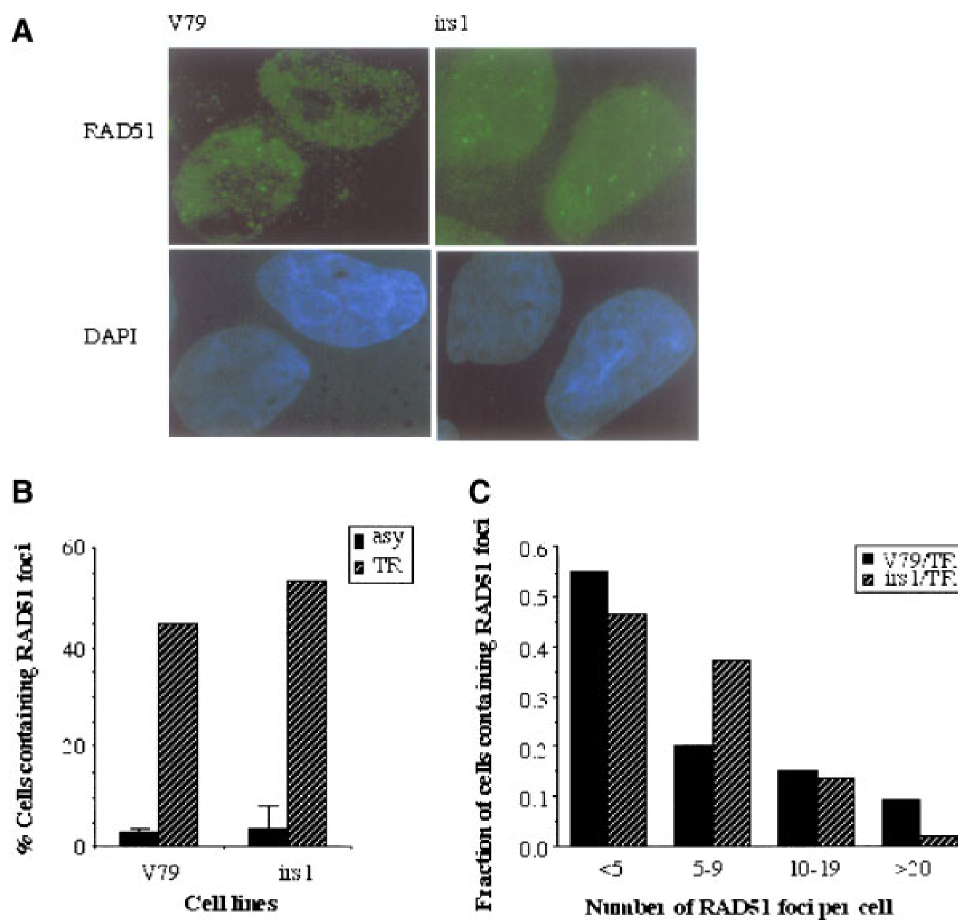


**Fig. 5.** RAD51 foci induced by thymidine in V79 and irs1 cells. **A**: Thymidine (TR) induced RAD51 foci in V79 and irs1 cells. **B**: Percentage of cells containing RAD51 foci in V79 and irs1 compared with that in asynchronous cells. **C**: Distribution of RAD51 foci per cell in thymidine treated cells. At least 200 nuclei were scored for each sample. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

synchronized with thymidine/aphidicolin block/release [Tarsounas et al., 2003]. This finding prompted us to investigate whether HU or thymidine induces the association of RAD51 with chromatin, and whether this process is affected in irs1 cells. To test this, the extracts of V79 and irs1 cells treated with HU or thymidine were fractionated and RAD51 protein level was determined in cytoplasmic, nuclear, and chromatin fractions. In the cytoplasmic and nuclei fractions, RAD51 level showed little difference before and after treatment with HU or thymidine in all the cell lines tested (Fig. 7A,B). The RAD51 level in chromatin fraction, however, is increased in V79 cells after either HU or thymidine treatment (Fig. 7C, lines 2 and 3; and 7D) compared to that in the asynchronous cells (Fig. 7C, line 1). In contrast, the HU-induced binding of RAD51 to chromatin is not observed in irs1 cells (Fig. 7C, line 5), but is restored in the *XRCC2* transfectant (Fig. 7C, line 8). These results suggest that irs1 cells are defective in loading of RAD51 onto stalled replication forks induced by HU. It is noticed that the thymidine-induced RAD51 association with chromatin is not affected in irs1 cells, compared to the *XRCC2* transfectant (Fig. 7C, lines 6 and 9). These results suggest that XRCC2 facilitates loading of RAD51 to HU-induced, but not thymidine-induced, stalled replication forks.

## DISCUSSION

Accumulating evidence suggest that homologous recombination is the primary repair mechanism in DNA synthesis to ensure a faithful replication by eliminating DSBs that are formed as consequences of replication fork collapse [Thompson and Schild, 2002; reviewed

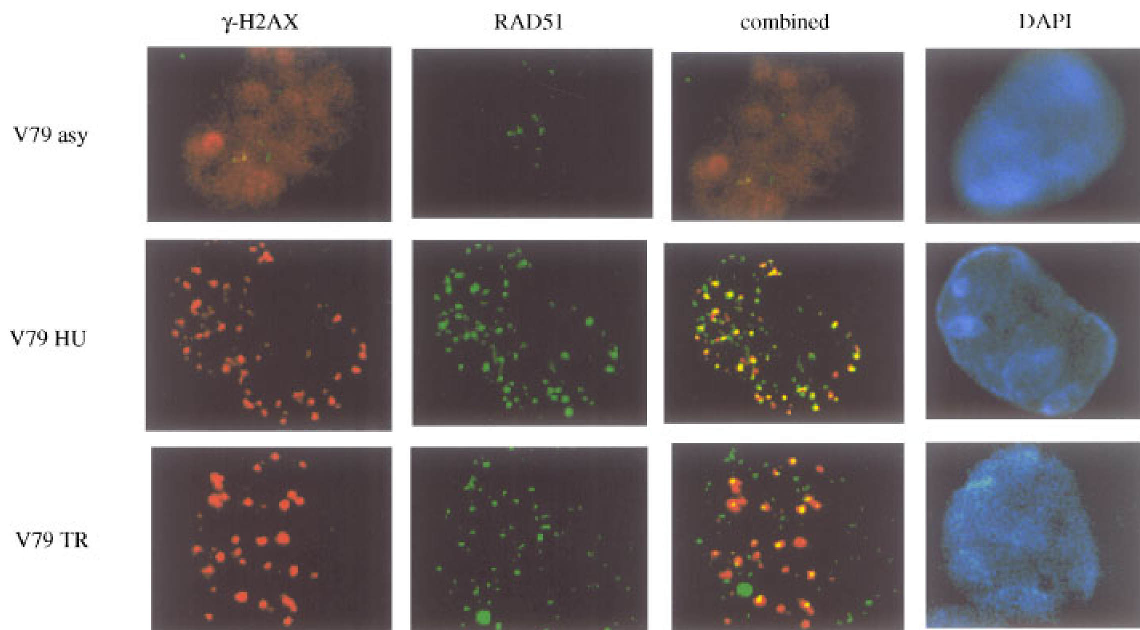


**Fig. 6.** Induction of γ-H2AX foci by HU or thymidine in V79 cells. Combined images show the colocalization of γ-H2AX foci with RAD51 foci. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in Lusetti and Cox, 2002; Helleday, 2003]. In this study, we provide evidence that XRCC2, a protein involved in the repair of DSB, plays a role in cellular recovery from HU-induced replication arrest by promoting loading of RAD51 onto damaged chromatin and assembly of RAD51 foci. The function of XRCC2, however, is not essential for the recovery of cells from replication arrest induced by thymidine. Our results also suggest that there are at least two sub-pathways in homologous recombination, XRCC2-dependent and -independent, for assembly of RAD51 foci following replication arrest in S-phase.

### Cell Cycle Dependent Protein Expression of XRCC2 and RAD51D

The first evidence supporting a role of XRCC2 in DNA replication is that the protein level of XRCC2, as well as its binding partner RAD51D, is dramatically increased in S- and $G_2$/M-phases compared to $G_1$- or $G_0$-phases (Fig. 1), implicating that expression of these proteins is regulated during the cell cycle and that these proteins play a function during and after DNA replication. In addition, the patterns of protein expression of XRCC2 and RAD51D are very similar to those for RAD51 (Fig. 1) [Chen et al., 1997], RAD52 [Chen et al., 1997], and RAD54 [Essers et al., 2002], suggesting that the protein expression of these HRR proteins is regulated coordinately. Interestingly, we did not observe any significant changes in the level of RAD51 following release from a thymidine/aphidicoline block in HeLa nor in hamster V79 cells. The observation for RAD51 is consistent with previous findings that the cell cycle-regulated expression of RAD51 was found in human peripheral blood lymphocytes [Flygare et al., 1996], normal human skin fibroblasts [Chen et al., 1997], and normal mouse skin cells [Yamamoto et al., 1996], but not in HeLa cells [Tarsounas et al., 2003]. These data suggest that some factors, such as p53 that is inactivated in HeLa and V79 cells, may be involved in the regulation of the expression of RAD51 and the RAD51 paralogs. It is of interest to further investigate these factors and to examine whether the cell cycle-dependent expression of HRR proteins is aberrant in cancer cells.

### Role of XRCC2 in Repair of HU-Induced DSBs at Stalled Replication Forks

XRCC2 deficiency results in increased survival sensitivity to HU in mutant irs1 cells, which also failed to form RAD51 foci after HU treatment (Figs. 3 and 4). The HU-hypersensitivity of irs1 and RAD51 focus formation are partially or fully corrected by introduction of a functional *XRCC2* gene, suggesting that *XRCC2* plays a

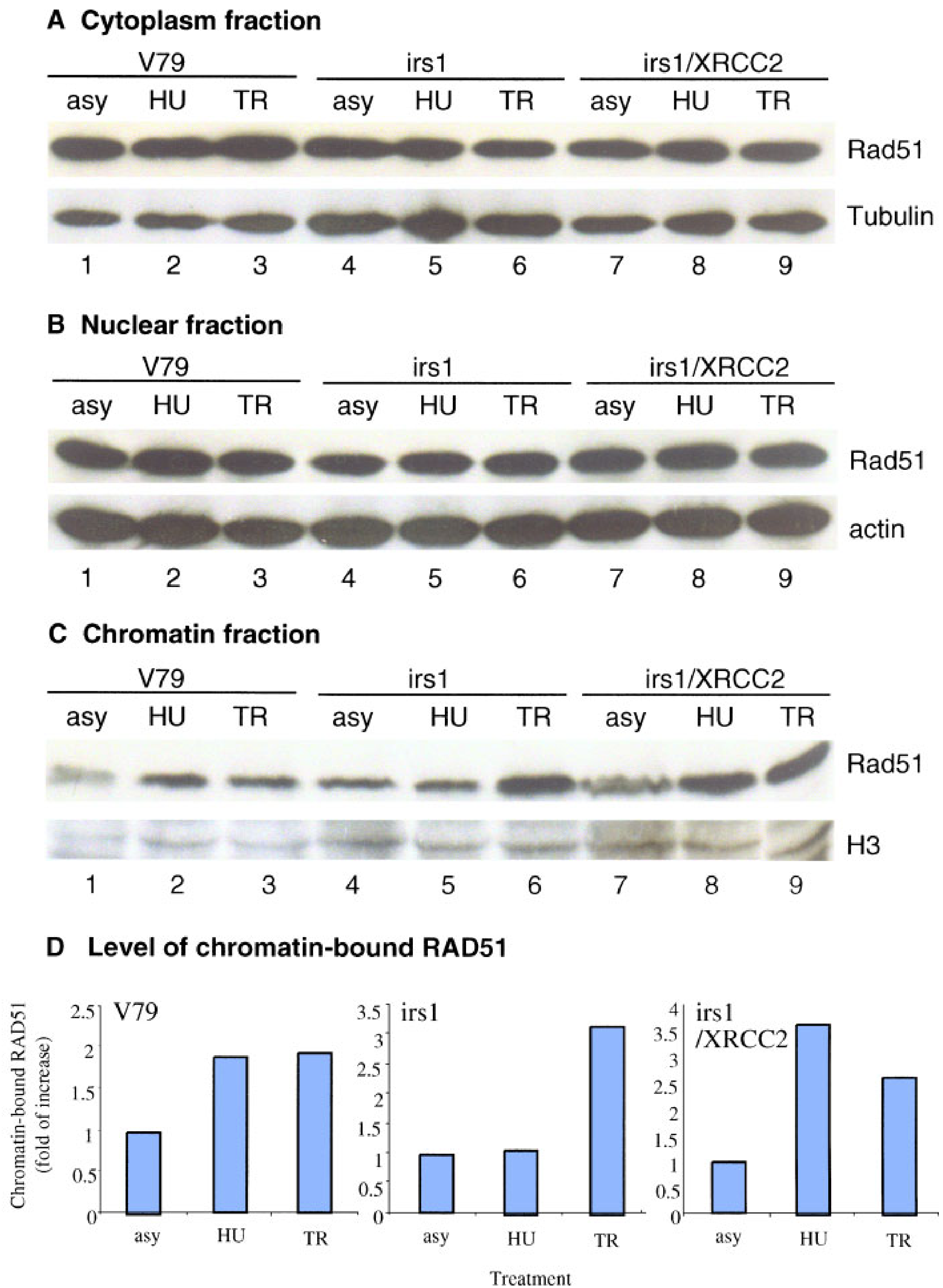


**Fig. 7.** RAD51 level in cytoplasmic (**A**), nuclear (**B**), and chromatin fractions (**C**). Forty microgram cytoplasmic or nuclear proteins were separated on SDS–PAGE gel. The amount of chromatin-associated protein loaded (16–22 μl) was adjusted based on the cytoplasmic protein concentration of the sample, which is related to the number of cells used for the fractionation. RAD51 protein was visualized by immunobloting with RAD51 antibody. Tubulin, actin, and H3 were used as loading controls for cytoplasmic, nuclear, and chromatin fractions, respectively. **D**: Quantitative analysis of the level of chromatin-bound RAD51 in HU- or thymidine-treated cells relative to that in asynchronous cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

role in repair of the arrested replication fork induced by HU. We and others have previously shown that irs1 cells are defective in RAD51 focus formation after exposure to ionizing irradiation and mitomycin C (MMC) [O'Regan et al., 2001; Liu, 2002]. The function of *XRCC2* in RAD51 focus assembly is likely linked to its role as a mediator in the repair of double strand breaks via RAD51-mediated homologous recombination [Johnson et al., 1999]. HU inhibits the synthesis of several nucleotide precursors, thus completely disrupting the incorporation of nucleotides into DNA and arresting replication fork elongation [Bianchi et al., 1986]. The stalled replication forks induced by HU result in DSBs, which can be detected either by γ-H2AX foci or by pulsed-field gel electrophoresis [Saintigny et al., 2001; Lundin et al., 2003]. The DSBs induced by HU stimulate homologous recombination in mammalian cells [Saintigny et al., 2001; Lundin et al., 2003]. During homologous recombination, RAD51 is loaded onto the DSB sites and the level of RAD51 associated with chromatin is increased after HU treatment (Fig. 7). XRCC2 promotes loading of RAD51 onto HU-damaged chromatin, as demonstrated by the evidence that the defect of *XRCC2* in irs1 cells resulted in neither RAD51 focus formation nor increase of chromatin-associated RAD51 (Figs. 4 and 7). Recently, it was shown that RAD51 in the nucleoplasm of living cells is compartmentalized into at least three distinct fractions [Yu et al., 2003]. The two relatively immobile fractions contain protein complexes either through RAD51 self-interaction or interaction with BRCA2, and the third fraction comprises mobile RAD51 [Yu et al., 2003]. Strikingly, HU reduces the immobile fraction of RAD51, particularly in the BRCA2-bound fraction, and the mobilized RAD51 may be targeted to the stalled replication forks [Yu et al., 2003]. These results suggest a mechanism for the dynamic control of RAD51 protein relocalization from nucleoplasm to chromatin, which can be triggered by arrested DNA replication. We speculate from our results that XRCC2 is also involved in such a mechanism to help targeting RAD51 onto damaged chromatin.

### XRCC2-dependent and -independent Pathways for Loading of RAD51 Onto Stalled Replication Forks

It is rather surprising that RAD51 foci are formed efficiently in irs1 cells undergoing replication arrest induced by thymidine (Fig. 5). Consistently, irs1 cells treated with thymidine showed only slightly increased survival sensitivity, compared to the wild-type (Fig. 3), and normal cell cycle progression after release from the blocking by the drug (Fig. 2). Our survival results of irs1 treated with thymidine differ from those reported for *xrcc3* mutant irs1SF, which showed hypersensitivity to thymidine [Lundin et al., 2002]. We also tested the survival sensitivity of irs1SF to thymidine and obtained similar results as with irs1 cells (data not shown). We noticed that in our experiments, thymidine at higher concentrations (>1 mM) severely inhibited cell growth for both the wild type and the irs1 mutant, and the incubation time for forming visible colonies (>50 cells) was almost doubled at 1 mM thymidine, compared to the control (7 and 12 days for wild-type and the mutant, respectively). Therefore, we did not use the thymidine concentrations higher than 1 mM as Lundin et al. [2002] used. In each experiment, we ensured that the thymidine solution (in PBS) was made no longer than 2 weeks.

Thymidine slows down replication chain elongation by depleting cells of deoxycytidine triphosphate (dCTP), causing a less stringent arrest of replication compared to HU [Bjursell and Reichard, 1973]. There is evidence that thymidine stimulates homologous exchanges [Lundin et al., 2002], suggesting that the recombigenic DNA structures are generated by thymidine. We observed that both HU and thymidine induce γ-H2AX foci (Fig. 6), although DSBs are not detected by PFGE in cells treated with thymidine [Saintigny et al., 2001; Lundin et al., 2002, 2003; Mohindra et al., 2004]. It may suggest that HU or thymidine induces different structures of stalled replication forks, and those induced by thymidine may not cause strand discontinuation, such as DSBs that are detectable by PFGE. The lesions induced by thymid appear to be rapidly recovered, as evidenced by the cell cycle progression data (Fig. 2). We also observed that RAD51 focus formation was not affected in S-phase irs1 cells synchronized with thymidine/aphidicolin double blocking and release (data not shown). These results suggest that sub-pathways of HRR are involved in repair of stalled replication forks. Recently, it was reported that BRCA2 defective Capan-1 cells synchronized at S-phase by thymidine/aphidicolin double blocking display RAD51 foci

as efficiently as the wild type cells [Tarsounas et al., 2003], although these cells are not capable of forming RAD51 foci after exposure to ionizing radiation [Chen et al., 1999]. These results suggest that there are at least two sub-pathways for loading of RAD51 at the stalled replication forks, and XRCC2 and BRCA2 may act in the same pathway.

It has been suggested that HRR can be triggered by either classical or non-classical DSBs or other abnormal structures at the stalled replication forks [Helleday, 2003]. The types of initiating DNA substrates for HRR may determine which of the pathways is to be used for RAD51 focus assembly. A classical DSB with two free ends occurring at the stalled replication fork may induce two-end recombination repair, which may require the same sets of HRR proteins as those involved in repair of DSBs induced by ionizing radiation. A single strand break at a collapsed replication fork can be converted to a DSB with one-end and trigger the one-end recombination [Helleday, 2003]. Other structures, such as chicken foot structure at the stalled forks, may also present in mammalian cells when an un-repaired base damage blocks replication fork progression [Braybrooke et al., 2003; Helleday, 2003]. The classical DSB can also be repaired by non-homologous end joining pathway (NHEJ). It is interesting to note that cells defective in NHEJ are more sensitive to HU than the wild type, suggesting that NHEJ is involved in the repair of HU-induced damage [Saintigny et al., 2001; Lundin et al., 2002]. In contrast, NHEJ deficient cells showed no increased sensitivity to thymidine [Lundin et al., 2002]. These results suggest that thymidine-induced lesions may be structurally different from the classical DSBs as those induced by HU and ionizing radiation. We speculate that XRCC2 participates in the HRR pathway for repair of the classical DSBs, but is not involved in that for non-classical DSB.

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