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Mutagenesis

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# Nuclear localization of Rad51B is independent of BRCA2

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**Abstract**

Human Rad51 is critical for the maintenance of genome stability through its role in the repair of DNA double-strand breaks. Rad51B (Rad51L1/hRec2) is one of the five known paralogs of human Rad51 found in a multi-protein complex with three other Rad51 paralogs, Rad51C, Rad51D and Xrcc2. Examination of EGFP-Rad51B fusion protein in HeLa S3 cells and immunofluorescence in several human cell lines confirms the nuclear localization of Rad51B. This is the first report to detail putative interactions of a Rad51 paralog protein with BRCA2. Utilization of a BRCA2 mutant cell line, CAPAN-1 suggests that Rad51B localizes to the nucleus independent of BRCA2. Although both Rad51B and BRCA2 are clearly involved in the homologous recombinational repair pathway, Rad51B and BRCA2 do not appear to associate directly. Furthermore, mutations in the KKLK motif of Rad51B, amino acid residues 4-7, mislocalizes Rad51B to the cytoplasm suggesting that this is the nuclear localization signal for the Rad51B protein. Examination of wild-type EGFP-Rad51B fusion protein in mammalian cells deficient in Rad51C showed that Rad51B localizes to the nucleus independent of Rad51C; further suggesting that Rad51B, like Rad51C, contains its own nuclear localization signal.

## 1. Introduction

Repair of DNA double-strand breaks in eukaryotic cells occurs primarily through two pathways, error prone non-homologous end joining (NJEH) or high fidelity homologous recombinational repair (HRR) (1,2). The HRR pathway involves the participation of many proteins including several members of the Rad52 epistasis group, Rad51, Rad52, Rad54, and the Rad51 paralog proteins (3). Rad51 is central to the catalysis of DNA strand exchange between identical DNA strands in an ATP-dependent manner (4). There are five human paralogs of Rad51, Rad51B (Rad51L1/hRec2), Rad51C (Rad51L2), Rad51D (Rad51L3), Xrcc2 and Xrcc3 (5-12). Rad51B has been shown to bind directly to Rad51C, and indirectly with Rad51D and Xrcc2 to form a multiprotein complex (BCDX2) that functions in HRR (13-16). Recently, Rad51C has been shown to be complexed with either Rad51B, BCDX2 or Xrcc3 in the resolution of Holliday junctions (17). Rad51B preferably binds to Holliday junction DNA substrates *in vitro* further indicating that it may have a role in Holliday junction resolution (18). Moreover, deficiency of any of the Rad51 paralog proteins in vertebrate cells results in increased sensitivity to ionizing radiation and DNA damaging agents similar to mutants of Rad51 and BRCA2 (9,19-22).

The breast cancer susceptibility protein, BRCA2, functions in the HRR pathway (23) and has been shown to interact directly with Rad51 (24). BRCA2 binds to Rad51 through a series of eight similar sequence motifs of approximately 30 amino acids each, referred to as the BRC repeats (24). X-ray crystallography has been used to detail the interaction of Rad51 with the BRC4 repeat of BRCA2 (25). It has been suggested that BRCA2 may be responsible for loading Rad51 onto single-stranded DNA during

homology pairing creating nucleoprotein filaments (26). A cancer-causing truncation mutant of the BRCA2 protein that is unable to localize to the nucleus inhibits Rad51 localization to the nucleus as well as foci formation following DNA damage, suggesting that Rad51 relies on its interaction with BRCA2 for proper subcellular localization (27,28). However, a BRCA2-independent association of Rad51 with chromatin during S-phase has recently been described suggesting that Rad51 may have multiple methods for nuclear localization (29).

The nuclear localization of a protein involves the recognition of targeting signals by members of the importin family and subsequent translocation by the nuclear pore complex (reviewed in (30). There are three classes of short modular peptide sequences sufficient for nuclear localization. The first of these is a short stretch of basic amino acids; the second, contains two short stretches of basic amino acids separated by a spacer of 10-12 amino acid residues (31,32), and the third, contains charged or polar residues interspersed with non-polar residues (33).

Rad51B has a short stretch of basic residues KKLK in the N-terminal sequence of the protein that has been suggested to act as a nuclear localization signal for this protein (10). A KKLK motif is found to independently localize the Ku80 DNA repair protein to the nucleus demonstrating the functionality of this motif as a nuclear localization signal (34). Recently it has been shown that Rad51C contains a nuclear localization signal at the extreme C-terminus of the protein (35). Studies have also placed Rad51D and Xrcc2 in the nucleus of the cell, although whether they transport independently to the nucleus is as yet not known.

This report is the first to directly localize Rad51B to the nucleus and furthermore demonstrate that the KKLK motif in Rad51B functions as a nuclear localization signal, allowing Rad51B to direct its subcellular localization independent of both BRCA2 and its partner protein Rad51C. Direct fluorescence of an EGFP-Rad51B fusion protein and immunofluorescence were used to localize Rad51B in several human cell types. Rad51B showed nuclear staining in the BRCA2-deficient pancreatic cancer cell line, CAPAN-1, demonstrating that Rad51B does not rely on BRCA2 for nuclear localization. Rad51B and a fragment of BRCA2 containing BRC repeats 1-3 failed to interact in yeast two-hybrid assay and no evidence for an interaction between BRCA2 and Rad51B was identified *in vivo*. Additional studies showed that mutation of the KKLK motif of Rad51B, amino acid residues 4-7, mislocalized Rad51B to the cytoplasm suggesting that this is the nuclear localization signal for the Rad51B protein. Examination of wild-type Rad51B in mammalian cells deficient in Rad51C (36) showed that Rad51B localizes to the nucleus independent of Rad51C further suggesting that Rad51B, like Rad51C, contains its own nuclear localization signal. Understanding the localization of Rad51 paralog proteins and the interactions involved between the paralogs will be necessary to fully understand their function *in vivo*.

## 2. Materials and Methods

### 2.1 *Cell culture*

HeLa S3, MCF10A, CAPAN-1 and MCF7 cells were obtained from the American Type Culture Collection (Manassas, VA). HeLa S3 cells were cultivated in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 300 μg/ml L-

glutamine, and antibiotics. MCF10A cells were cultured in DME:F12 supplemented with 5% heat-inactivated equine serum, 0.01 mg/ml bovine insulin (Sigma, St. Louis, MO), 0.5 μg/ml hydrocortisone and 20 ng/ml murine EGF (Becton-Dickinson; Franklin Lakes, NJ), 300 μg/ml L-glutamine and antibiotics. MCF7 cells were grown in Dulbecco's Modified Eagle's Media α supplemented with 10% heat-inactivated FBS, 300 μg/ml L-glutamine, 0.01 mg/ml insulin, and antibiotics. CAPAN-1 cells were grown in RPMI 1640 supplemented with 20% FBS, 300 μg/ml L-glutamine and antibiotics. V79 cells and the Rad51C-deficient V79-derived irs3 cells (36) were grown in monolayer culture in α–MEM supplemented with 10% FBS and antibiotics. Cells were maintained in a humidified incubator at 37°C and 5% $CO_2$. All media and supplements were obtained from Invitrogen (Carlsbad, CA) unless otherwise specified.

*2.2 Primary Antibodies*

Generation of a polyclonal antibody to Rad51B was previously described (15). Additional antibodies used in these studies include a BRCA2 monoclonal Ab-1 (Oncogene Research; San Diego, CA), an actin monoclonal antibody (Oncogene Research; San Diego, CA) and polyclonal antibodies RAD51 C-20 (Santa Cruz Biotechnology, Santa Cruz, CA) and RAD51 Ab-1 (Oncogene Research; San Diego, CA).

*2.3 Cellular lysates and immunoprecipitation*

Cell extracts were prepared from exponentially growing cells in lysis buffer (50 mM Tris pH 7.5, 50 mM NaCl, 0.5% Nonidet P-40 with protease inhibitors), incubated

on ice for 30 minutes, centrifuged at 14,000 x g for 5 minutes to pellet cellular debris and the supernatants collected. One milligram of whole cell extract was precleared with 30 µl of recombinant protein G agarose (Invitrogen, Carlsbad, CA). Samples were incubated with 25 µg of appropriate antibody or antibody preadsorbed with the appropriate peptide. (50 µg). Preimmune rabbit serum was used as a control for the anti-Rad51 immunoprecipitation and a non-related anti-actin monoclonal antibody was used as a control for the anti-BRCA2 monoclonal antibody immunoprecipitation. For each sample, 30 µl of recombinant protein G agarose was added, the samples were incubated for 1 hour then washed 3 times with 500 µl of cold lysis buffer. Subsequently, 2X SDS-PAGE loading buffer was added and samples were boiled for 5 minutes prior to SDS-polyacrylamide gel electrophoresis.

2.4 *Western blot analysis*

Immunoprecipitated samples or cell lysates were subjected to electrophoresis in 3-8% NuPAGE gels for BRCA2 immunoblots or 12% Tris-Glycine gels (Invitrogen; Carlsbad, CA) for Rad51 and Rad51B immunoblots as per manufacturer's instructions. The resolved polypeptides were transferred to a polyvinylidene difluoride (PVDF) membrane and blocked in 5% milk in phosphate-buffered saline (PBS) for 30 minutes followed by incubation with the appropriate primary antibody diluted in 5% PBS milk for at least one hour at room temperature. The Rad51B polyclonal antibody was diluted 1:1000, BRCA2 Ab-1 diluted 1:500, and Rad51 C-20 diluted 1:1000. Blots were washed three times with 1X TBS-T (Tris-buffered saline with 0.1% Tween 20 (Bio-Rad, Hercules, CA)), and a horseradish peroxidase-conjugated secondary antibody (Santa Cruz

Biotechnology, Santa Cruz, CA) diluted in 5% PBS milk was applied for 1 hour. The membranes were washed three more times in 1X TBS-T and visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech; Piscataway, NJ).

2.5 *Immunofluorescence*

For immunocytochemical analysis, cells were grown on coverslips for 24 hours, fixed with ice cold acetone for 2 minutes, and allowed to dry. The coverslips were then incubated overnight at 4°C with the appropriate primary antibody diluted 1:100 in 5% PBS milk. Coverslips were then washed 3 times in PBS and incubated for 1 hour with the appropriate secondary antibody, fluorescein-labeled donkey anti-mouse or rhodamine-labeled donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). The coverslips were washed three times with PBS, dipped in sterile distilled water and allowed to dry before mounting with Vectashield with DAPI (Vector Laboratories, Burlingame, CA). All slides were examined using a Zeiss Axioscope II fluorescence microscope (Thornwood, NY) and images captured using Cytovision software (Applied Imaging Systems; Santa Clara, CA).

2.6 *Yeast two-hybrid analysis*

Plasmid containing the full-length BRCA2 cDNA was kindly provided by Myriad Genetics (Salt Lake City, UT). Primers were created to amplify amino acid residues 638-1508 containing the BRCA2 BRC repeats 1-3 (BRC1-3) with unique restriction sites BamHI and XhoI and cloned into the pGADT7 vector (Clontech; Palo Alto, CA). Rad51 and Rad51B cloned into pGBT9 were kindly provided by Dr. David Schild (Lawrence Berkeley Laboratory, Berkeley, CA). Yeast two-hybrid analysis was performed using the

Matchmaker yeast two-hybrid kit as per manufacturer's instructions (Clontech, Palo Alto, CA). Yeast colonies were co-transformed with the BRC1-3 fragment in the activating domain vector and either Rad51, Rad51B, Rad51C, Rad51D, Xrcc2, Xrcc3 or control plasmid in the binding domain vector and assayed for β-galactosidase activity using O-nitrophenol-β-D-galactopyranoside as a substrate as outlined in the yeast protocols' handbook (Clontech, Palo Alto, CA).

2.7 *EGFP-fusion proteins and direct fluorescence*

The full-length Rad51B cDNA was fused to an enhanced green fluorescent protein by cloning Rad51B in-frame into the EcoRI and NotI restriction enzyme sites in the pEGFP-C2 plasmid (Clontech, Palo Alto, CA). The amino acid sequence KKLK, amino acids 4-7 of Rad51B, was mutated to NNLN using the Quikchange site-directed mutatgenesis kit (Qiagen, Valencia, CA) to create the pEGFP-Rad51B/NNLN vector as per manufacturer's instructions. The successful generation of the mutant construct was confirmed by sequencing (Biotech Core; Mountain View, CA). HeLa S3 cells grown on coverslips in 24 well plates were transfected with 1 ug of plasmid DNA using 6 µl Fugene transfection reagent (Roche Applied Science, Indianapolis, IN) as per manufacturer's instructions and grown for 48 hours. Cells were fixed in 4% formaldehyde in PBS for 30 minutes and mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Slides were examined using a Zeiss Axioscope II fluorescence microscope (Thornwood, NY) and images captured using Cytovision software (Applied Imaging Systems; Santa Clara, CA).

2.8 *Transfection of EGFP-labeled Rad51B in V79 and irs3 cells*

Hamster V79 cells and the Rad51C-deficient V79-derived irs3 cells were seeded on glass coverslips in 24-well dishes at a density of 2 x $10^4$ cells/well 24 hours prior to transfection. Transfection was performed in 500 μL growth medium (without antibiotics) with 2 μL Lipofectamine 2000 reagent (Invitrogen, CA) and 0.8 μg DNA. After 24 hours, the transfection medium was removed and the cells were fixed with 4% formaldehyde in PBS for 1 hour at 4°C. The coverslips were then mounted onto glass microscope slides with the addition of 3 μL Vectashield mounting medium with DAPI (Vector Laboratories). EGFP-labeled protein expression and intracellular localization was examined by fluorescence microscopy. Fluorescence images were captured as described above.

## **3**. **Results and discussion**

*3.1 Nuclear localization of Rad51B*

The function of the Rad51 paralogs in the HRR pathway suggests that these proteins exist primarily in the nucleus of the cell where they interact to repair damaged DNA. Immunostaining with a Rad51B-specific antibody localized the Rad51B protein primarily to the nucleus in HeLa S3 cells (Figure 1A). Preabsorption of the Rad51B antibody with an epitope-specific peptide abolished this immunostaining indicating that the staining was specific for Rad51B (data not shown).

Several cancer cell lines were examined to identify any variation in the subcellular localization of Rad51B across different cell types. The spontaneously immortalized normal breast epithelial cell line MCF10A, the MCF7 breast cancer cell

line, and CAPAN-1 pancreatic cancer cell line, which contains a C-terminal truncation of the BRCA2 protein, were examined. Since BRCA2 had been previously shown to be predominantly cytoplasmic in CAPAN-1 cells, this cell line was chosen to identify whether the localization of Rad51B would be altered in cells containing mutant BRCA2. Rad51B was localized to the nucleus in the MCF10A and MCF7 cells similar to the HeLa S3 cells (Figure 1B and 1C). Rad51B immunostaining showed that the Rad51B protein was localized to the nucleus in the CAPAN-1 cells suggesting that it can be transported into the nucleus independent of BRCA2 (Figure 1D). Merged images from dual labeling for both Rad51B and BRCA2 in CAPAN-1 cells showed no apparent co-localization of the two proteins as the BRCA2 immunostaining was predominantly cytoplasmic and Rad51B immunostaining was primarily nuclear (Figure 2). These results suggest that Rad51B does not require BRCA2 for nuclear localization and unlike Rad51, Rad51B may not interact with BRCA2.

*3.2 Rad51B does not directly or indirectly interact with BRCA2.*

Experiments were performed to determine whether there is a direct or indirect interaction between Rad51B and BRCA2. HeLa S3 cell extracts were immunoprecipitated with a Rad51B-specific antibody and examined for complex formation with BRCA2. Although BRCA2 was present in the extract, Rad51B did not co-immunoprecipitate BRCA2 (Figure 3A, Lanes 1 and 3). Immunoprecipiation of BRCA2 was observed using a Rad51-specific antibody demonstrating that the immunoprecipitatation of the high molecular weight BRCA2 protein was possible from the HeLa S3 cell extracts (Figure 3B, Lanes 1 and 3). Preimmune serum did not pull

down the BRCA2 protein demonstrating the specificity of the Rad51-BRCA2 interaction (Figure 3B, Lane 2). These data confirm previous results that demonstrated that Rad51 co-precipitates BRCA2 (24). Reciprocal co-immunoprecipitations were also performed using a BRCA2-specific antibody to identify complex formation with Rad51 or Rad51B. A significant amount of Rad51 was co-immunoprecipitated with BRCA2 antibody as compared to an unrelated antibody used as a control (Figure 3C, Lane 1), while Rad51B was not found to associate with BRCA2 (Figure 3D, Lane 1). Both the Rad51 and Rad51B proteins were detected by direct western blotting (Figure 3C and 3D, Lane 3). These results suggest that BRCA2 is not found in a complex with Rad51B *in vivo*.

To rule out transient or weak interactions that might be undetectable by immunoprecipitation, yeast two-hybrid analysis was used to test for a direct interaction between Rad51B and a fragment of the BRCA2 protein. The BRCA2 protein contains 8 BRC repeats. BRC repeats 1-3 (BRC1-3) comprising amino acids 638-1508 of the full-length BRCA2 protein that have been previously shown to bind to Rad51 by yeast two-hybrid analysis (24). This same fragment was tested against the full-length Rad51B protein. Full-length Rad51 was used in the assay as a positive control. The results in Figure 3E show that Rad51 interacts with the BRC1-3 fragment of BRCA2 while no interaction was observed between Rad51B and BRC1-3 in the yeast two-hybrid assay. Moreover, no direct interaction was observed between Rad51C, Rad51D, Xrcc2 or Xrcc3 and this fragment of BRCA2 by yeast two-hybrid analysis with all the paralogs proteins exhibiting binding activity close to that of background levels (Figure 3E).

*3.3 Rad51B independently localizes to the nucleus*

It has been suggested the KKLK motif in the extreme N-terminus of Rad51B, amino acid residues 4-7, acts as a nuclear localization signal for the Rad51B protein (37). To determine if this motif was responsible for the nuclear localization of Rad51B, several enhanced green fluorescent protein (EGFP) fusion protein constructs were generated and examined by direct fluorescence. A wild-type EGFP-Rad51B, a control construct expressing EGFP alone, and an EGFP-Rad51B NNLN mutant, in which the KKLK sequence was mutated to NNLN in the EGFP-Rad51B construct by site-directed mutagenesis, were individually transfected into HeLa S3 cells and examined for localization of the fusion proteins by direct fluorescence. The wild-type EGFP-Rad51B protein was specifically localized in the nucleus in HeLa S3 cells (compare Figure 4A and 4B). However, the EGFP-Rad51B NNLN mutant was not exclusively nuclear but rather found throughout the cell similar to the EGFP control (Figure 4C). Localization of the overexpressed EGFP-Rad51B fusion protein suggests that Rad51B contains an independent nuclear localization signal, as its nuclear import did not seem to rely on any partner protein that may be present in limiting amounts. The mislocalization of the KKLK mutation suggests that this motif functions as the independent NLS of Rad51B.

Rad51B has been found in a heterocomplex with Rad51C as well as in the larger BCDX2 complex *in vitro* and *in vivo* (13-16). Additional EGFP constructs were generated for each of the Rad51 paralog proteins in the BCDX2 complex. As shown in Figure 5, EGFP-Rad51C exhibits a nuclear localization consistent with the recent demonstration that Rad51C contains a nuclear localization signal at the C-terminal end of the protein (38). The EGFP-Xrcc2 protein was localized throughout the cell similar to findings by Liu et al., in which a GFP-Xrcc2 construct was localized in wild-type V79

cells and found not to be exclusively nuclear (39) in contrast to previous reports (40). The EGFP-Rad51D also appeared to be localized throughout the cell, further supporting sequence data that shows that Rad51D does not contain an NLS.

To further characterize the subcellular localization of the individual Rad51 paralog proteins, Hela cells were subjected to subcellular fractionation followed by SDS-PAGE and western blot analysis. The results in Figure 6 show that endogenous Rad51C is localized primarily to the nuclear fraction, whereas endogenous Xrcc2 and Rad51D were found in both the nuclear and cytoplasmic fractions suggesting that Xrcc2 and certainly Rad51D may rely on other partner proteins present in limiting quantities for transport into the nucleus.

As Rad51B has been shown to only bind Rad51C in the BCDX2 complex, studies were aimed to determine whether Rad51B is localized to the nucleus independently or whether it requires the formation of a heterocomplex with Rad51C prior to nuclear import. To determine whether Rad51C may play a role in recruiting Rad51B to the nucleus, localization of Rad51B was assessed in the V79-derived hamster mutant cell line, irs3, which is defective in Rad51C (36). As in Figure 4A, the EGFP control construct in Figure 7A was localized throughout the cell. The results in Figure 7B show that the EGFP-Rad51B fusion protein was localized to the nucleus in the Rad51C mutant cell line demonstrating that absence of Rad51C did not alter Rad51B localization and therefore Rad51C does not direct the subcellular localization of Rad51B. The EGFP-Rad51B NNLN mutant was observed throughout the irs3 cells similar to EGFP control confirming the previous results in the HeLa S3 cells (Figure 7C). Similar experiments performed in the V79 parental cell line showed that EGFP-Rad51B was nuclear while the

EGFP-Rad51B NNLN mutant was unable to localize to the nucleus in the V79 cells (data not shown). The nuclear localization of Rad51B in Rad51C null cells and the mis-localization of the Rad51B protein mutant in the putative nuclear localization signal indicates that Rad51B localizes to the nucleus independent of Rad51C through the use of the N-terminal KKLK motif.

### 3.4 Summary

Using both direct fluorescence and immunocytochemistry, this is the first study to show direct evidence that Rad51B localizes to the nucleus. Rad51C and Xrcc2 have already been identified in the nucleus of hamster cells, and more recently the nuclear localization signal for Rad51C has been identified to be at the C-terminal end of the protein. Further, it has been demonstrated that Rad51 is a nuclear protein whose subcellular localization is dependent on BRCA2. In this study, we were unable to demonstrate an association between Rad51B and the BRCA2 protein, although both proteins are common to the Rad51 double-strand break repair pathway.

It has been suggested through sequence homology that the Rad51 paralogs have diverged from Rad51 in such a way that they would be incapable of reproducing the same binding mode with the BRC repeat (41). Sequence alignment of the eight key amino acid residues of Rad51 that interact with the BRC4 of BRCA2 shows at least one polar or charged residue in the paralog sequences that would preclude BRC4 binding to any paralog. Previously, binding of the Rad51 paralogs has been tested against BRC3 that did not reveal an interaction between BRCA2 and any of the Rad51 paralog proteins (28). Our studies examined a larger region of BRCA2 containing BRC motifs 1-3 which

demonstrated that Rad51B and the other paralogs did not interact with this region of BRCA2 (Figure 3). It is still possible that the other paralogs may interact with BRCA2 through the other repeats or a different region of the BRCA2 protein, but is it unlikely that this would be true for Rad51B from the data presented here. It appears evident that if any of the paralogs do interact with BRCA2 it may be indirectly or under conditions not examined in this study. Our recent domain mapping studies of the BCDX2 complex suggest that the homologous region of Rad51 that binds BRCA2 on the Rad51 paralogs may have evolved for the purpose of forming unique multimeric complexes such as BCDX2 (42).

There is evidence that all of the members of the BCDX2 complex localize to the nucleus, although to date only Rad51B and Rad51C are implicated to do so independently. We found EGFP-Rad51C to localize exclusively to the nucleus in HeLa S3 cells confirming the previous report that Rad51C localizes to the nucleus through an independent nuclear localization signal (35). Rad51D and Xrcc2 co-elute from nuclear extracts from HeLa cells suggesting they can be found in the nucleus (43). Expression of a GFP-Xrcc2 fusion protein is nuclear in the irs1 CHO mutant that is Xrcc2 null (40). Preliminary evidence did not find Rad51D and Xrcc2 to be exclusively nuclear in EGFP-fusions in HeLa S3 cells (Figure 5). This suggests that when Xrcc2-GFP is expressed in irs1 cells, the fusion protein does not have to compete with wild type protein for binding to a partner protein for nuclear import while in HeLa S3 cells that contain endogenous Xrcc2, the partner protein is limiting and not all of the fusion protein will localize to the nucleus. As sequence analysis does not suggest nuclear localization signals in either Rad51D or Xrcc2, we propose that complex formation with a nuclear-targeted protein

such as Rad51C or even in a larger complex also including Rad51B is necessary for Rad51D and Xrcc2 to be found in the nucleus.

This study finds that, unlike Rad51, Rad51B is not found to directly or indirectly bind BRCA2. The independent nuclear localization of Rad51B found in this study suggests that Rad51B is able to enter the nucleus without the need for its partner protein Rad51C. This implies that Rad51B may have unique functions separate from its role in the BCDX2 paralog complex. Alternatively, Rad51B may aid in targeting the BCDX2 complex for nuclear localization. Further studies will be necessary to determine the role of Rad51B in the BCDX2 complex during the processes of homologous recombinational repair.

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**Figure Legends**

Figure 1. Nuclear localization of Rad51B in several cell types. Immunofluorescence with anti-Rad51B antibody of A. HeLa S3, B. MCF10A, C. MCF7, D. CAPAN-1 cells. Panels E-H are DAPI staining for panels A-D respectively.

Figure 2. Immunofluorescence of Rad51B and BRCA2 in CAPAN-1 cells. Panel A. Immunofluorescence of Rad51B detected with a secondary goat-anti-rabbit Rhodamine antibody. Panel B. Immunofluorescence of BRCA2 detected with secondary goat-anti-mouse FITC antibody. Panel C. Merge of images from A and B. Panel D. DAPI staining depicting cell nuclei.

Figure 3. Test of Rad51B-BRCA2 interactions. Panel A. Lane 1: Immunoprecipitation with anti-Rad51 antibody; Lane 2: Immunoprecipitation with rabbit preimmune serum; Lane 3: direct western of lysate showing BRCA2 expression in the cells. Samples were blotted with anti-BRCA2 antibody. Panel B. Lane 1: Immunoprecipitation with anti-Rad51B antibody; Lane 2: Immunoprecipitation with anti-Rad51B antibody preadsorbed with peptide serum; Lane 3: Direct western of cellular lysate showing BRCA2 expression. Panels C and D. Lane 1: Immunoprecipitation with monoclonal anti-BRCA2 antibody; Lane 2: Immunoprecipitation with non-related monoclonal antibody; Lane 3: Direct western blotting demonstrates expression of protein in cell lysates. Panel C was probed with anti-Rad51 polyclonal antibody. Panel D was probed with anti-Rad51B polyclonal antibody. Panel E. Yeast two-hybrid analysis of Rad51B binding BRCA2 does not find evidence of a direct interaction. Yeast two hybrid activating domain vector

containing amino acids 638-1508 of BRCA2 was co-transformed with DNA binding domain vectors for Rad51, Rad51B, Rad51C, Rad51D, Xrcc2, Xrcc3 or empty control vector and assayed for binding activity using ONPG as a substrate. Activity is measured in β-galactosidase units. Results are from a representative experiment performed in triplicate with error bars representing standard error.

Figure 4. Rad51B contains a nuclear localization signal. Detection of expression of EGFP-fusion protein in HeLa S3 cells by direct fluorescence. Panel A. EGFP control. Panel B. EGFP-Rad51B. Panel C. EGFP-Rad51B NNLN. Panels D, E and F show DAPI staining corresponding to A, B and C respectively.

Figure 5. Localization of Rad51C, Rad51D and Xrcc2 EGFP-fusion proteins. Detection of expression of A. EGFP-Rad51C, B. EGFP-Rad51D, and C. EGFP-Xrcc2 in HeLa S3 cells by direct fluorescence. Panels D,E and F show DAPI staining corresponding to A-C respectively.

Figure 6. Western Blot of subcellular fractions of endogenous Rad51 paralogs in HeLa S3 cell lysates. Nuclear and cytoplasmic fractions were blotted with antibodies to A. BRCA1 as a nuclear control protein, B. Rad51C, C. Rad51D, D Xrcc2 and E. Hsp70 as a cytoplasmic control protein.

Figure 7. Direct fluorescence of EGFP-Rad51B fusion proteins in irs3 Rad51C mutant CHO cells. Panel A. EGFP control. Panel B. EGFP-Rad51B. Panel C. EGFP-Rad51B NNLN mutant. Panels D, E and F are DAPI staining of Panels A, B and C respectively.

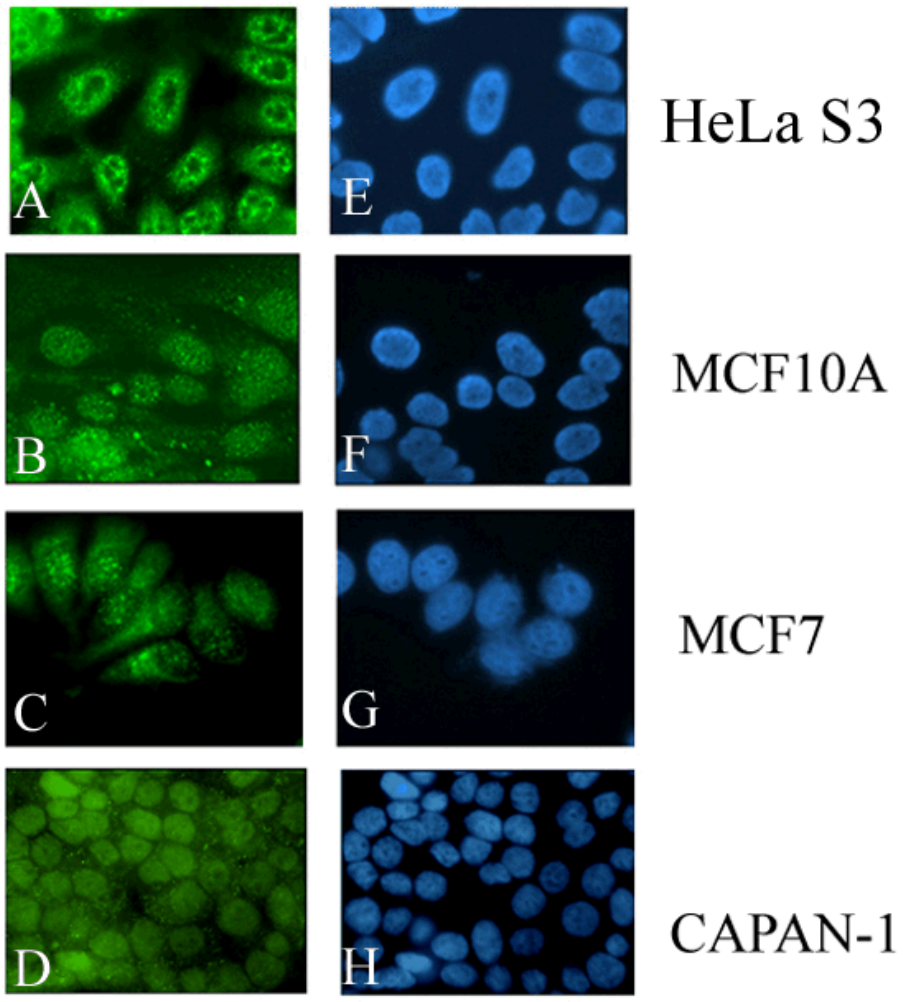


Figure 1

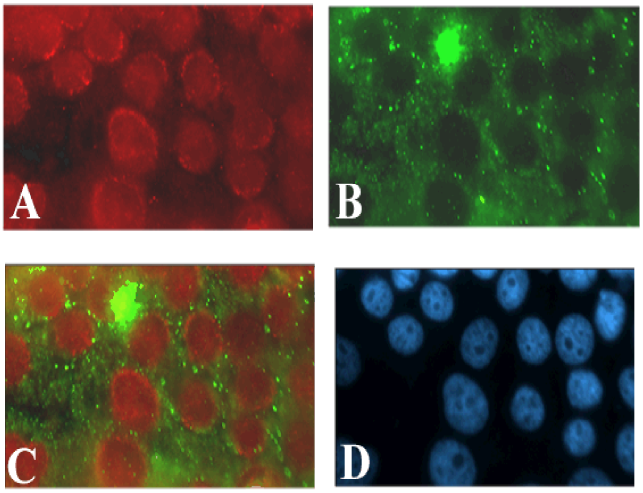


Figure 2

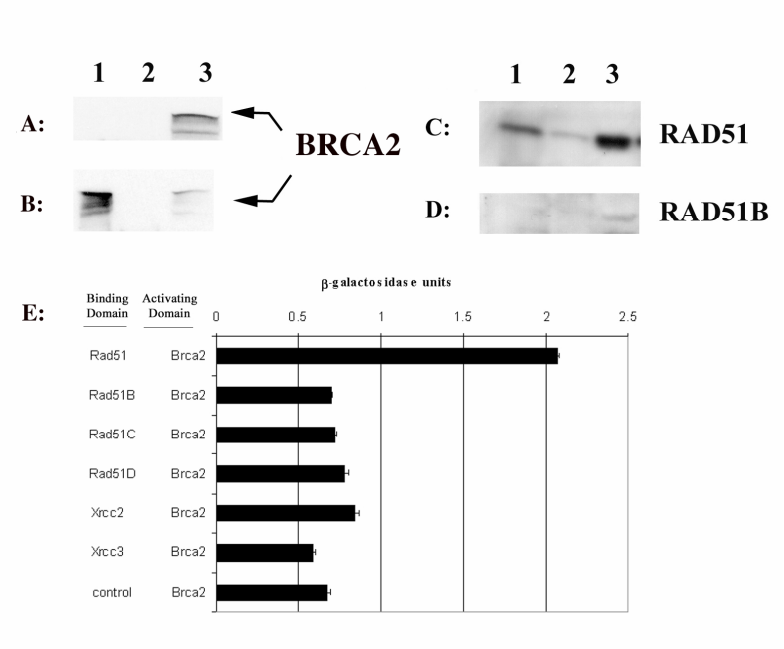
1 2 3
A:
BRCA2
B:
1 2 3
C:
RAD51
D:
RAD51B
E:
β-galactosidase units
Binding Domain
Activating Domain
0
0.5
1
1.5
2
2.5
Rad51 Brca2
Rad51B Brca2
Rad51C Brca2
Rad51D Brca2
Xrcc2 Brca2
Xrcc3 Brca2
control Brca2

Figure 3

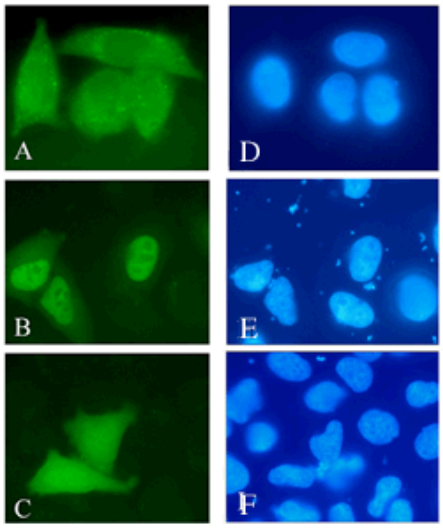


Figure 4

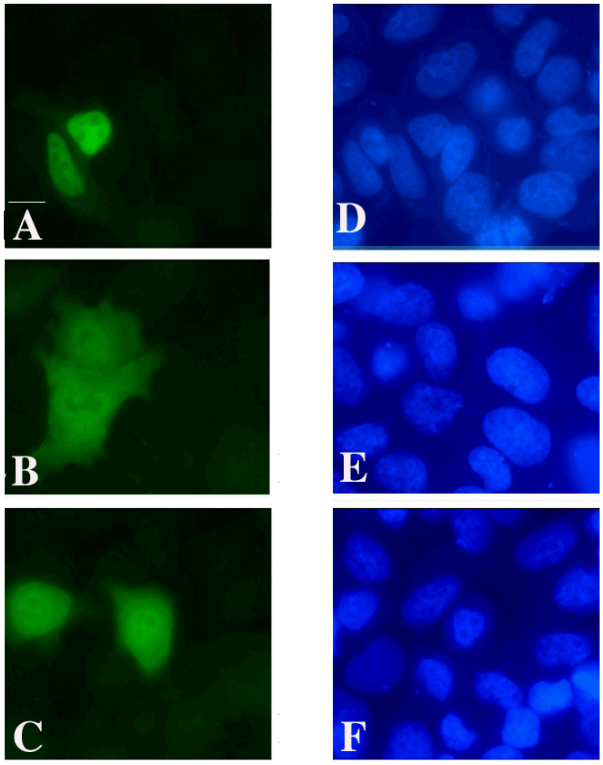


Figure 5

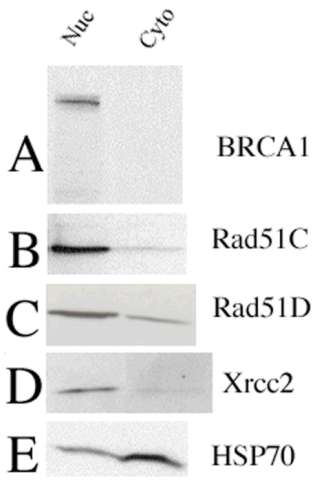


Figure 6

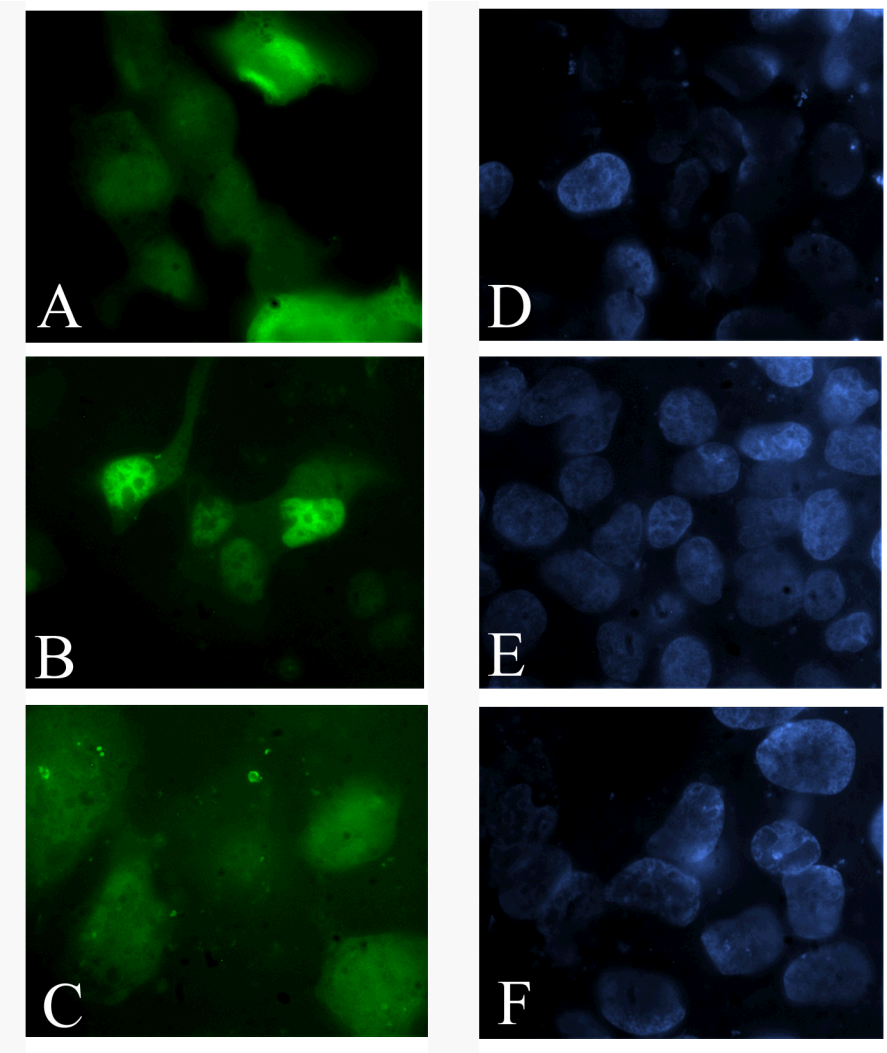


Figure 7