Published in final edited form as:

Cancer Genet Cytogenet. 2008 October 15; 186(2): 85–94. doi:10.1016/j.cancergencyto.2008.06.013.

Restoration of CAPAN-1 cells with functional *BRCA2* provides insight into the DNA repair activity of individuals who are heterozygous for *BRCA2* mutations

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Abstract

Mutations in the *BRCA2* gene are associated with inherited, early-onset breast cancer. CAPAN-1 cells have been very beneficial in studying how *BRCA2* mutations contribute to malignant transformation. They exhibit loss of heterozygosity (LOH), and the remaining copy of *BRCA2* has a 6174delT mutation, which causes a premature C-terminal truncation that removes the domains for DNA repair and the nuclear localization signals. The DNA repair protein RAD51, which interacts with *BRCA2*, exhibits impaired nuclear translocation in CAPAN-1. It has been speculated that RAD51 may require *BRCA2* for nuclear entry and C-terminally truncated *BRCA2* may retain RAD51 in the cytoplasm. This may cause heterozygous individuals to exhibit deficient DNA repair and cell viability comparable to individuals with LOH or bi-allelic *BRCA2* mutations. We simulated a heterozygous condition by employing stably transfected CAPAN-1 cells with wildtype *BRCA2*. Our studies determined that fusion of a nuclear localization signal to RAD51 did not increase its ability to independently enter the nuclei of CAPAN-1 cells. Furthermore, restoration of functional *BRCA2* did not significantly improve DNA repair, or re-establish cell viability in CAPAN-1 cells. The results imply that C-terminally truncated *BRCA2* hinders RAD51 nuclear translocation, possibly contributing to genetic instabilities in homozygous, as well as heterozygous individuals.

1. Introduction

The World Health Organization states that 1.2 million people per year will be diagnosed with breast cancer, worldwide [1], and among cancers is the second leading cause of death in women. Therefore, it is imperative to obtain a better understanding on the molecular level of the factors that predispose women to breast cancer, which can have an enormous impact on the search for new drug targets as well as therapeutic regimens. With respect to molecular determinants of breast cancer susceptibility, the *BRCA2* gene has been noted as dramatically increasing genetic predisposition. The *BRCA2* protein has been shown to be responsible for maintenance of

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genomic integrity by repairing DNA double-strand breaks (DSBs), specifically via its interaction with the recombinase and DNA repair protein RAD51 [2,3].

RAD51 catalyzes the strand exchange of DNA homologues to promote gene conversion and repair DSBs by homologous recombination (HR), [4,5]. HR is one of two pathways of repair of DSBs in mammals, the other being nonhomologous end-joining (NHEJ), [6–11]. HR requires the damaged DNA molecule to use the undamaged homologue as a template in order to repair the DSB. NHEJ involves ligation of the DNA ends at the breakpoint junction regardless of whether the original genetic information is still present. As a result, HR confers greater accuracy in repair than NHEJ[6–11]. Studies performed in mice where the *RAD51* gene was either mutated or completely knocked-out have shown its importance in genomic stability and cell viability [12,13]. Non-functional RAD51 fails to repair chromosome breaks and other DNA lesions which leads to an accumulation of DSBs and stalled replication forks [12,13]. Furthermore, inactivation of the *RAD51* gene causes embryonic lethality [14].

The interaction between RAD51 and BRCA2 was initially discovered from yeast two-hybrid screening assays [15–18]. The BRCA2 binding site on RAD51 is found within amino acids 98–339, which is a highly conserved region (Fig. 1) [16,19,20]. Moreover, studies examining the interaction between the two proteins have collectively shown that BRCA2 has two regions for RAD51 binding. The first region is located in the mid-portion of BRCA2, which consists of eight highly conserved amino acid motifs called BRC repeats (Fig. 2). The repeats have different binding affinities for RAD51—repeats 1–4, 7, and 8 all interact with RAD51; however, repeats 3 and 4 have the strongest interaction [16,21]. Observations imply that the purpose of this interaction ranges from possibly facilitating nuclear transport of RAD51 to loading of RAD51 onto sites of damaged DNA to form the nucleoprotein filaments required for strand invasion during HR-mediated repair of double-strand breaks [3,22–27]. The second RAD51 binding site is located on the CTD (C-terminal domain) of BRCA2, and is described as playing a major role in the regulation of RAD51 recombination activity by displacing the single-strand DNA binding protein replication protein A (RPA) from the exonucleolytically processed 3'-single-strand overhangs of the DSBs, thus allowing RAD51 to bind and form nucleoprotein filaments [28]. The CTD portion of BRCA2 has been shown to be highly active in HR-mediated repair with RAD51 [28]. This region consists of five domains—a helical domain, followed by three oligonucleotide/oligosaccharide binding domains (OB1, OB2, OB3) that have structural similarities with ssDNA binding proteins such as RPA, and a tower domain, which extends from OB2, and has structural similarities with the DNA binding domains of bacterial site-specific recombinases able to bind double-strand DNA [28], (Fig. 2). Also located on the C-terminus of BRCA2 are its two nuclear localization signals (NLSs), [22,29]. As a result, C-terminal mutations which disrupt, or truncations which remove, the NLSs are extremely detrimental to BRCA2 DNA repair functions, because they prevent nuclear localization. It has been strongly speculated that RAD51 relies on BRCA2 for nuclear translocation in response to ionizing radiation induced DNA damage. This idea is heavily based on the observation that RAD51 has no known NLSs [30] and cell lines which have non-functional or absent BRCA2 NLSs primarily exhibit cytoplasmic localization of RAD51 after induction of DSBs by ionizing radiation (IR) [22,23].

Therefore, we hypothesize that C-terminally truncated BRCA2 hinders RAD51 nuclear translocation necessary for repair of IR-induced DSBs, resulting in reduced DNA repair efficiency and cell viability after DNA damage. As a result, individuals who are heterozygous for *BRCA2* mutations may not recover from genomic insults at the same rate as individuals homozygous for the wildtype *BRCA2* allele and are at the same level of risk of loss of genomic integrity as individuals with bi-allelic mutations. And, this could account for the reduction in repair of DSBs observed in individuals heterozygous for *BRCA2* mutations [31]. For our study, we employed CAPAN-1 cells which have traditionally been used as an *in vitro* model to explore

the ramifications of mutated *BRCA2* on normal cellular function. They are derived from a human pancreatic carcinoma (*BRCA2* is expressed in pancreatic, ovarian and prostate tissues, in addition to breast tissues) and exhibit loss of heterozygosity with respect to the *BRCA2* gene [32,33]. One copy of *BRCA2* is missing and the remaining copy has a 6174delT mutation [34–38]. This mutation disrupts BRC repeats 7 and 8 and causes a frameshift that introduces a stop codon which prematurely truncates the protein. This premature truncation removes the domains for DNA repair, the second RAD51 binding site, and most notably, the protein's NLSs thereby preventing *BRCA2* from translocating into the nucleus (Fig. 2). Impaired nuclear transport of RAD51 after IR-induced DSBs, but not after S phase DSBs which result from stalled replication forks, has been consistently observed in CAPAN-1 cells, strongly supporting a role for *BRCA2* in the nuclear translocation of RAD51 after exposure to γ -irradiation [22, 23]. To simulate a heterozygous state for our studies, the CAPAN-1 cell line was stably transfected with recombinant human wildtype *BRCA2*. The results of our studies determined that fusion of a NLS to RAD51 did not allow nuclear translocation independent of *BRCA2* status to occur in CAPAN-1 cells, and stable transfection of CAPAN-1 cells with wildtype *BRCA2* did not significantly improve DSB repair efficiency and cell survival after irradiation when compared with controls.

2. Material and methods

2.1. Cell cultures, plasmids and transfections

CAPAN-1 cells (ATCC) were fed Dulbecco's Modified Eagle Medium (DMEM; Invitrogen-Gibco-BRL) supplemented with 20% fetal clone (Hyclone), 1% antibiotic/antimycotic solution, and 1% glutamine. HeLa cells were fed DMEM supplemented with 10% fetal clone (Hyclone), 1% antibiotic/antimycotic solution, and 1% glutamine. WI38 cells were a generous gift from Dr. Daohong Zhou (MUSC), and were maintained in Modified Eagle's Medium (MEM; Invitrogen-Gibco-BRL) supplemented with 10% fetal bovine serum (Invitrogen-Gibco-BRL), 1% antibiotic/antimycotic solution, and 1% glutamine. All cell lines were maintained at 37°C, with 5% CO₂ in a humidified incubator.

The construct of *RAD51* subcloned into pEGFP-C1 (denoted as GFP-RAD51) was a generous gift from Dr. Yinyin Huang (Dana-Farber Cancer Institute, Harvard Medical Institute). To generate the GFP (green fluorescent protein)-NLS-RAD51 construct, in which a nuclear localization signal (NLS) was placed on the GFP-RAD51 construct, we subcloned a linker containing the DNA sequence (5'-CCAAAGAAAAAGAGGAAAGTC-3'; Integrated DNA Technology) of the following NLS sequence (PKKKRKV) [39] annealed to its DNA complement, into the GFP-RAD51 construct via Sma I and BamH I restriction enzyme sites that were also included on the 5' and 3' ends of the linker. The nuclear localization signal was subcloned into the construct to be expressed C-terminal to the GFP protein sequence and N-terminal to the RAD51 protein sequence. Transient transfections were performed in CAPAN-1 and HeLa cells by using Targefect F-2 and Virofect transfection reagents according to manufacturer's published procedure (Targeting Systems). Assays were performed 48 hours post-transfection. Stable transfection of CAPAN-1 cells with wildtype full-length human *BRCA2* cDNA (confirmed by DNA sequence) sub-cloned into the pREP4 expression vector (denoted as CAPAN-1/*BRCA2*) was performed using the Targefect F-1 transfection reagent (Targeting Systems), according to the manufacturer's procedure. *BRCA2* expression was confirmed by western blot analysis, as described in section 2.2.

2.2. Western blot analysis

Cell lysates were obtained as described [40] and the protein concentrations were determined by using a commercial protein assay kit (Biorad). Sub-cellular fractionation was performed using NE-PER Cytoplasmic and Nuclear Extraction Reagents (Pierce), according to the

manufacturer's protocol. The lysates (200 µg per sample for probing with anti-BRCA2 and 50 µg per sample for probing with anti-GFP) were suspended in Laemmli buffer, resolved on SDS-PAGE gels, and transferred to PVDF filters (Millipore, Immobilon-P) overnight at 35 volts. To assess loading and transfer efficiency, the membrane was stained with Ponceau S prior to blocking. The membrane was blocked using 5% nonfat dry milk, 0.2% Tween 20 in Tris-buffered saline (TBS-T) for 1 hour at room temperature. After blocking and subsequent washing twice for 10 minutes in TBS-T, the membrane was exposed to the BRCA2 primary antibody (1:50; Ab-2; EMD Biosciences), anti-GFP primary antibody (1:1000; JL-8; Clontech) or anti-Lamin B (1:200; C-20; Santa Cruz). The membrane was washed twice with TBS-T before being exposed to a horseradish peroxidase-conjugated secondary antibody which accommodated the primary antibody (1:5,000; Jackson ImmunoResearch), followed by washing with TBS-T four times. Blot analyses were visualized using the Mosal western detection protocol employing Luminol (3-aminophthalhydrazide; Sigma-Aldrich) and P-coumaric acid (Sigma-Aldrich).

2.3. Immunofluorescence and identification of DNA double-strand breaks (DSBs)

Cells were seeded and allowed to grow at low confluency on cover slips in six well tissue culture plates. After irradiation using a ^{137}Cs source (calibrated at 1.52 gray [Gy]/min), the cells were fixed in 2% paraformaldehyde, neutralized with 1M glycine-tris, pH 7.4, and washed with PBS. For cells transfected with GFP-tagged constructs, the nuclei were stained with TOPRO-3 (1:1000 in PBS; Molecular Probes), and placed on glass slides with Anti-fade Aqua Poly Mount (Molecular Probes) for immunofluorescence analysis. For γ -H2AX staining, after the cells were fixed, they were blocked in Blocking solution containing 5% normal donkey serum, 1% BSA (Fraction V), 0.1% Triton X-100 in Phosphate-Buffered Saline (PBS) for 1 hour. The primary antibody anti- γ -H2AX (2 µg/µl; JWB301; Upstate Biotechnology) was diluted in blocking solution and cells were incubated for 1 hour, followed by three 5 minute washes in PBS. The Cy-3 conjugated anti-mouse secondary antibody (1:400; Jackson ImmunoResearch) was diluted in blocking solution and cells were incubated for 45 minutes, washed with PBS, and placed on glass slides as described above. The fixed cells were analyzed with a Zeiss LSM 5 Pascal Vario 2 Laser Scanning Confocal Microscope System (MUSC, Department of Pathology & Lab Medicine). The number of DSBs was determined by counting the number of γ -H2AX foci per cell for 100 cells in randomly chosen fields and the average number of foci per cell was determined.

2.4. Cell viability analysis

Cell viability was assessed as published [41], with the following modifications. Cells were plated in 6-well plates at a density of 1×10^5 cells per well. Twenty-four hours later, the cells were exposed to 15 Gy of ionizing radiation, and controls were non-irradiated cells plated at the same density. At two weeks post-irradiation, detection of viable cells was performed by first washing with PBS, followed by staining with 0.5% crystal violet in 50% methanol for 15 minutes. The wells were gently rinsed with water twice, and allowed to dry. Next, the cells were incubated for 30 minutes with 0.1 M citrate sodium, pH 5.4/20% methanol solution. The absorbance of each well was read at 570 nm. The percentage of cell survival was defined as the relative absorbance of irradiated *versus* non-irradiated cells. Statistical analyses and plots were performed using 2 tailed t-tests from <http://faculty.vassar.edu/lowry> and SigmaPlot 10.0. P values < 0.05 were considered statistically significant.

3. Results

3.1. A nuclear localization signal placed on RAD51 does not enable it to independently translocate into the nuclei of CAPAN-1 cells

RAD51 has no known NLSs [30] and previous studies have shown that it remains in the cytoplasm of CAPAN-1 cells after exposure to ionizing radiation [23]. We wanted to examine if a NLS placed on RAD51 would allow it to independently translocate into the nuclei of CAPAN-1 cells after induction of IR-induced DSBs. Furthermore, we wanted to compare the nuclear localization of RAD51 fused with a NLS in CAPAN-1 cells stably transfected with wildtype *BRCA2* (denoted as CAPAN-1/*BRCA2* cell line; Fig. 3) with CAPAN-1 cells. We subcloned the DNA sequence encoding the following functional nuclear localization signal, Pro-Lys-Lys-Lys-Arg-Lys-Val (PKKKRKV) [39], N-terminal to the RAD51 protein sequence and C-terminal to the GFP protein sequence in the pEGFP-C1 vector (denoted as GFP-NLS-RAD51; Figs. 4A, 4B). We subsequently transfected the construct into CAPAN-1, CAPAN-1/*BRCA2* and HeLa cells (used as a control because they have endogenous wildtype *BRCA2*), exposed them to 15 Gy of ionizing radiation, and used immunofluorescence and sub-cellular fractionation to probe the sub-cellular localization of GFP-NLS-RAD51 before and 6 hours post-irradiation (Fig. 5A–C, Fig. 6). The results show that at 6 hours after irradiation, RAD51 remains primarily cytoplasmic in CAPAN-1 cells (Fig. 5A, Fig. 6), as expected. However, CAPAN-1/*BRCA2* cells showed slightly more nuclear localization of GFP-NLS-RAD51 than observed in CAPAN-1 cells after IR (Fig. 5B, Fig. 6), yet there was an appreciable amount still localized in the cytoplasm. And, HeLa cells, which have endogenous wildtype *BRCA2*, showed predominately nuclear localization of RAD51 after irradiation (Fig. 5C, Fig. 6).

3.2. Restoration of CAPAN-1 cells with wildtype *BRCA2* does not significantly increase the resolution of DSBs

We observed that placement of a functional NLS on RAD51 did not enable it to translocate into the nucleus independent of *BRCA2* in CAPAN-1 cells. As a result, we wanted to compare DSB repair in CAPAN-1 cells with CAPAN-1/*BRCA2* cells, since they have both mutated and wildtype *BRCA2*, which simulates a heterozygous state. CAPAN-1/*BRCA2* cells, along with CAPAN-1 and WI38 cells (a control cell line, which exhibits efficient repair of DSBs) were irradiated at 15 Gy, and probed for phosphorylation of histone 2AX (γ -H2AX) by immunofluorescence. Histone 2AX phosphorylation is a reliable marker for the recognition and resolution of DNA DSBs [42,43]. As expected, the control cell line, WI38 showed fewer DSBs than the CAPAN-1 and CAPAN-1/*BRCA2* cell lines, due to more efficient repair, at the 6 and 24 hour time points for recovery after IR (Fig. 7A). At the 24 hour time point, WI38 cells showed an average of 19 ± 5.0 DSBs per cell, whereas CAPAN-1 and CAPAN-1/*BRCA2* cells showed an average of 35 ± 12.0 and 30 ± 12.0 DSBs per cell, respectively (Fig. 7B). The sub-cellular localization results for GFP-NLS-RAD51 showed a slightly higher nuclear distribution in CAPAN-1/*BRCA2* cells (Fig. 5B, Fig. 6), than what was observed in CAPAN-1 cells (Fig. 5A, Fig. 6), yet an appreciable amount of GFP-NLS-RAD51 remained in the cytoplasm. These observations were also reflected in the DSB repair results because there was minimal difference between the average number of repaired DSBs between CAPAN-1 and CAPAN-1/*BRCA2*, with the difference at the 24-hour time point not being statistically significant.

3.3. Restoration of CAPAN-1 cells with wildtype *BRCA2* does not significantly increase cell viability after irradiation

The *BRCA2* mutation in CAPAN-1 cells renders them radiosensitive; therefore, dosages of ionizing radiation that a normal cell can withstand are more detrimental to CAPAN-1 cells, due to their inability to efficiently repair DSBs, which results in reduced cell viability after irradiation [44,45]. To determine if there is a significant difference in cell survival between CAPAN-1 and CAPAN-1/*BRCA2* after irradiation, we plated cells at equal densities, irradiated

them at 15 Gy, and performed crystal violet assays two weeks later to assess the percentage of cell viability after IR-induced DNA damage. HeLa cells, which have endogenous wildtype *BRCA2*, were included as a control. Two weeks after irradiation, the results showed that CAPAN-1 cells exhibited the lowest level of cell viability (64.5 ± 2.12), HeLa cells exhibited the highest (95.0 ± 7.07), and CAPAN-1/*BRCA2* exhibited results intermediate to the other cell lines (71.0 ± 1.41), (Fig. 8). However, in spite of CAPAN-1/*BRCA2* cells having cell viability levels that were intermediate to CAPAN-1 and HeLa, the percentage was not significantly higher than what was observed for CAPAN-1. Conversely, there was a marked difference in the percentage of cell viability between CAPAN-1/*BRCA2* and HeLa cells that was statistically significant ($p < 0.05$). And, the difference in cell viability between CAPAN-1/*BRCA2* and CAPAN-1 cells was not statistically significant, implying that restoration of wildtype *BRCA2* had a very marginal affect on improvement of cell viability.

4. Discussion

Previous studies have shown that CAPAN-1 cells exhibit impaired nuclear translocation and predominately cytoplasmic localization of RAD51 after IR-induced double-strand breaks [22,23]. RAD51 has no known NLSs [30]; therefore, it must require the aid of additional proteins and/or complexes in order to translocate into the nucleus, and previous reports strongly support *BRCA2* as serving a major role in this function, in response to IR-induced DSBs. This prompted us to analyze if placement of a nuclear localization signal on RAD51 would enable it to independently enter the nuclei of CAPAN-1 cells in response to IR-induced DSBs.

Results observed in CAPAN-1 cells showed impaired nuclear translocation of GFP-NLS-RAD51 after irradiation (Fig. 5A). Although the last two BRC repeats (BRC 7–8) of *BRCA2* in CAPAN-1 cells are disrupted due to the 6174delT mutation, BRC repeats 1–6 remain intact and 1–4 are able to bind RAD51; therefore, binding would presumably still occur between the intact repeats of *BRCA2* and GFP-NLS-RAD51. The premature truncation of *BRCA2* in CAPAN-1 cells results in removal of its nuclear localization signals, thereby resulting in *BRCA2* being localized to the cytoplasm. Furthermore, we wanted to examine if restoration of wildtype *BRCA2* in CAPAN-1 cells would improve nuclear localization of RAD51 after IR. Stable transfection of wildtype *BRCA2* in CAPAN-1 cells would now cause them to mimic a heterozygous state, and the observations would provide implications of what occurs in carriers of *BRCA2* mutations. CAPAN-1 cells stably transfected with wildtype *BRCA2* (CAPAN-1/*BRCA2*) exhibited slightly more nuclear sub-cellular localization of GFP-NLS-RAD51 after IR-induced DSBs than CAPAN-1 cells (Fig. 5B). But the amount of GFP-NLS-RAD51 remaining in the cytoplasm of CAPAN-1/*BRCA2* cells was greater than observed in HeLa cells (control), which have wildtype endogenous *BRCA2* and exhibited predominately nuclear localization of GFP-NLS-RAD51 after IR (Fig. 5C). In HeLa cells, the expression of GFP-NLS-RAD51 in the immunofluorescence images appeared to be lower than what was observed in the CAPAN-1 and CAPAN-1/*BRCA2* cell lines, but we were able to better observe predominately nuclear localization of GFP-NLS-RAD51 in the sub-cellular fractionation images (Fig. 6). Therefore, restoration of CAPAN-1 cells with wildtype *BRCA2* cannot fully compensate for the hindered nuclear translocation of RAD51 that occurs from the presence of C-terminally truncated *BRCA2*. Few attempts have been made to incite RAD51 nuclear translocation independent of *BRCA2* status, which is what we did by fusing RAD51 with a functional NLS. We observed that placement of a NLS on RAD51 did not enable it to independently enter the nuclei of CAPAN-1 cells. Our results strongly support the implications of other observations [46] that truncated *BRCA2* is likely binding and retaining RAD51 in the cytoplasm.

Furthermore, our results showed that at 24 hours after irradiation, despite restoration of wildtype *BRCA2*, the average number of DSBs remaining in CAPAN-1/*BRCA2* cells ($30 \pm$

12.0) was not statistically significantly lower than the number observed in CAPAN-1 (35 ± 12.0), or even comparable to the control WI38 cell line (19 ± 5.0). Our studies employed IR dosages at 15 Gy, which would incite substantial levels of DNA damage. Furthermore, CAPAN-1 cells are radiosensitive; therefore, it was of interest to compare the rate of cell viability among CAPAN-1, CAPAN-1/BRCA2 and HeLa (control) cells, to determine how the combination of wildtype and truncated BRCA2 protein in CAPAN-1/BRCA2 cells influences cell viability. At two weeks after irradiation, the CAPAN-1/BRCA2 cells consistently exhibited cell viability rates (71.0 ± 1.41) that were only slightly higher than CAPAN-1 cells (64.5 ± 2.12), and considerably lower than HeLa cells (95.0 ± 7.07), (Fig. 8). In fact, the rate of cell survival in CAPAN-1/BRCA2 cells was not statistically significantly higher than what was observed in CAPAN-1 cells; however, it was statistically significantly lower than the rate observed in HeLa cells ($p < 0.05$). Taking into consideration the values obtained from the number of DSBs remaining 24 hours after IR, along with those obtained from the cell viability assays, restoration of CAPAN-1 cells with wildtype *BRCA2* in the CAPAN-1/BRCA2 cell line did not significantly improve DNA repair efficiency and cell viability after IR-induced DSBs. It must be reiterated that in this study, DSBs were induced at an IR dosage of 15 Gy which would cause substantial levels of DNA damage. And, there are no known mutations of *RAD51* in CAPAN-1 cells. Therefore, our results imply that in CAPAN-1/BRCA2 cells, and other cell types that are heterozygous for *BRCA2* mutations, the efficiency of DNA repair after *severe* DNA damage may not be considerably higher than in CAPAN-1 cells. In fact, this has been observed in other cell lines that either have or mimic heterozygous *BRCA2* phenotypes [31,47–49]. Moreover, individuals who are heterozygous carriers of *BRCA2* mutations could be at risk for loss of genomic integrity at a similar rate as those who are homozygous for *BRCA2* mutations or who exhibit loss of heterozygosity.

Evidence from prior studies strongly supports that BRCA2 transports RAD51 into the nucleus to repair DSBs caused by IR. However, mutations of *BRCA2* that adversely affect the NLSs—such as C-terminal truncations in CAPAN-1 cells—prevent BRCA2 from translocating into the nucleus in response to IR-induced DSBs. C-terminally truncated BRCA2 has the ability to bind RAD51 via its BRC repeats and potentially retain it in the cytoplasm, even when mutated cell lines have been restored with wildtype *BRCA2*, such as the CAPAN-1/BRCA2 cell line. As previously described, the model which proposes that RAD51 relies on BRCA2 for nuclear translocation in response to IR-induced DNA damage, is heavily based on the observation that RAD51 has no known NLSs [30] and cell lines having C-terminally truncated BRCA2 primarily exhibit cytoplasmic localization of RAD51 after induction of DSBs by IR exposure [22,23]. Furthermore, an additional model states that BRCA2 may hold RAD51 in a sequestered state in the cytoplasm until DNA damage, and then proceeds with mobilization of RAD51 to sites of DNA damage [50]. Collectively, these models support the role of the BRC repeat region of BRCA2 potentially binding and sequestering RAD51 in the cytoplasm of C-terminally truncated BRCA2 mutants. We plan to further examine the dynamics of the RAD51-BRCA2 interaction after IR-induced DSBs, by determining the upstream signaling pathways that specifically induce the interaction between the two proteins and provoke their mobilization into the nucleus. Our future studies will provide even greater incite into the molecular mechanisms involved in the development of breast carcinomas that are the result of *BRCA2* mutations.

Acknowledgments

This work was supported in part by NIH grants K01 CA96944 (ETB), CA85269 (JTH) and MUSC-Hollings Cancer Center Seed Funding.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001;94:153–156. [PubMed: 11668491]
2. Sharan SK, Morimatsu M, Albrecht U, Lim DS, Regel E, Dinh C, Sands A, Eichele G, Hasty P, Bradley A. Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. *Nature* 1997;386:804–810. [PubMed: 9126738]
3. Yuan SS, Lee SY, Chen G, Song M, Tomlinson GE, Lee EY. BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex in vivo. *Cancer Res* 1999;59:3547–3551. [PubMed: 10446958]
4. Ogawa T, Yu X, Shinohara A, Egelman EH. Similarity of the yeast RAD51 filament to the bacterial RecA filament. *Science* 1993;259:1896–1899. [PubMed: 8456314]
5. Benson FE, Stasiak A, West SC. Purification and characterization of the human Rad51 protein, an analogue of E. coli RecA. *Embo J* 1994;13:5764–5771. [PubMed: 7988572]
6. Derbyshire MK, Epstein LH, Young CS, Munz PL, Fishel R. Nonhomologous recombination in human cells. *Mol Cell Biol* 1994;14:156–169. [PubMed: 8264583]
7. Jackson SP, Jeggo PA. DNA double-strand break repair and V(D)J recombination: involvement of DNA-PK. *Trends Biochem Sci* 1995;20:412–415. [PubMed: 8533154]
8. Roth DB, Porter TN, Wilson JH. Mechanisms of nonhomologous recombination in mammalian cells. *Mol Cell Biol* 1985;5:2599–2607. [PubMed: 3016509]
9. Roth DB, Wilson JH. Nonhomologous recombination in mammalian cells: role for short sequence homologies in the joining reaction. *Mol Cell Biol* 1986;6:4295–4304. [PubMed: 3025650]
10. Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, Yamaguchi-Iwai Y, Shinohara A, Takeda S. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *Embo J* 1998;17:5497–5508. [PubMed: 9736627]
11. Johnson RD, Jasin M. Sister chromatid gene conversion is a prominent double-strand break repair pathway in mammalian cells. *Embo J* 2000;19:3398–3407. [PubMed: 10880452]
12. Taki T, Ohnishi T, Yamamoto A, Hiraga S, Arita N, Izumoto S, Hayakawa T, Morita T. Antisense inhibition of the RAD51 enhances radiosensitivity. *Biochem Biophys Res Commun* 1996;223:434–438. [PubMed: 8670299]
13. Sonoda E, Sasaki MS, Buerstedde JM, Bezzubova O, Shinohara A, Ogawa H, Takata M, Yamaguchi-Iwai Y, Takeda S. Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. *Embo J* 1998;17:598–608. [PubMed: 9430650]
14. Tsuzuki T, Fujii Y, Sakumi K, Tominaga Y, Nakao K, Sekiguchi M, Matsushiro A, Yoshimura Y, Morita T. Targeted disruption of the Rad51 gene leads to lethality in embryonic mice. *Proc Natl Acad Sci U S A* 1996;93:6236–6240. [PubMed: 8692798]
15. Mizuta R, LaSalle JM, Cheng HL, Shinohara A, Ogawa H, Copeland N, Jenkins NA, Lalonde M, Alt FW. RAB22 and RAB163/mouse BRCA2: proteins that specifically interact with the RAD51 protein. *Proc Natl Acad Sci U S A* 1997;94:6927–6932. [PubMed: 9192668]
16. Wong AK, Pero R, Ormonde PA, Tavtigian SV, Bartel PL. RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. *J Biol Chem* 1997;272:31941–31944. [PubMed: 9405383]
17. Chen PL, Chen CF, Chen Y, Xiao J, Sharp ZD, Lee WH. The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment. *Proc Natl Acad Sci U S A* 1998;95:5287–5292. [PubMed: 9560268]
18. Marmorstein LY, Ouchi T, Aaronson SA. The BRCA2 gene product functionally interacts with p53 and RAD51. *Proc Natl Acad Sci U S A* 1998;95:13869–13874. [PubMed: 9811893]
19. Aihara H, Ito Y, Kurumizaka H, Yokoyama S, Shibata T. The N-terminal domain of the human Rad51 protein binds DNA: structure and a DNA binding surface as revealed by NMR. *J Mol Biol* 1999;290:495–504. [PubMed: 10390347]
20. Matsuo Y, Sakane I, Takizawa Y, Takahashi M, Kurumizaka H. Roles of the human Rad51 L1 and L2 loops in DNA binding. *Febs J* 2006;273:3148–3159. [PubMed: 16780572]
21. Bignell G, Micklem G, Stratton MR, Ashworth A, Wooster R. The BRC repeats are conserved in mammalian BRCA2 proteins. *Hum Mol Genet* 1997;6:53–58. [PubMed: 9002670]

22. Spain BH, Larson CJ, Shihabuddin LS, Gage FH, Verma IM. Truncated BRCA2 is cytoplasmic: implications for cancer-linked mutations. *Proc Natl Acad Sci U S A* 1999;96:13920–13925. [PubMed: 10570174]
23. Davies AA, Masson JY, McIlwraith MJ, Stasiak AZ, Stasiak A, Venkitaraman AR, West SC. Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. *Mol Cell* 2001;7:273–282. [PubMed: 11239456]
24. Stark JM, Pierce AJ, Oh J, Pastink A, Jasin M. Genetic steps of mammalian homologous repair with distinct mutagenic consequences. *Mol Cell Biol* 2004;24:9305–9316. [PubMed: 15485900]
25. Pellegrini L, Yu DS, Lo T, Anand S, Lee M, Blundell TL, Venkitaraman AR. Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. *Nature* 2002;420:287–293. [PubMed: 12442171]
26. Shin DS, Pellegrini L, Daniels DS, Yelent B, Craig L, Bates D, Yu DS, Shivji MK, Hitomi C, Arvai AS, Volkmann N, Tsuruta H, Blundell TL, Venkitaraman AR, Tainer JA. Full-length archaeal Rad51 structure and mutants: mechanisms for RAD51 assembly and control by BRCA2. *Embo J* 2003;22:4566–4576. [PubMed: 12941707]
27. Yu VP, Koehler M, Steinlein C, Schmid M, Hanakahi LA, van Gool AJ, West SC, Venkitaraman AR. Gross chromosomal rearrangements and genetic exchange between nonhomologous chromosomes following BRCA2 inactivation. *Genes Dev* 2000;14:1400–1406. [PubMed: 10837032]
28. Yang H, Jeffrey PD, Miller J, Kinnucan E, Sun Y, Thoma NH, Zheng N, Chen PL, Lee WH, Pavletich NP. BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure. *Science* 2002;297:1837–1848. [PubMed: 12228710]
29. Yano K, Morotomi K, Saito H, Kato M, Matsuo F, Miki Y. Nuclear localization signals of the BRCA2 protein. *Biochem Biophys Res Commun* 2000;270:171–175. [PubMed: 10733923]
30. Boulikas T. Nuclear import of DNA repair proteins. *Anticancer Res* 1997;17:843–863. [PubMed: 9137418]
31. Arnold K, Kim MK, Frerk K, Edler L, Savelyeva L, Schmezer P, Wiedemeyer R. Lower level of BRCA2 protein in heterozygous mutation carriers is correlated with an increase in DNA double strand breaks and an impaired DSB repair. *Cancer Lett* 2006;243:90–100. [PubMed: 16448746]
32. Goggins M, Schutte M, Lu J, Moskaluk CA, Weinstein CL, Petersen GM, Yeo CJ, Jackson CE, Lynch HT, Hruban RH, Kern SE. Germline BRCA2 gene mutations in patients with apparently sporadic pancreatic carcinomas. *Cancer Res* 1996;56:5360–5364. [PubMed: 8968085]
33. Ozcelik H, Schmocker B, Di Nicola N, Shi XH, Langer B, Moore M, Taylor BR, Narod SA, Darlington G, Andrulis IL, Gallinger S, Redston M. Germline BRCA2 6174delT mutations in Ashkenazi Jewish pancreatic cancer patients. *Nat Genet* 1997;16:17–18. [PubMed: 9140390]
34. Oddoux C, Struwing JP, Clayton CM, Neuhausen S, Brody LC, Kaback M, Haas B, Norton L, Borgen P, Jhanwar S, Goldgar D, Ostrer H, Offit K. The carrier frequency of the BRCA2 6174delT mutation among Ashkenazi Jewish individuals is approximately 1%. *Nat Genet* 1996;14:188–190. [PubMed: 8841192]
35. Neuhausen S, Gilewski T, Norton L, Tran T, McGuire P, Swensen J, Hampel H, Borgen P, Brown K, Skolnick M, Shattuck-Eidens D, Jhanwar S, Goldgar D, Offit K. Recurrent BRCA2 6174delT mutations in Ashkenazi Jewish women affected by breast cancer. *Nat Genet* 1996;13:126–128. [PubMed: 8673092]
36. Roa BB, Boyd AA, Volcik K, Richards CS. Ashkenazi Jewish population frequencies for common mutations in BRCA1 and BRCA2. *Nat Genet* 1996;14:185–187. [PubMed: 8841191]
37. Abeliovich D, Kaduri L, Lerer I, Weinberg N, Amir G, Sagi M, Zlotogora J, Heching N, Peretz T. The founder mutations 185delAG and 5382insC in BRCA1 and 6174delT in BRCA2 appear in 60% of ovarian cancer and 30% of early-onset breast cancer patients among Ashkenazi women. *Am J Hum Genet* 1997;60:505–514. [PubMed: 9042909]
38. Levy-Lahad E, Catane R, Eisenberg S, Kaufman B, Hornreich G, Lishinsky E, Shohat M, Weber BL, Beller U, Lahad A, Halle D. Founder BRCA1 and BRCA2 mutations in Ashkenazi Jews in Israel: frequency and differential penetrance in ovarian cancer and in breast-ovarian cancer families. *Am J Hum Genet* 1997;60:1059–1067. [PubMed: 9150153]
39. Yoneda Y, Semba T, Kaneda Y, Noble RL, Matsuoka Y, Kurihara T, Okada Y, Imamoto N. A long synthetic peptide containing a nuclear localization signal and its flanking sequences of SV40 T-

- antigen directs the transport of IgM into the nucleus efficiently. *Exp Cell Res* 1992;201:313–320. [PubMed: 1322314]
40. Brown ET, Fuller GM. Detection of a complex that associates with the Bbeta fibrinogen G-455-A polymorphism. *Blood* 1998;92:3286–3293. [PubMed: 9787165]
41. Hao CM, Redha R, Morrow J, Breyer MD. Peroxisome proliferator-activated receptor delta activation promotes cell survival following hypertonic stress. *J Biol Chem* 2002;277:21341–21345. [PubMed: 11927585]
42. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 1998;273:5858–5868. [PubMed: 9488723]
43. Rogakou EP, Boon C, Redon C, Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J Cell Biol* 1999;146:905–916. [PubMed: 10477747]
44. Morimatsu M, Donoho G, Hasty P. Cells deleted for Brca2 COOH terminus exhibit hypersensitivity to gamma-radiation and premature senescence. *Cancer Res* 1998;58:3441–3447. [PubMed: 9699678]
45. Abbott DW, Freeman ML, Holt JT. Double-strand break repair deficiency and radiation sensitivity in BRCA2 mutant cancer cells. *J Natl Cancer Inst* 1998;90:978–985. [PubMed: 9665145]
46. Kowalczykowski SC. Cancer: catalyst of a catalyst. *Nature* 2005;433:591–592. [PubMed: 15703734]
47. Tannenbaum B, Mofunanya T, Schoenfeld AR. DNA damage repair is unaffected by mimicked heterozygous levels of BRCA2 in HT-29 cells. *Int J Biol Sci* 2007;3:402–407. [PubMed: 17940634]
48. Warren M, Lord CJ, Masabanda J, Griffin D, Ashworth A. Phenotypic effects of heterozygosity for a BRCA2 mutation. *Hum Mol Genet* 2003;12:2645–2656. [PubMed: 12928478]
49. Wiegant WW, Overmeer RM, Godthelp BC, van Buul PP, Zdzienicka MZ. Chinese hamster cell mutant, V-C8, a model for analysis of Brca2 function. *Mutat Res* 2006;600:79–88. [PubMed: 16643964]
50. Yu DS, Sonoda E, Takeda S, Huang CL, Pellegrini L, Blundell TL, Venkitaraman AR. Dynamic control of Rad51 recombinase by self-association and interaction with BRCA2. *Mol Cell* 2003;12:1029–1041. [PubMed: 14580352]

Figure 1. Schematic diagram of the human RAD51 protein

RAD51 has 339 amino acids. Amino acids 1–95 comprise the N-terminal domain, which as a HHH sequence (amino acids 58–77). The conserved ATPase domain is located within amino acids 119–306. And, amino acids 307–339 comprise the C-terminal domain. The region between amino acids 98–339 is where RAD51 interacts with BRCA2, and is highly conserved.

Figure 2. Schematic diagram of the human BRCA2 protein

The mid-portion of BRCA2 has eight highly conserved amino acid residues that are the BRC repeats, which facilitate one of two sites for RAD51 binding (blue line/arrow). The CTD (C-terminal domain) of BRCA2 contains the domains for DNA repair, which are the helical, tower, OB1, OB2, and OB3 domains (location is shown by brown line/arrow). The CTD also contains the protein's two nuclear localization signals (red rectangles/arrow) and the second RAD51 binding site (blue line/arrow). The approximate location of the 6174delT mutation in CAPAN-1 cells is shown (green arrow) which causes a frameshift that introduces a stop codon (white dotted line) which prematurely truncates BRCA2.

Figure 3. Expression of recombinant wildtype BRCA2 in stably transfected CAPAN-1 cells

Lane 1: Absence of 480-kDa wildtype BRCA2 expression in CAPAN-1 cells. Lane 2: Expression of 480-kDa wildtype BRCA2 protein after stable transfection of CAPAN-1 cells (denoted as CAPAN-1/BRCA2). Lane 3: Expression of endogenous wildtype BRCA2 in HeLa cells for comparison of recombinant BRCA2 expression in CAPAN-1/BRCA2. Samples were resolved on 6% SDS-PAGE gels.

Figure 4. Expression of GFP-NLS-RAD51 protein

(A). Schematic diagram of the GFP-tagged RAD51 protein containing a nuclear localization signal (NLS). (B). Expression of the ~75 kDa GFP-NLS-RAD51 protein, Mock transfected (M), 2 days (2), 4 days (4), and 6 days (6) post-transfection. Samples were resolved on 12% SDS-PAGE gels.

A

CAPAN-1

CON

6 hr

GFP-NLS-RAD51

GFP-NLS-RAD51
+
TOPRO-3

B

CAPAN-1/BRCA2

CON

6 hr

GFP-NLS-RAD51

GFP-NLS-RAD51
+
TOPRO-3

Figure 5. GFP-NLS-RAD51 protein is unable to enter the nuclei of CAPAN-1 cells after IR-induced DNA damage

Six hours after irradiation (15 Gy) the sub-cellular localization of GFP-NLS-RAD51 (green) was detected by immunofluorescence analyses in (A) CAPAN-1, (B) CAPAN-1/BRCA2, and (C). HeLa cells. Nuclei were identified by TOPRO-3 staining (blue). Controls (CON) were non-irradiated samples.

Figure 6. Subcellular fractionation of GFP-NLS-RAD51

The subcellular localization of GFP-NLS-RAD51 is shown 6 hours post-IR (15 Gy) by subcellular fractionation (*N*, nuclear extract; *C*, cytoplasmic extract). Lanes 1,2: CAPAN-1 lysate; lanes 3,4: CAPAN-1/BRCA2 lysate; lanes 5,6: HeLa lysate. Lamin B antibody was used as a nuclear protein marker to confirm separation of nuclear and cytoplasmic fractions. Samples were resolved on 12% SDS-PAGE gels.

Figure 7. Identification of DSBs by γ -H2AX staining

(A). DSBs were detected at 6 hours and 24 hours post-irradiation (15 Gy) by staining of γ -H2AX foci (pink) in CAPAN-1, CAPAN-1/BRCA2 and WI38 cells. Nuclei were identified by TOPRO-3 counterstaining (blue). Controls (CON) were non-irradiated samples. (B). Quantification of the number of γ -H2AX foci remaining 24 hours post-irradiation was 35.0 ± 12.0 for CAPAN-1, 30.0 ± 12.0 for CAPAN-1/BRCA2, and 19.0 ± 5.0 for WI38 cells.

Figure 8. Cell viability after irradiation

Cell survival was assessed two weeks after irradiation (15 Gy) in CAPAN-1, CAPAN-1/BRCA2, and HeLa cells by a crystal violet/methanol solution assay. Four individual experiments were performed with triplicate samples in 6 well plates. Controls were non-irradiated cells harvested as normal for two weeks along with the irradiated samples. The percentage of surviving cells (two weeks after irradiation) compared with controls was quantified, which was 64.5 ± 2.12 for CAPAN-1, 71.0 ± 1.41 for CAPAN-1/BRCA2, and 95.0 ± 7.07 for HeLa. * denotes that the percent viability of HeLa cells is significantly higher than CAPAN-1/BRCA2 cells ($p < 0.05$).