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Differential roles of XRCC2 in S-phase RAD51 focus formation induced by DNA replication inhibitors

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ABSTRACT

RAD51 proteins accumulate in discrete nuclear foci in response to DNA damage. Previous studies demonstrated that human RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) are essential for the assembly of RAD51 foci induced by ionizing radiation and cross-linking agents. Here we report that XRCC2 also plays important roles in RAD51 focus formation induced by replication arrest during S-phase of cell cycle. In wild-type hamster V79 cells treated with hydroxyurea (HU), RAD51 protein form punctate nuclear foci, accompanied by increased RAD51 protein level in both cytoplasmic and nuclear fractions, and increased association of RAD51 with chromatin. In contrast, *xrcc2* hamster mutant irs1 cells are deficient in the formation of RAD51 foci after HU treatment, suggesting that the function of XRCC2 is required for the assembly of RAD51 at HU-induced stalled replication forks. Interestingly, we found that irs1 cells are able to form intact RAD51 foci in S-phase cells treated with thymidine (TR) or aphidicolin, although irs1 cells are hypersensitive to both HU and TR. Our findings suggest that there may be two distinct pathways (XRCC2-dependent or XRCC2-independent) involved in loading of RAD51 onto stalled replication forks, probably depending upon the structure of DNA lesions.

Introduction

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 [34]. The assembly of RAD51-ssDNA nucleoprotein complex is a rate-limiting step, as RAD51 has 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 nucleonization (reviewed in [34;37-39]. 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 [16;30]. 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 DNA damaging agents [4;15;22;29;35]. 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 HPRT loci as a reporter gene, it was demonstrated that XRCC3 is essential for the repair

of camptothecin (CPT)-induced DSBs following replication fork arrest [2]. In addition, it was found that irs1SF cells show an increased sensitivity to DNA replication elongation inhibitors HU and thymidine, both of which induce replication arrest-associated homologous recombination *in vivo* [24]. 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 [18]. The defects in slowing replication fork of *xrcc3* mutants can be corrected by introduction of purified human RAD51C-XRCC3 complex or RAD51 [18]. These data suggest that XRCC3 and RAD51 cooperatively modulate the progression of replication forks on damaged chromosomes [18]. Recently, XRCC3 was found to directly interact and co-immunoprecipitate in human cell extracts with RPA [43], which plays an essential role in DNA synthesis (reviewed in [41]). Moreover, RAD51D physically and functionally interacts with BLM, the causal gene for Bloom's Syndrome [6]. BLM interacts and colocalizes with RPA and RAD51 [8;42], and plays a role in recovery from S-phase replication arrest [12].

In this study, we investigated the role of XRCC2 in the S-phase RAD51 focus formation following DNA replication fork arrest induced by replication inhibitors, hydroxyurea (HU), aphidicolin and thymidine (TR). Our data showed that *xrcc2* mutant irs1 cells are hypersensitive to HU and TR, compared to the wild type V79 cells. RAD51 foci are induced in S-phase V79 cells after treatment with HU, TR or aphidicolin. The function of XRCC2 is essential for HU-induced RAD51 focus formation as RAD51 foci

are missing in *xrcc2* deficient irs1 cells. However, RAD51 foci are formed normally in irs1 cells after treatment with aphidicolin or thymidine, indicating that RAD51 foci induced by these agents are independent of XRCC2. Our data suggest that there are at least two pathways for assembly of RAD51 foci following replication arrest in S-phase, probably depending upon the types of lesions or HRR substrates at the stalled replication forks.

Materials and Methods

Cell lines

Hamster cell lines V79 (the wild type), irs1 (*xrcc2* mutant) and irs1/XRCC2 (*XRCC2* complemented irs1 cell line) were cultured at 37 ºC in an atmosphere of 5% CO_2 in α-MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics as described [22]. 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 [23]. 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, and 2 mM glutamine and penicillin/streptomycin.

Cell synchronization

For the normal human cells (MJ90), we used serum starvation and aphidicolin blocking/release method as described previously [13]. To obtain G0 phase cells, cells were grown in medium containing 0.2% serum for 72 h. G1 cells were obtained by stimulating

G0 phase of cells with medium containing 15% serum for 12 h. To prepare S and G2/M cells, G0 cells were stimulated with 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), medium containing 15% serum was then added for 2 or 6 h to collect S- and G2/M phase cells, respectively. To synchronize hamster cells in S-phase, cells were plated 24 h before incubation with 2 mM thymidine for 16 h. Cells were then released in regular medium supplemented with 10% serum for 8 h followed by incubation with 2 mM hydroxyurea, 1 mg/ml aphidicolin or 2 mM thymidine for another 16 h. Cells were washed with PBS extensively and released in regular medium for 1 h before harvest.

Analysis of cell cycle distribution by FACScan.

To monitor quality of the cell synchrony, cell cycle profiles were analyzed by flow cytometry. Cells were trypsinized and fixed in 70% ethanol in PBS at 4C overnight, followed by treatment with RNase A and staining with propidium iodide (PI). 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 are then fixed with ethanol and stained with FITC-conjugated anti-BrdU antibody following manufacturer's instruction (Becton-Dickinson, San Jose, CA). Data were acquired on a FACScan and analyzed with Cell Quest™ 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

The 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% precast SDS-PAGE gels and immunoblotted with the designated antibodies. Rabbit anti-human XRCC2 and rabbit anti-human RAD51 antibodies were described previously [23]. 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 secondary 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

The similar procedures were followed as described {Liu, 2002 #5362} with some modification. Cells grown on monolayer were trypsinized and resuspended at $1x10^5$ cell/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 acetone-methanol mixture (1:1 v/v) on ice for 5 min, blocked with 1X 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 for 10 min for three times with gentle agitation, cells were incubated with Alexa-fluor 546 conjugated anti-Rabbit IgG (Molecular Probes, Eugene, OR) for 1 hour followed by washing with PBS for three times. 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 5 foci were recorded as positive.

Cell fractionation

Subcellular fractionation was carried out using the methods described by Tarsounas et al. {Tarsounas, 2003 #7109} with some modifications. Briefly, exponentially growing hamster V79 and irs1 cells were harvested by trypsinization. A total of 3 x 10^6 cells were pelleted and resuspended in hypotonic Buffer A (10mM HEPES-KOH pH 7.1, 50mM NaCl, 0.3M Sucrose, 0.1mM EDTA, 0.5% Triton X-100, 1mM DTT and protease inhibitors) and incubated on ice for 15 min. Nuclei were pelleted by centrifugation at 1500 x 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, protease inhibitors) and then resuspended in Buffer C (10 mM HEPES NaOH pH 7.1, 0.5 M NaCl, 0.1 mM EDTA, 0.5% NP40, 1 mM DTT and protease inhibitors) and incubated on ice for 15 min with occasional vertex. The extracts were centrifuged at 16,000 x g at 4°C for 10 min. Supernatant (nuclear fraction) was collected in fresh tubes. After washing with Buffer C once, the pellet (chromatin fraction) was then resuspended in SDS-PAGE loading buffer, sonicated to shear chromatin DNA and boiled for 5 min. Proteins were separated by SDS-PAGE and analyzed by western blotting. To quantify the proteins, the western blot bands of RAD51 on the X-ray film were analyzed using KODAK 1D Imaging Systems. The levels of RAD51 were normalized with actin, and the ratios of RAD51 induction relative to asynchronous cells were calculated.

Results

Protein levels of XRCC2 and RAD51D are increased in S- and G2/M-phases

To elucidate the function of RAD51 paralogs in HRR and DNA replication, we first examined the cell cycle regulation of the protein expression for XRCC2 and RAD51D in human normal fibroblast MJ90 cells. As shown in Figure 1, the protein expression levels of XRCC2 and RAD51D are very low and almost undetectable in cells synchronized at G0- or G1-phases, but are dramatically increased in S- through G2/M-phases. The expression patterns of XRCC2 and RAD51D are consistent with that of RAD51, which also peaks at S- and G2/M-phases (Figure 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 (Figure 1), as in the case of asynchronous cells [4;22]. These results suggest that

expression of XRCC2 and RAD51D is regulated during the cell cycle and are probably coordinated with that of RAD51. The increased expression in S- and G2-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, as the levels of these proteins showed no change throughout the cell cycle (data not shown). A similar finding was reported for RAD51 in HeLa cells, in which RAD51 levels remained unchanged at different phases of cell cycle [36]. In human lymphocytes and normal fibroblasts, however, the RAD51 expression increased at S- and G2-phases [9;14], in agreement with our data (Figure 1). These results suggest that the expression of RAD51 and RAD51 paralogs is regulated by cell cycle check-points, which are probably inactive in HeLa cells.

Recovery of cell cycle progression from replication block

We treated *xrcc2* mutant irs1 and wild type V79 cells with HU, aphidicolin or TR following incubation with TR to enrich cells at G1/S boarder. HU inhibits the synthesis of several nucleotide precursors, thus completely disrupting the incorporation of nucleotides into DNA and arresting replication fork elongation [3]. Thymidine and Aphidicolin slow down replication chain elongation by depleting cells of dCTP and inhibiting DNA polymerases, respectively [5;20]. Thymidine and aphidicolin induce a less stringent arrest of replication compared to HU. After treatment with the replication inhibitors, cells were grown in drug-free medium for 1.5 h to allow cells progress into S-phase. We achieved >90% S-phase cells using this approach as determined by FACS analysis of cell cycle profiles (Figure 2). In asynchronous cells, when cells were grown

at low density (<60% confluence), we consistently obtained a high level (50-60%) of S-phase cells, compared to 15-20% of S-phase cells in confluent cultures (data not shown). To compare the recovery of V79 and irs1 cells from replication arrest induced by HU and TR, we examined the cell cycle profiles in cells that were released to drug-free medium for 6, 9 and 24 h. The results show that either V79 or irs1 cells treated with HU are accumulated in G2 phase even after 24 h (Figure 2A). In contrast, the cells treated with TR progress through G2 phase normally (Figure 2B). No obvious differences were observed between V79 and irs1 after HU or TR treatment (Figure 2). These results suggest that although both HU and TR inhibit replication chain elongation, the damages induced by HU at the stalled replication forks are apparently not repaired efficiently that prevents cell division. The treatment with TR, however, does not alter the cell cycle progression V79 nor in irs1 cells.

Hypersensitivity of irs1 cells to HU and TR

Since the cell cycle progression in irs1 cells showed little difference compared to the wild type V79 cells after HU or TR treatment, we wondered whether the stalled replication forks induced by these inhibitors are repaired efficiently in irs1 cells. Then we examined the survival sensitivity of irs1 cells to HU or TR. Figure 3 shows the survival curves of V79 and irs1 cells grown in the medium containing HU or TR at various concentrations. Irs1 cells were more sensitive to either HU (Figure 3A) or TR (Figure 3B) compared to V79 cells. These results indicate that the DNA damages at the replication forks induced by HU or TR are not repaired efficiently in irs1 cells, and the damage caused by TR seems not to alter the cell cycle progression in irs1 cells.

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, RAD51 foci are induced in V79 nuclei after HU treatment. In untreated asynchronous V79 cells, only <3% of cells contained RAD51 foci, while in HU treated cells, 86% of cells became RAD51 foci positive (Figure 4B). Most of these RAD51 focus-positive V79 cells contain >20 foci per nucleus (Figure 4C). In contrast, irs1 cells lacked this response and showed no induction of RAD51 foci by HU (Figure 4). The defect in irs1 cells can be complemented by expression of a functional *XRCC2* gene (Figure 4). Both the percentage of RAD51 focus positive cells and the distribution of the foci per cell are corrected in XRCC2 complemented irs1 cells to near wild-type level (Figure 4B and 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 more than 50% of cells are in S-phase. These results are consistent with that reported before [22], 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.

HU-mediated up-regulation of RAD51 expression and chromatin-association are reduced in S-phase irs1 cells.

It was recently shown that HU treatment induces RAD51 expression in normal human fibroblasts [33]. These findings prompted us to investigate whether the level of RAD51

expression is altered in HU-treated V79 or irs1 cells, and whether XRCC2 is involved in regulating the induction of RAD51 by HU. To test this, V79 and irs1 cells synchronized in S-phase with HU were fractionated and RAD51 protein level was determined in cytoplasmic, nuclear and chromatin fractions. In asynchronous cells, the levels of RAD51 in cytoplasmic and nuclei fractions showed little difference between V79 and irs1 cells (Figure 5A-D). The RAD51 levels in both cytoplasmic and nuclear fractions are increased in V79 cells after HU treatment, compared to the asynchronous cells (Figure 5A-D). In irs1 cells, the RAD51 level is increased in cytoplasmic fraction (Figure 5A and B) that is comparable to that of V79 (Figure A and B), but the induction of RAD51 expression by HU in the nuclear fraction is significantly reduced compared to V79 cells (Figure 5C and D). Our results also show that HU induces an increased association of RAD51 with chromatin in both V79 and irs1 cells, but the level of the chromatin-associated RAD51 in irs1 is much lower compared to V79 cells (Figure 5E and F). These results suggest a defect in irs1 cells in redistribution of RAD51 to nuclei and targeting RAD51 to chromatin following treatment with HU.

XRCC2 is not required for the formation of RAD51 foci induced by thymidine

Since irs1 cells show increased cellular sensitivity to TR (Figure 3), we investigated whether XRCC2 is essential for RAD51 assembly at the stalled replication forks induced by TR. As shown in Figure 6A, TR treatment induces RAD51 foci in V79 cells. Surprisingly, RAD51 foci are also readily detected in irs1 cells (Figure 6A). 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 TR. We then scored the number of foci per cell and found that there is still no significant difference between V79 and irs1 cells (Figure 6C). We noticed that TR induces fewer RAD51 foci per cell in V79 cells compared to HU, as most of the TR-treated V79 cells contain less than 20 foci per cell (Figure 6C). These results indicate that XRCC2 is not required for the assembly of RAD51 foci in S-phase nuclei arrested by TR.

Since RAD51 foci are induced by TR treatment, we questioned whether the RAD51 foci in HU treated V79 cell (Figure 4) are also resulted from the incubation with TR in the double blocking/release procedure to synchronize cells at S phase (see Materials and Methods). Because the RAD51 foci are not formed in TR/HU treated irs1 cells (Figure 4), the foci induced by TR in irs1 cells (Figure 5) seem to be disappeared after 8 h release and 16 h incubation in TR-free medium. This indicates that the RAD51 foci seen in HU-treated V79 cells are solely induced by HU, but are not resulted from the TR incubation.

XRCC2 is not required for the formation of RAD51 foci induced by aphidicolin

We next tested whether XRCC2 is required in RAD51 focus formation after treatment with aphidicolin. As we mentioned above, thymidine-induced RAD51 foci disappear after cells are released from thymidine for 24 h, so the RAD51 foci observed should be solely induced by aphidicolin. As shown in Figure 7A, aphidicolin clearly induces RAD51 foci in both V79 and irs1 cells. Specifically, 65% of V79 cells and 59 % of irs1 cells show RAD51 foci (Figure 7B) after aphidicolin treatment. Compared to HU-treated cells, cells treated with aphidicolin contain much less foci, most of which are less than 20

foci per cell (Figure 7C). Like in the case of thymidine, neither the total number of cells containing RAD51 foci nor the distribution of the foci per cell shows significant differences between V79 and irs1 cells (Figure 7). These results indicate that XRCC2 is not required for RAD51 focus formation induced by treatment with aphidicolin.

Discussion

Accumulating evidence suggests that homologous recombinational repair is the primary mechanism in DNA synthesis to ensure a faithful replication by eliminating DSBs that are formed as consequences of replication forks collapse (reviewed in [1, 11, 17, 19]. In this paper, we report that XRCC2, a protein involved repair of DSB in HRR, plays an important role in DNA replication. We found that XRCC2 protein level is particularly higher in S and G2/M phases than in G1 or G0 phases, implicating a function of this protein during and after DNA replication. Interestingly, the level of RAD51D, which directly binds to XRCC2 [7;32], is also increased in S and G2/M phases, suggesting that the expression of other RAD51 paralogs may also be regulated during cell cycle. In addition, the expression of XRCC2 and RAD51D seems to be regulated by the same cell cycle checkpoints as those for RAD51, since the patterns of protein expression in cell cycle for these proteins are very similar. Moreover, *Xrcc2* mutant irs1 are hypersensitivity to HU and TR, the agents that block replication chain elongation and cause damages at the stalled replication forks. This result is consistent with the finding that *xrcc3* hamster mutant irs1SF cells exhibit increased sensitivity to HU and TR [24]. XRCC2 and XRCC3 are both members of RAD51 paralog family, but are involved in different RAD51

paralog complexes [23;26;27;40]. It is plausible to speculate that both of the RAD51 paralog complexes are involved in replication arrest-associated HRR.

We and others have previously shown that irs1 cells are defective in RAD51 focus formation after exposure to ionizing irradiation and mitomycin C (MMC) [22;29]. The function of XRCC2 in RAD51 focus assembly is likely linked to its mediator role in repair of double strand breaks (DSB) via RAD51-mediated homologous recombination [21]. In this study, we found that irs1 cells failed to form RAD51 foci after HU treatment but expression of XRCC2 in the mutant could rescue the formation of RAD51 foci. These results suggest that XRCC2 is required for the RAD51 assembly at the arrested replication fork induced by HU. Previous study has shown that HU induces DSBs and stimulates homologous recombination in mammalian cells [25;31]. Thus, XRCC2 is suggested to be involved in the RAD51-mediated homologous recombination for repair of HU-induced DSBs.

We observed that treatment with HU increased the protein level of RAD51 in both irs1 and V79 cells, which results are in consistence with that in human fibroblasts [33]. It is not known whether the up-regulation of RAD51 expression is caused by the enrichment of cells in S-phase, or by HU-induced replication arrest. The increased RAD51 level associated chromatin in HU treated cells may suggest the targeting of RAD51 at the stalled replication forks. Remarkably, the level of RAD51 in nuclear and chromatin fractions of irs1 is significantly reduced and compared to the wild type. These results suggest a defect in irs1 in RAD51 re-location and targeting to damage-containing chromatin. Recently, it was shown that Rad51 in the nucleoplasm of living cells is compartmentalized into at least three distinct fractions [44]. The two relatively immobile

fractions contain protein complexes either through RAD51 self-interaction or interaction with BRCA2, and the third fraction comprises mobile RAD51 [44]. 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 [44]. 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.

It is rather surprise that RAD51 foci are formed normally in irs1 cells undergoing replication arrest induced by thymidine or aphidicolin. These results suggest that XRCC2 is not essential for the RAD51 focus formation induced by these agents and that RAD51 can form foci in the absence of XRCC2. Recently, it is 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 [36], although these cells are not capable of forming RAD51 foci after exposure to ionizing radiation [10]. These results suggest that RAD51 focus formation can be either BRCA2-dependent or independent [36]. Since neither XRCC2 nor BRCA2 seem to be involved in the formation of RAD51 foci induced by thymidine/aphidicolin treatment, these proteins may act in the same pathway for *de novo* assembly of RAD51. Taken together, these results may suggest that there are at least two pathways for loading of RAD51 at the stalled replication forks.

Different from HU, TR does not generate detectable DSBs in cells even after incubation with TR for an extended period of time [24;25;28]. However, TR treatment

induces homologous exchanges in the absence of detectable DSBs [24]. It may be argued that RAD51 foci can be formed in the absence of DSB in S phase and that XRCC2 is not essential for the RAD51 focus assembly in this case. However, there is evidence that aphidicolin induces DSBs in mammalian cells [31]. Aphidicolin treatment also stimulates homologous recombination, and aphidicolin and HU in tandem could stimulate recombination as efficiently as aphidicolin or HU alone [31]. These results suggest that aphidicolin and HU act on a common pathway to inhibit replication elongation. However, it is not certain whether the DSBs induced by aphidicolin are processed in the same way as those induced by HU or ionizing radiation, since different sets of proteins seem to be required for repairing those damages.

Interestingly, NHEJ is involved in the repair of HU-induced damage [24;31], suggesting that HU-induced DSBs possess similar features to ionizing radiation-induced DSBs, which are substrates for both NHEJ and HRR. However, NHEJ is apparently not involved in repair of TR-induced damage, since mutant defective in NHEJ showed no increased sensitivity to TR [24]. TR may induce some abnormal structures or lesions, which may be different from the classical DSBs as those induced by HU and ionizing radiation. These lesions at stalled replication forks can serve as the substrates for HRR and recruit RAD51 by a mechanism that is not understood. Our results suggest that RAD51 focus formation induced by TR, which is independent of XRCC2, are operated by a different mechanism or pathway from that for HU- or ionizing radiation-induced foci.

It has been suggested that HRR can be triggered either by classical or non-classical DSBs or other abnormal structures at the stalled replication forks [17]. The types

of initiating DNA substrates for HRR may determine which of the pathways to be used for RAD51 focus assembly. A classical DSB with two free ends occurred 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. The classical DSB can also be repaired by NHEJ. The fact that HRR can be induced and that RAD51 can form foci in the absence of DSBs intermediates suggest that non-classical DSBs can also become the substrates for HRR. A single strand break at the collapsed replication forks can be converted to a DSB with one-end and trigger the one-end recombination [17]. 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 [6;17]. In this case, HRR can be triggered without a DSB. It is speculated that XRCC2 participates in the HRR pathway for the classical DSBs, but is not involved in the HRR pathways for non-classical DSB.

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Figure Legends

Figure 1. Increased expression of XRCC2, RAD51D and RAD51 in S- and G2/M phases of cell cycle. The normal human diploid somatic fibroblasts were synchronized as described in Material and Methods, and irradiated or unirradiated with 10 Gy X-ray. A total of 25 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.

Figure 2. RAD51 focus formation induced by HU in S-phase cells. **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 2-3 independent experiments.

Figure 3. Hydroxyurea-mediated up-regulation of RAD51 expression in cytoplasmic (**A and B**), nuclear (**C and D**) and chromatin fractions (**E and F**). A total of 3.8×10^6 cells were used for sub-cellular fractionation and equal amount of cell extracts were loaded onto SDS-PAGE gel. RAD51 protein was visualized by immunobloting and actin was

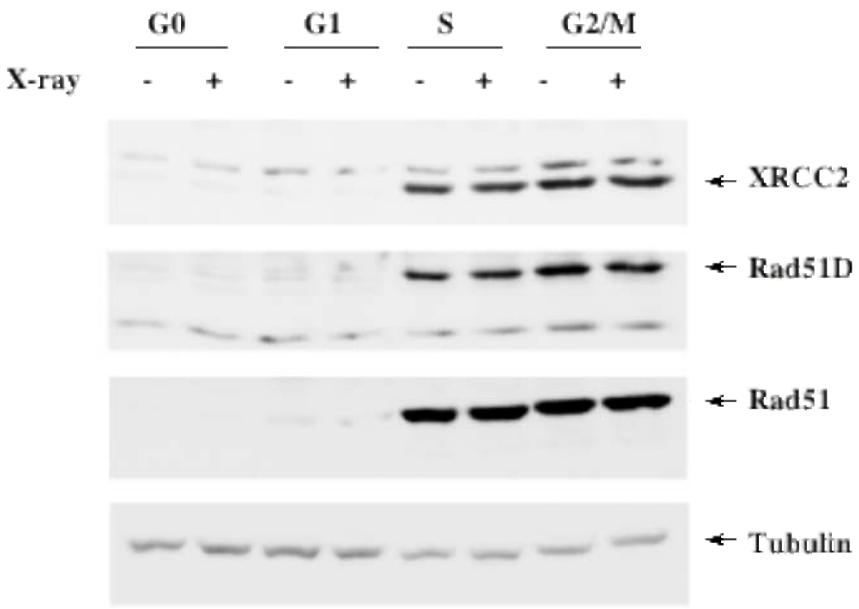
used as a loading control (**A, C and E**). The ratios of RAD51 protein levels in HU-treated cells relative to respective asynchronous control cells are shown as folds of RAD51 induction (**B, D and F**).

Figure 4. RAD51 foci induced by thymidine in S-phase 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 TR treated cells. At least 200 nuclei were scored for each sample. Data are from one (TR treated) or two (asynchronous) independent experiments.

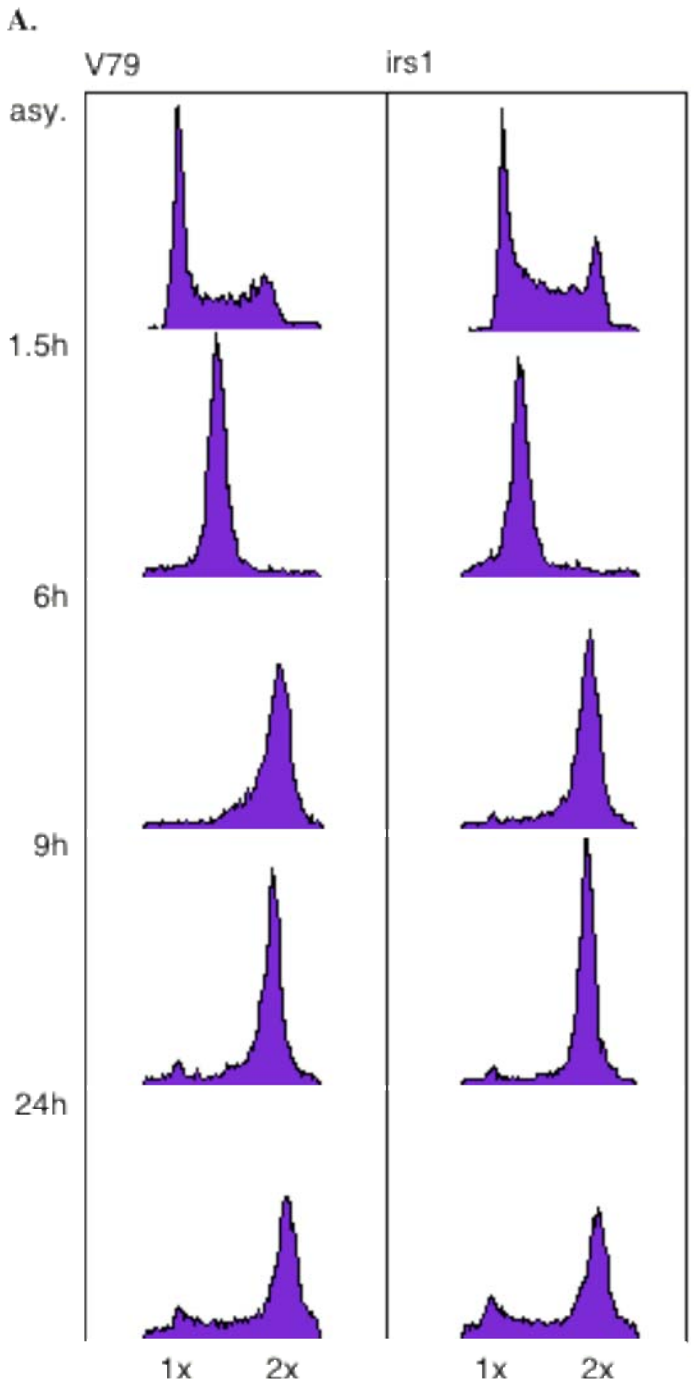
Figure 5. RAD51 foci induced by aphidicolin in S-phase V79 and irs1 cells. **A.** aphidicolin induced RAD51 foci in V79, and irs1 cells. **B.** Percentage of cells containing RAD51 foci in V79 and irs1 compared with that in asynchronous cells. **C.** Distribution of RAD51 foci per cell in aphidicolin treated cells. At least 200 nuclei were scored for each sample. Error bars represent standard errors from the mean of two independent experiments.

Figure 6. Survival sensitivity of V79 and irs1 cells to HU (**A**) and TR (**B**). Data are from 2-3 (for HU) or 1-2 (for TR) independent experiments.

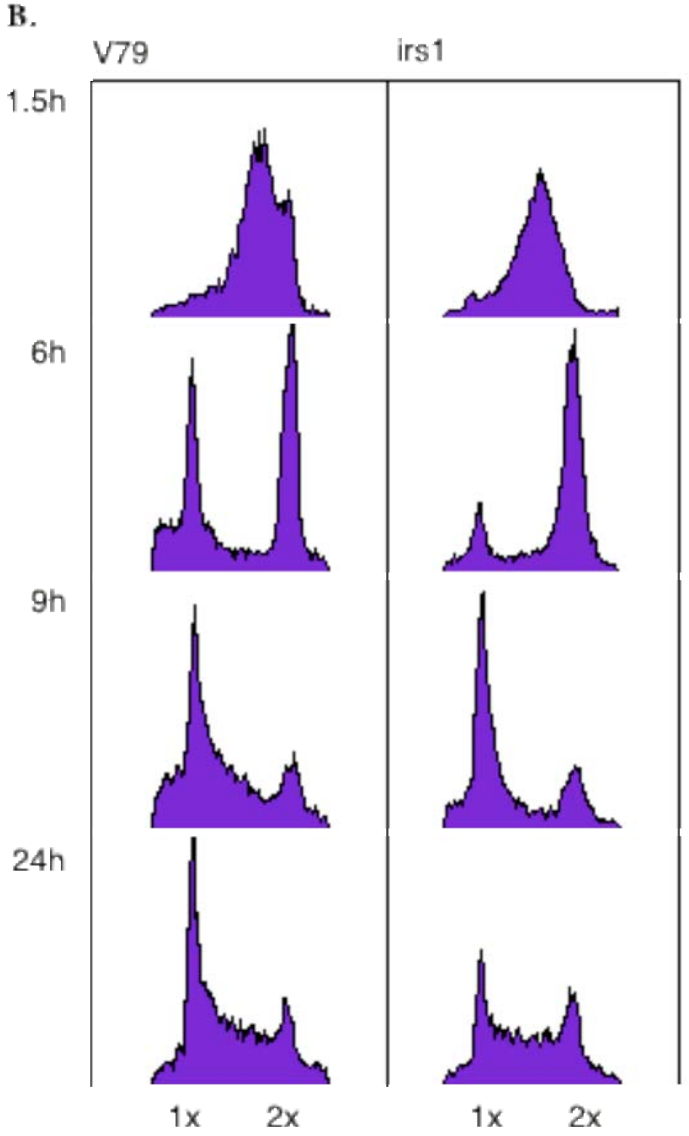
Figure 7. Cell cycle progression of V79 and irs1 cells after HU and TR treatment. Cells were harvested at 1.5, 6, 9 and 24 h after releasing from HU (**A**) or TR (**B**) blocking, and cell cycle distributions were analyzed on FACScan.



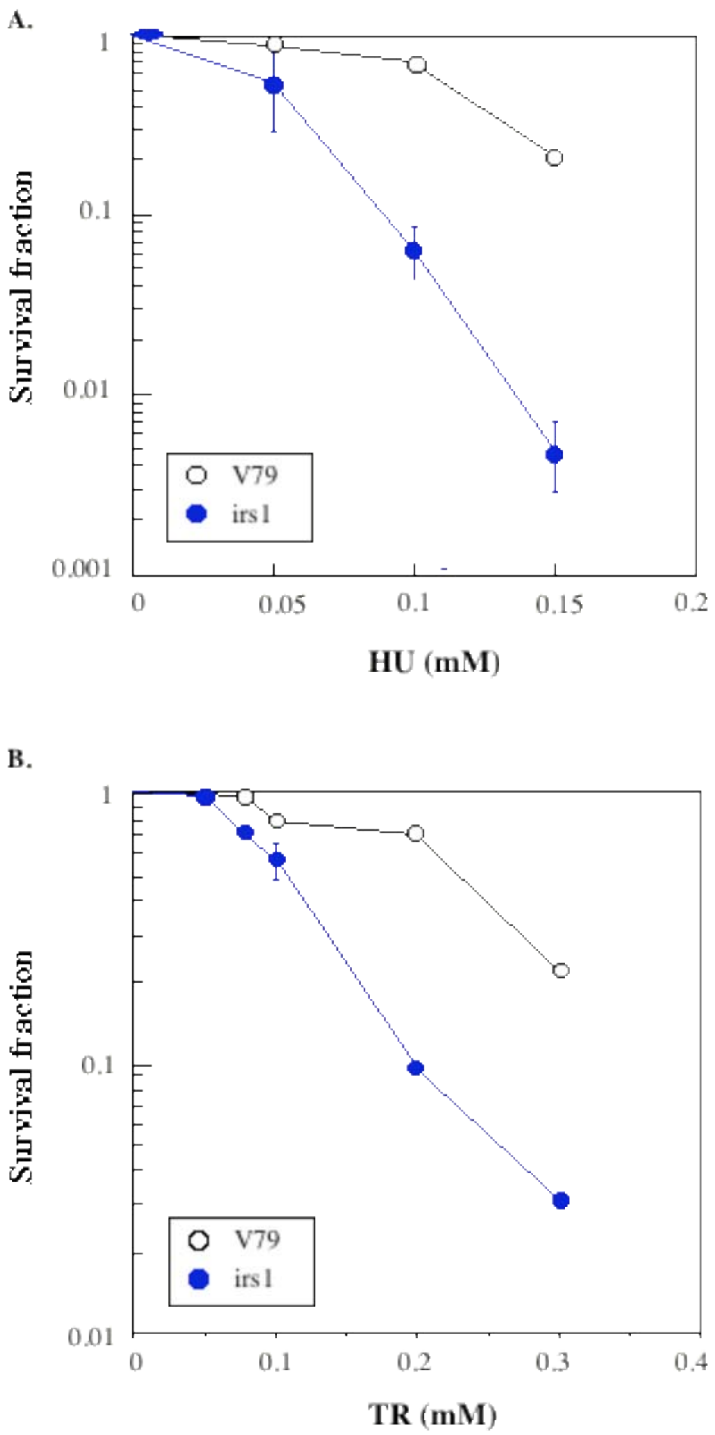
Lim and Liu, Figure 1



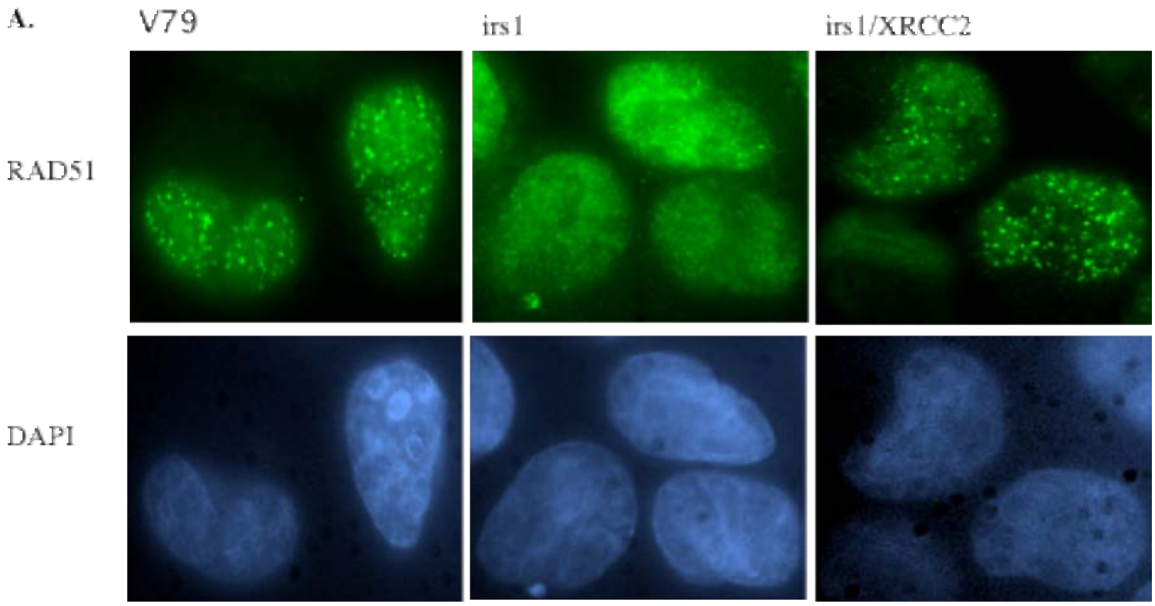
Lim and Liu, Figure 2A

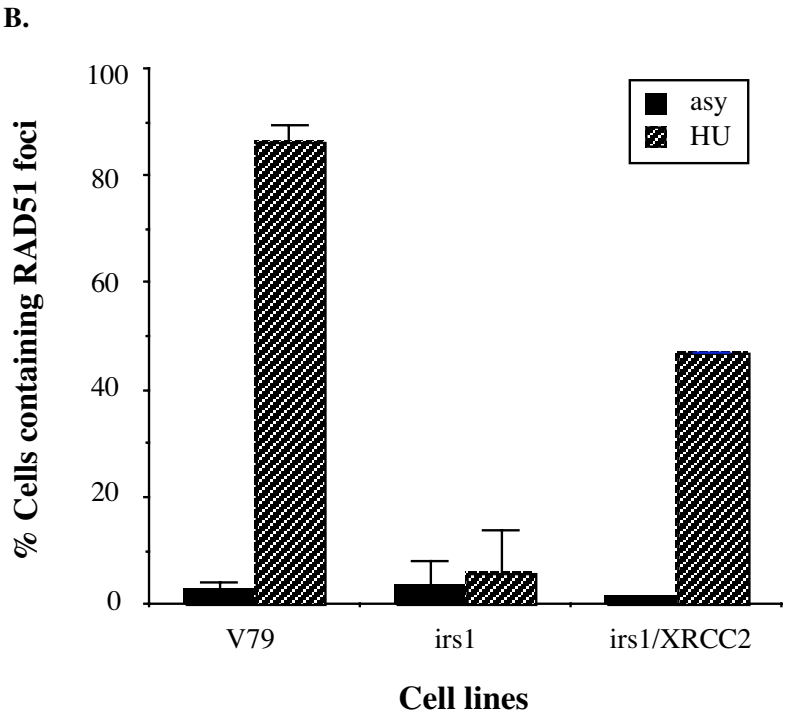


Lim and Liu, Figure 2B

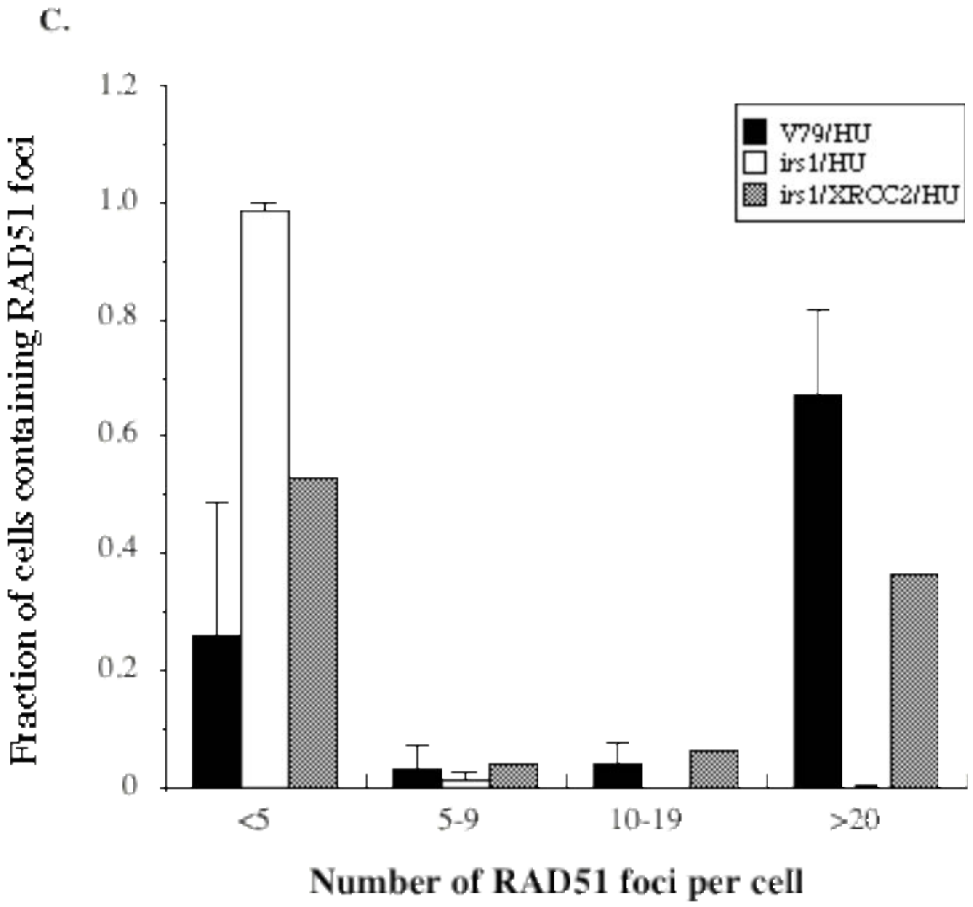


Lim and Liu, Figure 3A and B



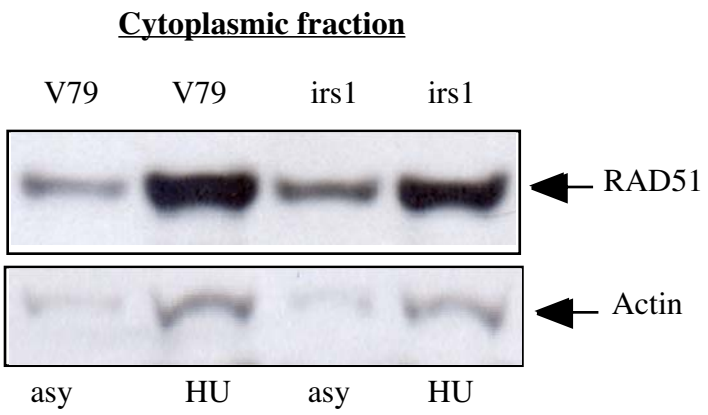


Lim and Liu, Figure 4A and B



Lim and Liu, Figure 4C

A.



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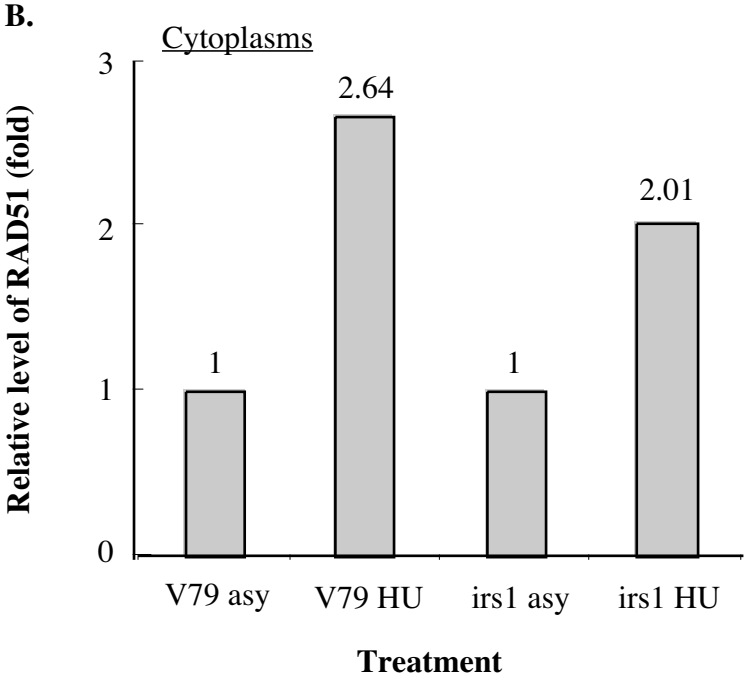
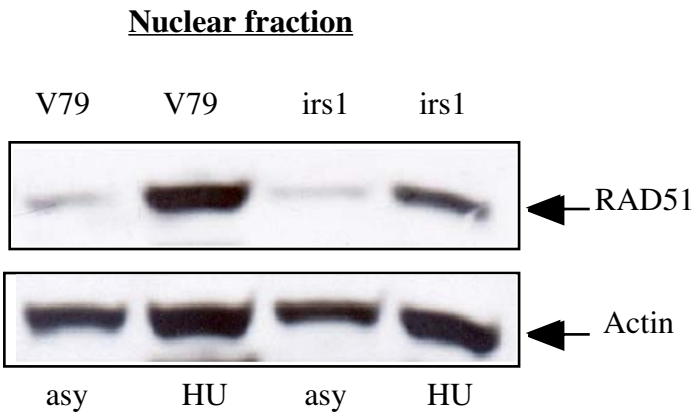
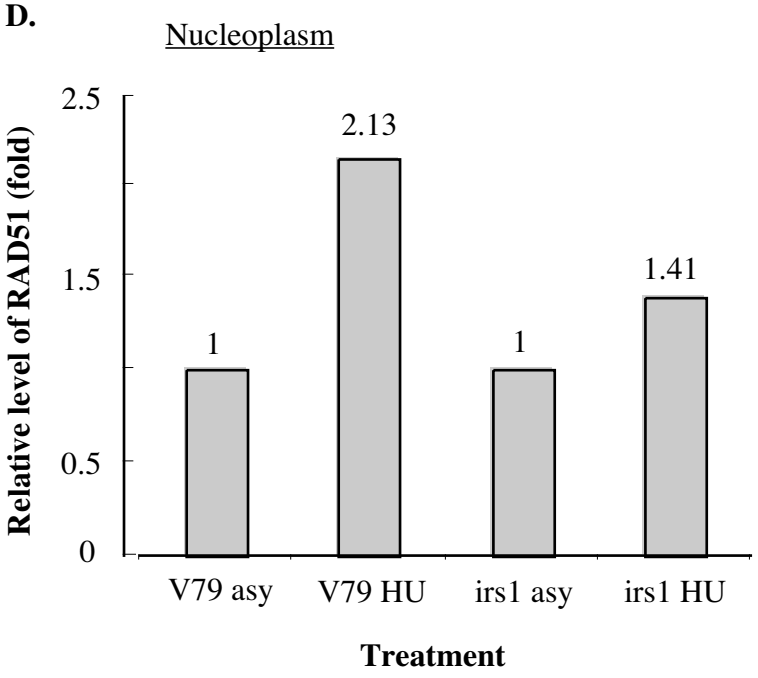


Lim and Liu, Figure 5A and B

C.

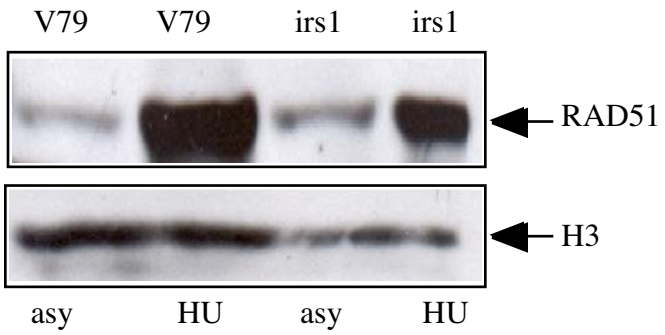


D.

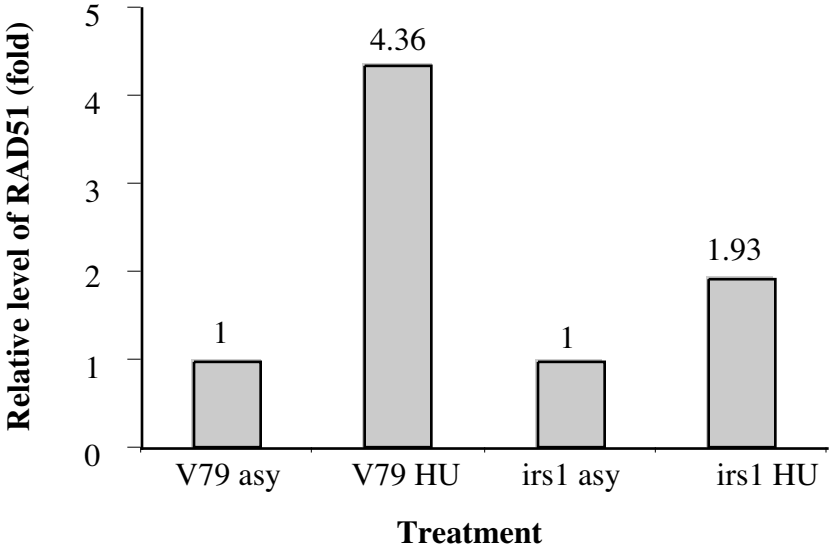


Lim and Liu, Figure 5C and D

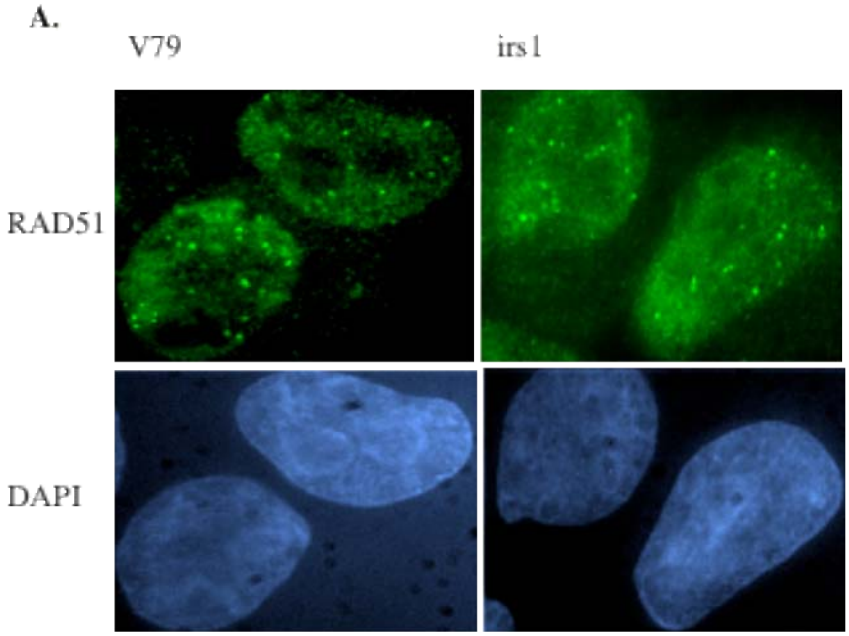
E.

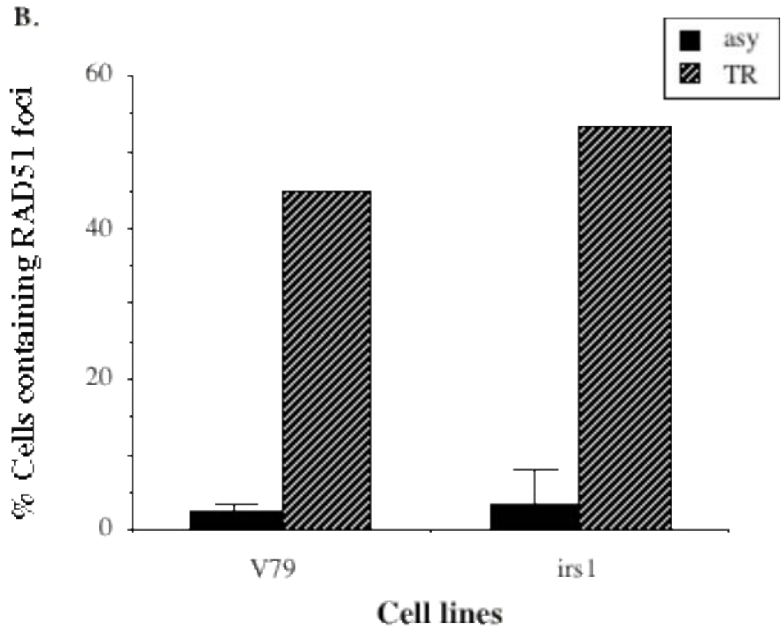


F.

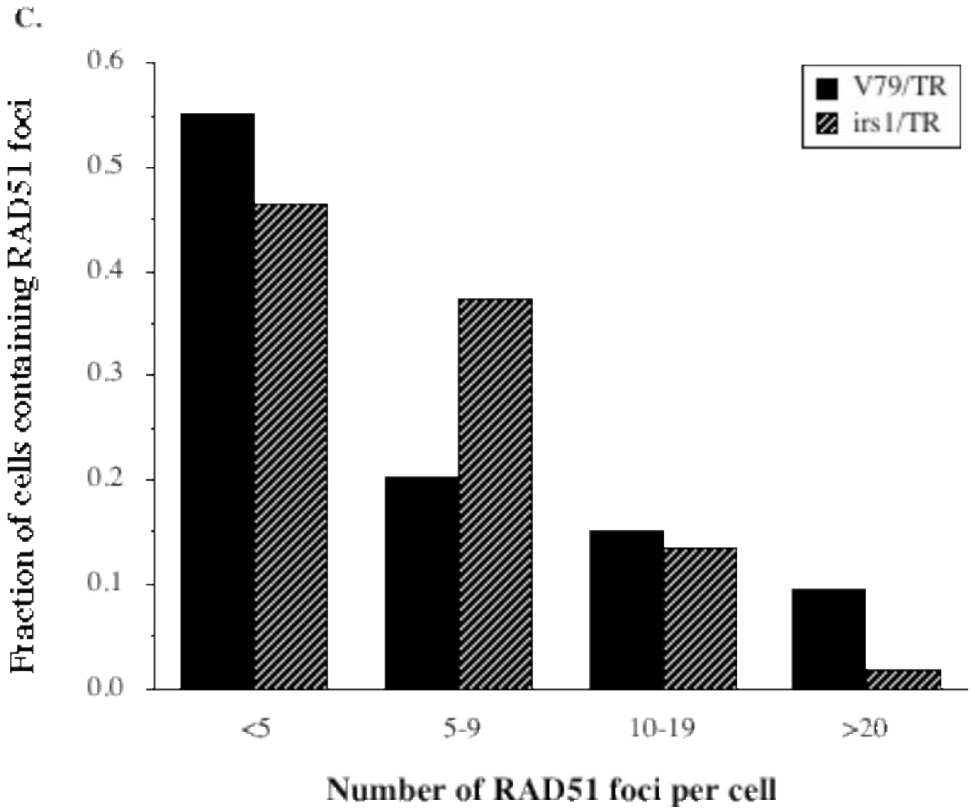


Lim and Liu, Figure 5E and F

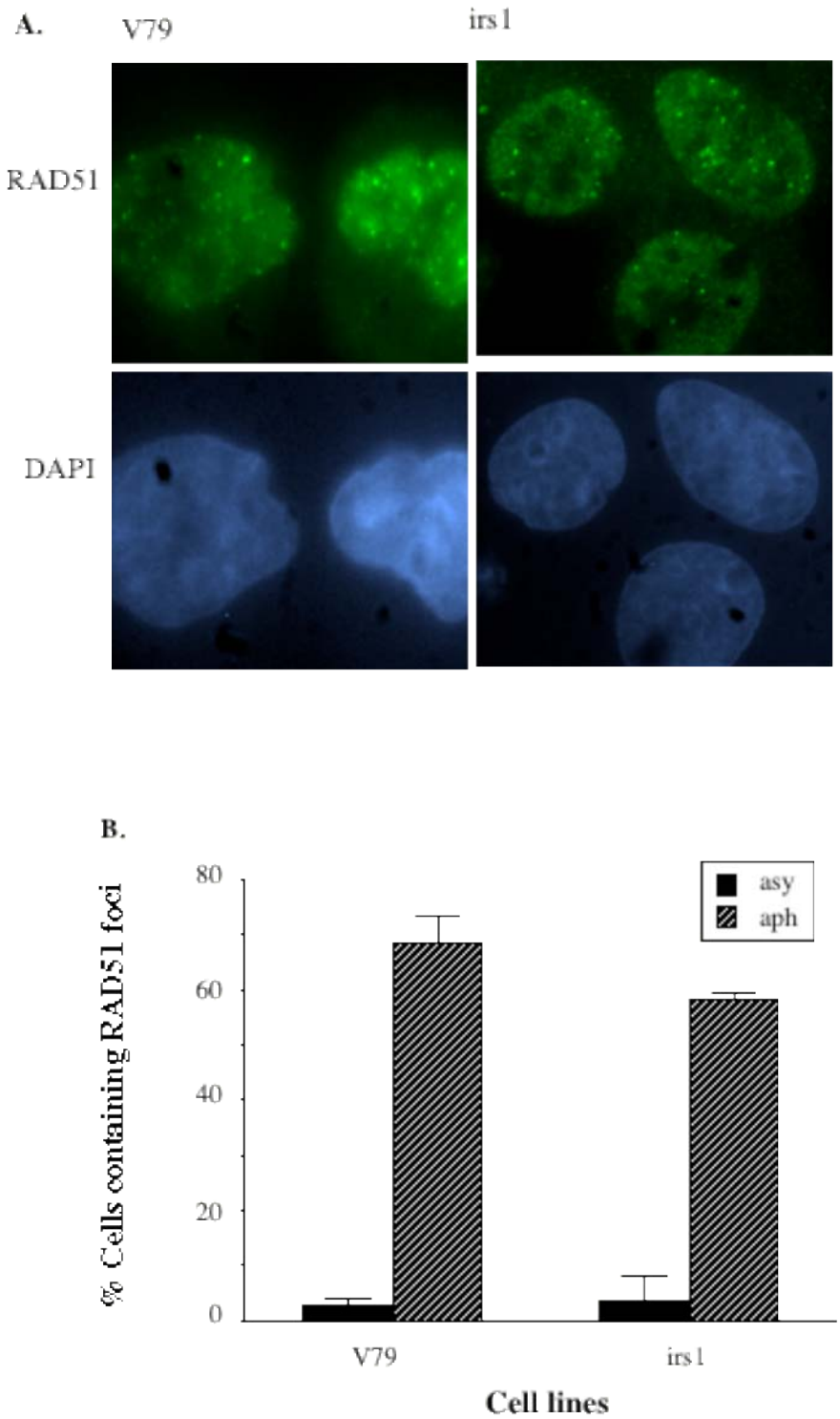




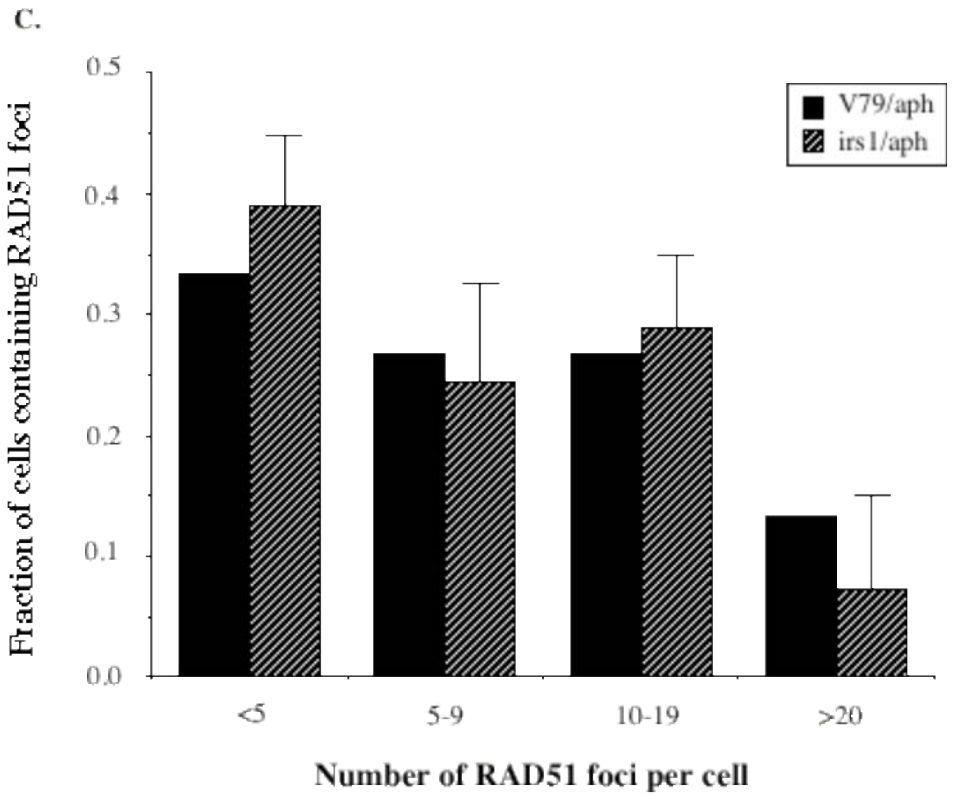
Lim and Liu, Figure 6A and B



Lim and Liu, Figure 6C



Lim and Liu, Figure 7A and B



Lim and Liu, Figure 7C