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14. ABSTRACT

The significant mortality associated with metastatic breast cancer suggests a clear need to improve current therapeutic strategies. Breast tumor cells with defective BRCA1 are believed to be more sensitive to the DNA-damage based therapies. We propose that the aberrant expression (gain or loss) or activity of protein(s) in BRCA1-associated pathways will lead to a BRCA1 null-like phenotype and DNA damage hypersensitivity in breast cancer cells. Previous studies have demonstrated that BRCC36 is over-expressed in the vast majority of invasive breast cancers and that depletion of BRCC36 sensitizes breast cancer cells to IR via the BRCA1 DNA repair pathway. Therefore, we are examining if abrogation of BRCC36 will sensitize breast tumors to the DNA-damage based therapies. We have tested a cancer cell-specific or "smart" therapeutic approach utilizing the conjugation of anti-HER2 antibodies and protamine to deliver BRCC36 siRNA to HER2 positive breast cancer cells. This approach should lead to improving the targeting of breast tumor cells while reducing non-specific toxicity.

15. SUBJECT TERMS

BRCA1, BRCC36, breast cancer, DNA repair, and target therapies

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INTRODUCTION

Breast Cancer

Breast cancer is the most common cancer affecting women, with a lifetime risk of ~10% by the age of 80 years. In the United States 207,090 new breast cancer cases and about 40,000 breast cancer related deaths are estimated for 2010 (American Cancer Society, 2010). It is estimated that 13.2 % of all American women (1 in 8) will develop breast cancer and 3.0 % will die from this disease (Ries, et al., 2008). Despite the advances in treatment and early detection, the mortality rate from breast cancer in women only decreased by 2.2% per year between 1990 and 2002 (Jemal, et al., 2008). Current estimates from previous studies (Collaborative Group on Hormonal Factors in Breast Cancer, 2001; Margolin, et al., 2006) indicate that family history is associated with 15% to 20% of breast cancer cases in the U.S. *BRCA1* (OMIM: 113705) and *BRCA2* (OMIM: 600185) are the two most important breast cancer susceptibility genes and deleterious mutations in these two genes account for about only 15-30% of familiar breast cancer (King, et al., 2003; Walsh, et al., 2006). Therefore, most familial aggregation of breast cancer remains unexplained. Furthermore, the majority of tumors occur in women with little or no family history, and because somatically acquired *BRCA1* mutations in these tumors have very rarely been reported, the contribution of BRCA1 to sporadic breast cancer is still poorly defined. We hypothesize that functional inactivation of the normal BRCA1 cellular activity may be vastly underestimated and that loss of BRCA1 activity is critical in the development of breast cancer.

BRCA1-Associated Proteins: Functional Modifiers of BRCA1

BRCA1 is one of the most intensively studied genes in breast cancer research field. The *BRCA1* gene encodes for a 220 kDa nuclear phosphoprotein that has been suggested to play a role in maintaining genomic stability and to act as a tumor suppressor (Miki, et al., 1994). Findings from mouse studies demonstrated that *Brca1* knockout mice, generated by removal of exon 11, have a defective G_2/M cell cycle checkpoint and extensive chromosomal abnormalities, and developed mammary tumors (Xu, et al., 2001; Xu, et al., 1999). Furthermore, recent findings of phenotypic overlap between *BRCA1*-associated and sporadic basal-like breast cancers suggest that the latter might have an underlying defect in BRCA1-related pathways (Foulkes, et al., 2003; Lakhani, et al., 2005; Sioud, 2006; Turner, et al., 2007). Therefore, dysfunction of other genes, which code for proteins in complementary pathways as BRCA1, could be important in the pathogenesis of a significant proportion of sporadic breast cancers.

BRCA1 interacts directly or indirectly with other tumor suppressors (such as p53 and BRCA2), DNA damage sensors (such as RAD51, RAD50, MRE11 and NBS1), ubiquitin ligase partners (BARD1, BRCC45, BRCC36), and signal transducers (such as p21 and cyclin B) to form multi-subunit protein complexes, such as BASC (BRCA1-associated genome surveillance complex) and BRCC [**Figure 1,** (Chen, et al., 2006a)]. These multi-subunit protein complexes are involved in a broad range of biological processes including DNA repair, cell cycle control, ubiquitination, and chromatin remodeling (Chen, et al., 2010). However, the number of these BRCA1-associated protein complexes and their complexity have yet to be fully elucidated. Thus, much of the current scientific effort involving BRCA1 centers around the biochemical functions of these BRCA1-associated protein complexes (Dong, et al., 2003; Wang, et al., 2007). The majority of BRCA1 functional studies have focused on its potential role in DNA damage responses. The

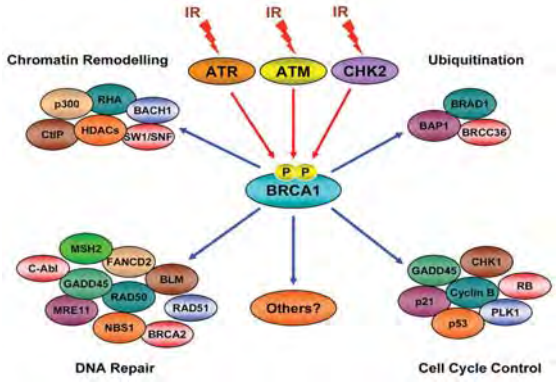

Figure 1. BRCA1-associated Protein Network. BRCA1 interacts with a number of proteins to form multi-subunit protein complexes. BRCA1-associated protein complexes are involved in DNA repair, protein ubiquitination, cell-cycle-checkpoint control, and chromatin remodeling (Chen et al, 2006).

implication that BRCA1 is a direct component of DNA damage response pathways comes from evidence of its interactions with BRCA2 and RAD51. The protein complex comprised of BRCA1, BRCA2, and RAD51 has been shown to activate DNA double-strand break (DSB) repair and to initiate homologous recombination, an observation which links the maintenance of genomic integrity to tumor suppression (Chen, et al., 1999). In addition, the BRCA1-associated MRE11-RAD50-NBS1 (MRN) complex has recently been demonstrated to activate CHEK2 downstream from ATM in response to replication-mediated DSBs (Takemura, et al., 2006). Disruption of any of these pathways may contribute to increased genomic instability and potentially sensitize cells to the effects of ionizing radiation (IR), specifically through the induction of cellular apoptosis. BRCA1 also interacts with a number of proteins and displays significant ubiquitin ligase activities. Importantly, deleterious mutations affecting the BRCA1 RING-finger domain, which were found in clinical specimens, abolish the ubiquitin ligase activity of BRCA1 (Ruffner, et al., 2001; Wu, et al., 1996). These findings support a relationship between the ligase activity of BRCA1 and the predisposition to breast cancer. In addition, BRCA1 has also been reported to interact with the RNA Pol II holoenzyme (Scully, et al., 1997). Two recent reports have suggested that BRCA1 and BARD1 may be involved in the degradation of RNA polymerase II complex and siRNA-mediated knockdown of BRCA1 and BARD1 results in stabilization of RNAP II in the cells following UV exposure (Kleiman, et al., 2005; Starita, et al., 2005). These studies reported that BRCA1/BARD1 appears to initiate the degradation of stalled RNAP II and thus disrupts the coupled transcription by inhibiting RNA processing machinery in cells exposed to DNA damage.

BRCA1-Associated Proteins as Potential Targets of Breast Cancer Therapies

In the last several decades, efforts have been made toward understanding the mechanism of the response to both cytotoxic chemotherapy and radiation therapy in the treatment of breast cancer. Because of the important role of BRCA1 in DNA repair, breast tumor cells with defective BRCA1 are believed to be more sensitive to DNA-damage based therapies (Farmer, et al., 2005). This speculation is supported by the recent development of the inhibitors of poly (ADP-ribose)-polymerase-1 (PARP). The PARP enzyme is involved in base excision repair which is a critical pathway in the repair of DNA single-strand breaks (Ratnam and Low, 2007; Schreiber, et al., 2002). Farmer and colleagues have shown that defects in BRCA1 or BRCA2 profoundly sensitize cells to the inhibition of PARP enzymatic activity, resulting in chromosomal instability, cell cycle arrest, and subsequent apoptosis (Farmer, et al., 2005). PARP inhibitors are currently in clinical trials of patients with breast cancer or other malignancies who are *BRCA1* or *BRCA2* mutation carriers. Two phase I studies have shown that AZD2281 (AstraZeneca, UK), a potent orally active PARP inhibitor, is well tolerated and leads to significant PARP inhibition in patients carrying *BRCA1* and *BRCA2* mutations with breast or ovarian cancer (Fong, et al., 2008; Yap, et al., 2007). Importantly, clinical responses have been observed in all cohorts evaluated thus far, and future phase II studies are planned (Fong, et al., 2008; Yap, et al., 2007). Findings from these recent studies further suggest that the design of novel therapies, which inhibit components of particular DNA repair pathways, may provide effective and more tolerable therapeutic options for breast cancer patients with BRCA1 defects. There is growing evidence suggesting that disruption of the BRCA1-associated complexes either through mutations or the aberrant expression of a key member(s) of these multiprotein complexes may result in loss of normal BRCA1 activity (Chen, et al., 2006b; McCarthy, et al., 2003; Wang, et al., 2007; Wu, et al., 2007). Therefore, these BRCA1-associated proteins are likely to be involved in tumorigenesis and are potential therapeutic targets.

Summary

Since tumor cells in general are genomically unstable and have defects in DNA damage responses, it has been proposed that targeting DNA repair pathways may lead to a therapeutic index in tumor cells over "normal" cells. Previous studies have demonstrated that BRCC36 is over-expressed in the vast majority of invasive breast cancers and that depletion of BRCC36 sensitizes breast cancer cells to IR via the BRCA1 DNA repair pathway. Therefore, we hypothesize that abrogation of BRCC36 will sensitize breast tumors to the DNA-damage based therapies.

BODY

Task 1: To establish siRNAs targeting BRCC36 specifically to HER-2 positive breast cancer cells in vitro using C6.5db-protamine/siRNA conjugates.

BRCC36 siRNA delivery via anti-HER2 antibodies and protamine conjugates sensertizes the HER2-positive cells to IR.

Considerable research efforts have been focused on applying siRNA to human disease therapy, including cancer therapy. A novel method for *in vivo* delivery of siRNAs to specific cell types has recently been developed, and it takes advantages of the nucleic-acid binding properties of protamine as well as the specificity of fragment antibodies (Fab) (Sioud, 2006). This method shows that systemically administered siRNA can be targeted to cells that express a specific cell-surface receptor (Peer, et al., 2007; Song, et al., 2005). Compared to other siRNA delivery systems, antibody-based siRNA targeting provides many advantages (Sioud, 2006), including that (i) the siRNA is stable in the blood with a prolonged half-life; (ii) the siRNA can be transported across capillary endothelial walls; (iii) the siRNA can be specifically bound to the plasma membranes of target cells ("smart drug"); and (iv) the siRNAs can be efficiently delivered into the target cells through endocytosis. Here, we will apply a cancer cell-specific or "smart" therapeutic approach utilizing diabody-P/siRNA conjugates that should lead to an improvement in the targeting of breast tumor cells, while reducing non-specific toxicity.

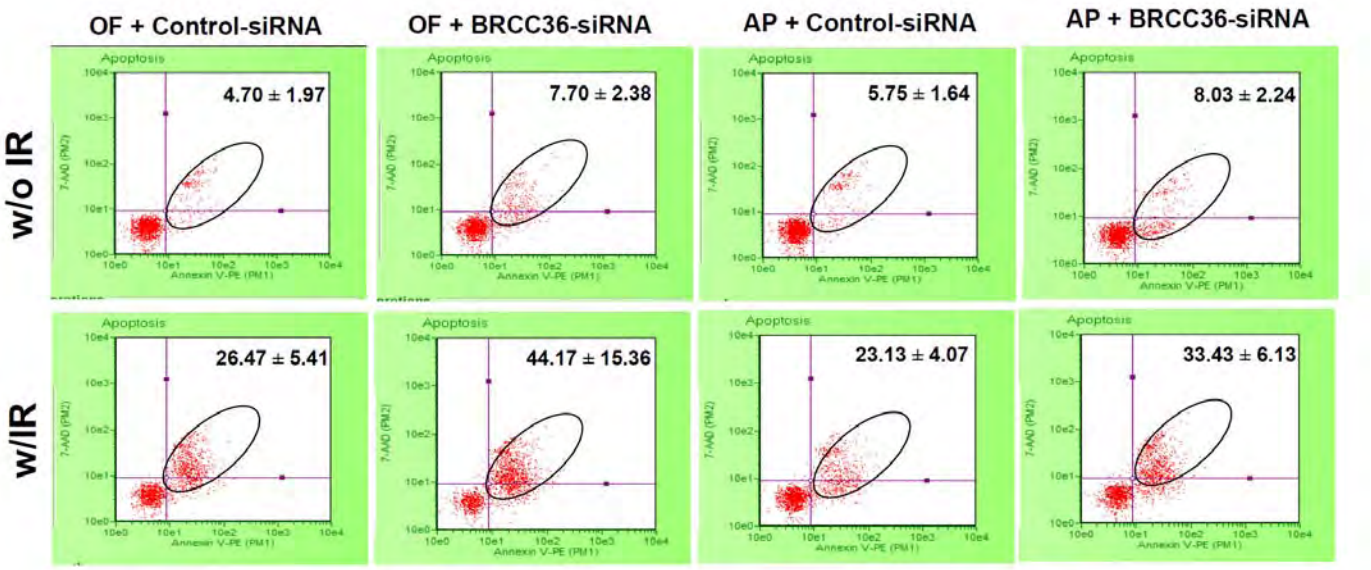

Figure 2. Depletion of BRCC36 by siRNA delivery via anti-HER2 antibodies and protamine conjugates enhances IR-induced apoptosis. SK-BR-3 cells were transfected with control- or *BRCC36*-siRNAs delivered via either oligofactamine or antibodies and protamine conjugates prior to IR exposure. The proportion of apoptotic cells was measured following annexin V and 7-amino actinomycin D staining using a Guava Personal Cytometer (OF: oligofactamine or AP: antibodies and protamine conjugates).

In the previous report, we have shown that BRCC36 siRNA delivered via the conjugates of Herceptin and protamine peptide enables knock-down of the level of BRCC36 in the HER2-positive breast cancer cells. We next access if BRCC36 siRNA delivery via antibody and protamine conjagates will achieve samiliar effects as the siRNA delivery via oligofactemine to enhance the IR-induced apoptosis. For the siRNA delivery studies, SK-BR-3 cells were plated at a density $5x10^3$ cells/cm^2. After reaching 30% to 40% confluence, cells were transfected with *BRCC36* siRNA using either oligofectamine or antibody/protamine

conjugates in OPTI reduced serum medium. Following depletion of *BRCC36* via siRNA, cells received 4 -Gy total IR utilizing a Cesium 137 Irradiator (Model 81-14R). Cells were then cultured for an additional 72 hours prior to harvesting and were examined for DNA damage-induced cell apoptosis via Annexin V and 7-amino actinomycin D staining. The proportion of apoptotic cells was determined utilizing a Guava Personal Cytometer (Guava Technologies) according to the manufacturer's instructions. As shown in **Figure 2**, no significant difference in the fraction of cells undergoing apoptosis in mock treated, siRNA-control transfected, or siRNA-*BRCC36* transfected cells was observed in the absence of IR, indicating that depletion of BRCC36 alone is not lethal. However, when combined with *BRCC36* knock-down delivered by either oligofactamine or antibodies and protamine conjugates, IR exposure led to a significant increase in the percentage of SK-BR-3 cells that undergo apoptosis (44.2% ± 15.4% or 33.4% ± 6.1%) when compared to the siRNA control group (26.5% ± 5.4%, or 23.1 ± 4.1, $p<0.05$), respectively. As a result, *BRCC36* siRNA delivery via anti-HER2 antibodies and protamine conjugates appears to sensitize the HER2-positive cells to IR.

Exogenous BRCC36 expression stimulates colony formation

Since BRCC36 is over-expressed in majority of breast tumor, we evaluated whether exogenous expression of BRCC36 alone could transform MCF-10F, a non-tumorigenic mammary epithelial cell line, initially by assessing anchorage-independent growth. As shown in **Figure 3**, FLAG-tagged BRCC36-overexpressing MCF-10F cells formed more colonies (>30 cells after 3 weeks) in soft agar, as compared to vector-control MCF-10F cells. The breast tumor cell line, MCF7 was included as a positive control. After quantification, BRCC36-overexpressing MCF-10F cells are ~5-times more efficient in colony formation than vector-control cells (18.5 ± 2.1 vs. 3.5 ± 0.7, $p< 0.01$) (**Figure 3**). Therefore, over-expression of BRCC36 leads to cellular transformation.

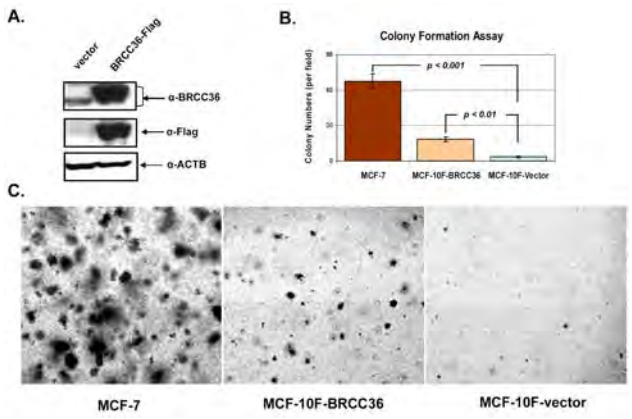

Figure 3. Exogenous overexpression of BRCC36 stimulates colony formation in MCF-10F cells. (**A**) The expression of BRCC36 in MCF-10F cells transfected with either a control vector (pFLAG-CMV2-5a) or a BRCC36-expression vector (pFLAG-CMV2-BRCC36) was determined by immunoblotting with either anti-BRC36 or -FLAG antibodies. (**B**) 5 x 10^5 of MCF-7 (positive control), BRCC36 transfected- or vector control MCF-10 cells were plated in soft agar (6-well plates). After 3 weeks, images from 5 independent fields of each well were taken and colonies containing >30 cells were scored. The numbers of colonies shown are the means ± standard deviations (SD) of triplicate results from two independent experiments. (**C**) Representative images of colony formation in soft agar by MCF-7, BRCC36-transfected and vector control MCF-10F cells.

Identify the novel substrates of BRCC complex

Much of the current scientific effort involving BRCA1 is being directed to defining the biochemical functions of BRCA1 and its interacting-proteins. Using a combination of affinity purification of anti-FLAG and mass spectrometric sequencing, we have reported a novel multiprotein complex, termed BRCC (BRCA1/2 Containing Complex), which contains seven polypeptides including BRCA1, BRCA2, BARD1 and RAD51 (Dong, et al., 2003). We first reported that BRCC was an E3 ubiquitin ligase complex

exhibiting activities in the E2-dependent ubiquitination of the tumor suppressor p53. In this multiprotein complex, three proteins, referred to as BRCC36, BRCC45, and BRCC120 have been found to be associated with BRCA1 and BRCA2. Among these novel BRCA1-associated proteins, *BRCC36* is located at the Xq28 locus, a chromosomal break point in patients with prolymphocytic T-cell leukemia (T-PLL) (Fisch, et al., 1993). The chromosomal break occurred in two different introns of *BCC36* and the fusion transcripts were expressed at high levels in the leukaemic cells from T-PLL patients (Fisch, et al., 1993). The *BRCC36/C6.1A* gene is highly conserved between species and bears sequence homology with both human Poh1/Pad1 subunit of the 26S proteasome and subunit 5 (Jab1) of the COP9 signalosome (Dong, et al., 2003). Despite its homology to POH1 and Jab1, BRCC36 represents a distinct branch in the evolutionary tree. We have demonstrated that depletion of BRCC36 resulted in increased sensitivity in breast cancer cells to IR and disruption of IR-induced BRCA1 phosphorylation and nuclear foci formation (Chen, et al., 2006b). RNA interference of BRCC36 also resulted in a defect in G2/M checkpoint arrest (Dong, et al., 2003). Cancer-associated truncations in BRCA1 have been found to reduce the association of BRCC36 with the BRCC complex. In addition, our previous study has shown that a recombinant four-subunit BRCC complex containing BRCA1-BARD1-BRCC45-BRCC36 revealed an enhanced E3 ubiquitin ligase activity compared to that of BRCA1-BARD1 heterodimer. Therefore, BRCC36 appears to be a positive regulator of BRCA1/BARD1 E3 ligase activity. Furthermore, BRCC36 has recently been reported to also be present in a novel protein complex, BRCA1-RAP80-ABRAXAS-BRCC36 (BRCA1 A complex), and displays deubiquitinating (DUB) activities (Sobhian, et al., 2007; Wang and Elledge, 2007). The recruitment of BRCC36 to this complex is via the interaction between the coiled-coil domains of BRCC36 and ABRAXAS. BRCC36 plays an important role in BRCA1 A complex, and it is essential for the localization of RAP80, ABRAXAS, and BRCA1 to sites of DNA damage. These findings suggest that the balance between synthesis and turnover of certain polyubiquiquitinated structure by BRCA1-BARD1 E3 and BRCC36 DUB activities, respectively, could be dynamic and mediated by other protein partners (e.g., BRCC45 or RAP80) in the same complexes.

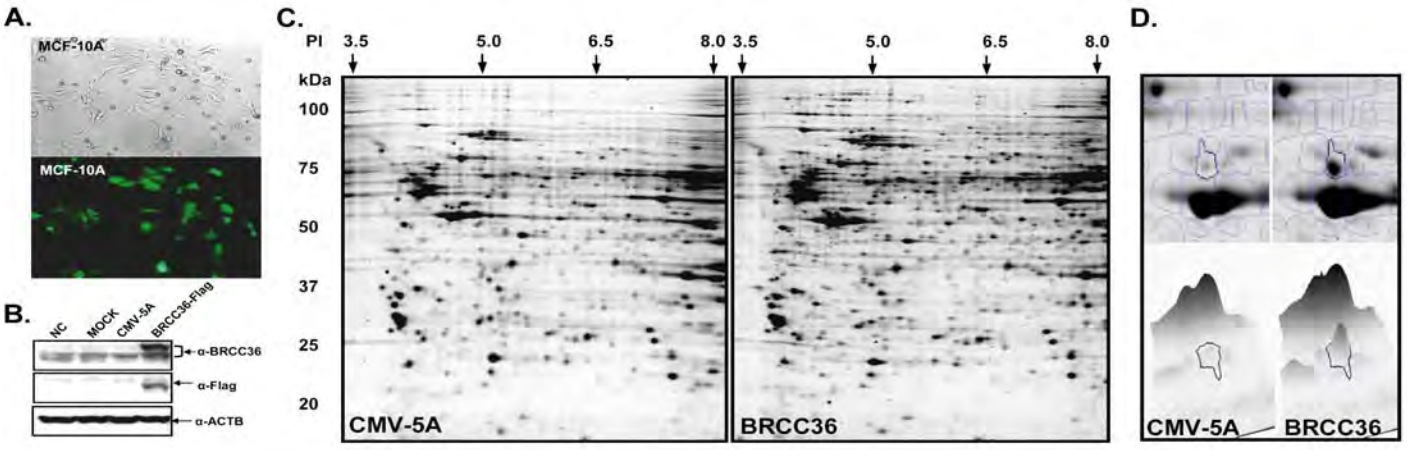

Figure 4. Two-dimensional Gels Analyses between BRCC36-overexpression MCF-10A and Parental Lines. (**A**) One million MCF-10A cells were electroporated with an eGFP construct and either 2 μg of a control plasmid (i.e., pFLAG-CMV2-5a) or a pFLAG-CMV2-BRCC36 plasmid via Nucleofector kit V (Amaxa). Transfection efficiency was determined to be ~60% using eGFP as a marker. (**B**) The expression of BRCC36 and Flag were determined by immunoblotting with specific antibodies. Protein loading levels were evaluated with anti-β-actin antibody. (**C**) Two-dimensional gels were run using protein lysates from MCF-10A cell transfected with CMV-5a empty vector (left) or BRCC36-Flag (right). Molecular weight markers are indicated in kDa and approximate isoelectric point is indicated across the top of the gels. (**D**) An example of 2D-analyses using Progenesis software: one protein (MW: ~60; PI: ~4.5) has been found to be overexpressed more than 3-fold in MCF-10A cell transfected with BRCC36 in comparison to the cell transfected with control vector.

In this capacity, BRCC36 has the potenti al to interact with nu merous protein substrates and subsequently affects their stabilization, potenti ally explaining the possible oncoge nic and tum or suppressor phenotypes associated with overexpression as observed in breast tu mors or m utations found in hereditary diseases. Therefore, we have studied to determ ine if BRCC36 can mediate protein stability u sing 2D protein gels. In this study, MCF-10A ce lls were transfected w ith a GFP reporter plasm id and either BRCC36-flag or the control vector. Transfection effi ciency was determ ined by eGFP and BRCC36 expression were determ ined by immunoblotting (**Figures 4A** and **B**). Forty-eight hours after transfection, cells were harvested and lysed in 2D-buffer. Fifty m icrogram protein from BRCC36 or control vector transfected cell lysates was separated by IEF over a pH 3–10 range followed by gradient SDS- PAGE. After fixing and staining, the gels were scanned with ProXPRESS™ Proteom ic Im aging System . Exam ple im ages from one of t he triplicate comparisons are shown in **Figure 4C**. After analyzing by Progenesis (Nonlinear Dynam ics, Inc.) (**Figure 4D**), the den sity of 22 sp ots was iden tified to b e increased at least 3-fold in cel ls transfected with BRCC36 alone than those transfected with empty vector, while 9 spots were 3-fold lower in BRCC36-transfected cells ($p<0.05$). Since BRCC36 displays DUB activ ity, it is expected that the substrates of BRCC36 m ay be up-regulated when BRCC36 is over-ex pressed. However, based on our previous stud y and recen t advance (Dong, et al., 2003; Sobhian, et al., 2007), the DUB activities of BRCC36 m ay be dependent on which BRCA1 complexes BRCC36 participating in, and ther efore, different BRCA1 com plexes m ay either stabilize or prom ote degrad ation of their various substrates. Thes e prelim inary 2D-gel analyses would suggest that both scenarios m ight be in effect following exogenous BRCC36 expression. Although 2D-ge l protein analysis is limited to more abundant proteins, we are able to resolve 1000s of i ndividual proteins and their isoform s. LC-M S/MS is being used to identify the protein spots consiste ntly alte red in repea ted experiments through our Biotechnology Core Facility at FC CC to identify potential substrates of the BRCC complex or BRCC36.

Task 2: To Determine if Abrogation of BRCC36 by C6.5-P siRNA Delivery can Sensitize Breast Tumors to DNA Damage-Based Therapies in Mouse Xenograft Models.

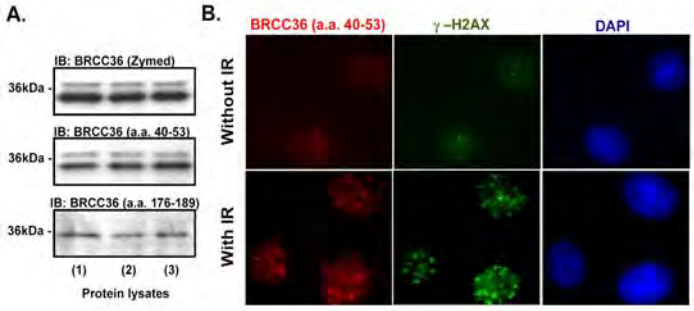

Figure 5. Characterization of New BRCC36 Antibodies. (**A**) Immunoblot analysis of BRCC36 in three independent lymphoblastoid cell lines using the Zymed polyclonal antibody (upper panel), antibodies derived against a.a. 40-53 (middle panel), and antibodies derived against a.a. 176-189 (lower panel). (**B**) IR-induced BRCC36 nuclear foci formation detected with antibodies derived against a.a. 40-53. MCF-10F cells were treated with 4-Gy IR and allowed to recover for 2 hours before performing immunoflorescence staining as described in our previous studies (Chen, et al, 2006). MCF-10F cells were also co-stained with γ-H2AX and DAPI for the nuclear foci positive control and nuclear visualization, respectively.

<u>Characterization of new polyclonal anti-BRCC36 antibodies</u>

In the previous study, we have i dentified a *BRCC36* fr ameshift mutation (c.880insGGGd up148) in a *BRCA1*/2 mutation-negative but *CHEK2*-c.1100delC positive fa mily with st rong indication of hereditary breast cancer history. This fram eshift mutation is predic ted to result in expression of a m utant protein [i.e., 72 new residues beginning at 294 and a stop codon at residue 366 (p.Arg294T hrfsX73)]. Since the commercially available antibodies (Zymed) is against to the C-terminus antibody and is not able to detect the mutant BRCC36. We have developed two new polyclo nal antibodies specific to different epitopes of BRCC36. Our antibodies derived against the N-term inus (a.a. 40-53) (NP_001018065, NCBI) detect both isoforms of BRCC36, while antibodies derived against am ino acids encoded by sequences in exons 7 and 8, i.e., a.a. 1 76-189 (NP_001018065, NCBI), uniquely de tect isoform 2 of BRCC36. In addition, the

commercial BRCC36 antibodies do not work for immunofluorescence (IF)-based assays (**data not shown**). As shown in **Figure 5**, BRCC36 forms discrete nuclear foci in MCF-10F cells following exposure to IR. These data are consistent with a recent study reporting the nuclear foci formation of exogenous HA tagged-BRCC36 in U2OS cells in response to DNA damage (Sobhian, et al., 2007). These findings continue to indicate that BRCC36 plays an important role in DNA damage/repair pathways.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that BRCC36 siRNA delivery via anti-HER2 antibodies and protamine conjugates sensitized the HER2-positive cells to IR.
- Established FLAG-tagged BRCC36-overexpressing MCF-10F and MCF-10A cell lines.
- Demonstrated exogenous BRCC36 expression stimulates colony formation, and this result supports the previous findings that BRCC36 over-expressed in the majority of breast tumors.
- Identified the novel substrates of BRCC complex using 2D-LC/MS.
- Demonstrated that BRCC36 may either stabilize or promote degradation of various substrates, dependent on which BRCA1 complexes BRCC36 participating in.
- Characterized two new polyclonal anti-BRCC36 antibodies, which is able to detect the mutant BRCC36.
- Demonstrated that BRCC36 forms discrete nuclear foci in MCF-10F cells following exposure to IR.

REPORTABLE OUTCOMES

Abstracts

1 **Chen, X.**, Klimowicz, C., Vanderveer, L., Weaver, J., Amin, N., Ouellette, T., Liao, C., Daly, M.B., Nathanson, K.L., Godwin, A.K. A BRCA1 5'non-coding variant influences breast cancer risk among African-Americans; In: Annual meeting of American Association of Cancer Research, 2009.

2. **Chen, X.,** Schaeffer A., Devarajan, K., Liao, C., Zhou, Y., Slater, C.M., Vanderveer, L., Conroy, J., Godwin, A.K. Defects in BRCA1 contribute to global differential allele-specific expression; In: 34 th San Antonio Breast Cancer Symposium, 2010.

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1. **Chen, X.,** Kistler, J.L., Godwin, A.K. BRCA1-associated proteins: novel targets for breast cancer radiation therapy, pp 121-141. In: DeFrina, R.H., editor. Chapter 4, Aggressive Breast Cancer. Hauppauge, NY: Nova Science Publishers, Inc., 2010.

CONCLUSIONS

The significant mortality associated with metastatic breast cancer suggests a clear need to improve current therapeutic strategies. Previous studies have demonstrated that BRCC36 is over-expressed in the vast majority of invasive breast cancers and that depletion of BRCC36 sensitizes breast cancer cells to IR via the BRCA1 DNA repair pathway. Therefore, we are examining if abrogation of BRCC36 will sensitize breast tumors to the DNA-damage based therapies. We have tested a cancer cell-specific or "smart" therapeutic approach utilizing the conjugation of anti-HER2 antibodies and protamine to deliver BRCC36 siRNA to HER2 positive breast cancer cells. This approach should lead to improving the targeting of breast tumor cells while reducing non-specific toxicity.

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APPENDICES

Abstracts

1 **Chen, X.**, Klimowicz, C., Vanderveer, L., Weaver, J., Amin, N., Ouellette, T., Liao, C., Daly, M.B., Nathanson, K.L., Godwin, A.K. A BRCA1 5'non-coding variant influences breast cancer risk among African-Americans; In: Annual meeting of American Association of Cancer Research, 2009.

2. **Chen, X.,** Schaeffer A., Devarajan, K., Liao, C., Zhou, Y., Slater, C.M., Vanderveer, L., Conroy, J., Godwin, A.K. Defects in BRCA1 contribute to global differential allele-specific expression; In: 34 th San Antonio Breast Cancer Symposium, 2010.

Publications

1. **Chen, X.,** Kistler, J.L., Godwin, A.K. BRCA1-associated proteins: novel targets for breast cancer radiation therapy, pp 121-141. In: DeFrina, R.H., editor. Chapter 4, Aggressive Breast Cancer. Hauppauge, NY: Nova Science Publishers, Inc., 2010.

A *BRCA1 5'non-coding variant* influences breast cancer risk among *African-Americans*

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Mutations in *BRCA1* and *BRCA2* have been implicated in the development of breast and ovarian cancer. Mutations (e.g., frameshifts, nonsense, splice site, large deletions/insertions) in the coding regions of these genes are associated with ~30% of hereditary breast cancer, a proportion which is less than originally estimated. Recent studies have suggested that the alterations in non-coding DNA within or near promoter regions are able to mediate the transcription factor binding and thus disrupt the expression of genes such as EGFR and BCL3. Therefore, we hypothesized that sequence variants in conserved, but non-coding regions of *BRCA1* and/or *BRCA2* may contribute to increase breast cancer risk. To test this hypothesis, we first identified 3 and 9 evolutionarily conserved regions in the 5' non-coding of *BRCA1* and *BRCA2*, respectively by conducting both comparative genomic analysis and phylogenetic footprinting of transcription factor binding site analysis. We then sequenced these conserved regions in 75 women (Caucasian: 58, African-American: 17) with a personal and family history of breast cancer. Four sequence variants that would be predicted to alter transcription factor binding were detected. One novel mutation, *BRCA1*-IVS1-85del5, was identified in the affected probands from two unrelated African-American breast cancer-prone families. No affected probands from 58 unrelated Caucasian breast cancer-prone families carries this variant. To determine the functional significance of this variant, we first employed a luciferase-reporter assay and demonstrated that RNA and protein expression from the *BRCA1*-IVS1-85del5 mutant allele is significantly decrease as compared to the wild-type allele *($p<0.05$)*. Results from electrophoretic mobility shift assays further confirmed that this sequence variant disrupts the binding of at least two transcription factors to this DNA site. To assess whether this variant may be associated with breast cancer risk, an African-American population-based screen of 263 women with breast cancer and 215 cancer-free controls unselected for family history identified a potential risk (O.R. = 1.91, 95% CI: 0.49-7.47) associated with the *BRCA1*-IVS-85del5 allele. In summary, this study provides evidence that a novel mutation in a non-coding region of *BRCA1* can alter its expression by blocking the transcription factor binding and appears to increase breast cancer risk. These findings will ultimately help to better define the role of sequence variants within highly conserved non-coding regions of *BRCA1* as they relate to cancer susceptibility. This work was supported in part by the Eileen Stein-Jacoby Fund; grants from the Congressionally Directed Medical Research Programs, Department of Defense, W81XWH-07-1-0685 and W81XWH-08-1-0361.

Defects in *BRCA1* contribute to global differential allele-specific expression.

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Differential allele-specific expression (DASE) has been shown to contribute to phenotypic variability in humans and more recently to the pathogenesis of cancer. DASE is associated with X-chromosome inactivation and genomic imprinting and is relatively common among non-imprinted autosomal genes. The DASE phenotype can also be transmitted by Mendelian inheritance. We have previously reported that nonsense-mediated mRNA decay (NMD) of mutant *BRCA1* as well as other epigenetic mechanisms can lead to DASE of *BRCA1* and enhanced susceptibility to breast cancer. BRCA1 has been implicated in many cellular processes including DNA repair, cell-cycle-checkpoint control, protein ubiquitination, and chromatin remodeling. Importantly, cells carrying a deleterious *BRCA1* mutation exhibit increased genome instability, therefore, we hypothesize that defects in *BRCA1* lead not only to DASE of itself, but increase genome-wide DASE and thus contribute to increased breast cancer susceptibility. To test this hypothesis, we employed a genome-wide ASE assay (Illumina Human Omni1-Quad BeadChip) using primary mammary epithelial cells [3 *BRCA1* wild-type vs. 3 *BRCA1* mutant carrying (2800delAA, 4154delA and R1751X)]. As shown in **Table 1**, cells carrying a *BRCA1* mutation had significantly more DASE events as compared to wild-type cells ($P<10^{-7}$). In addition, we identified 351 genes demonstrating DASE that were unique to the *BRCA1* mutant cells. The cellular functions of these genes are wide-ranging, including DNA repair, cell cycle control, lipid metabolism and protein degradation. In summary, this study provides the first evidence that mutant *BRCA1* can lead to global DASE, which in turn may contribute to the development of breast cancer in mutation carriers. This work was supported in part by the Eileen Stein-Jacoby Fund and a grant from the Congressionally Directed Medical Research Programs, Department of Defense, W81XWH-08-1-0361 (XC).

Table 1. *BRCA1* mutations increase the global DASE in PMECs

	w/o DASE†	w/ DASE	OR (95%CI)	*P*
BRCA1(-)	16494	2615	1.8 (1.70, 1.90)	$<10^{-7}$
BRCA1(+)	14989	4274		

†: A DASE event is defined as that the Log_2 ratio of allele-specific expression level of one gene is more than 1 or less than -1, i.e. the expression level from one allele is at least 50% less than the level of another allele.

Charpter 4. BRCA1-Associated Proteins: Novel Targets for Breast Cancer Radiation Therapy

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Abstract
Tumor cells, in general, are genom ically unstab le and have defects in DNA repair pathways, which subsequently hinder DNA dam age responses . It has been proposed that therapeutic strategies specifically targe ting DNA repair pathway proteins m ay lead to an increased therapeutic index in tum or cells versus norm al cells. The BR CA1 pathways are know n to play a critical role in DNA repair ; thus, breast tum ors with def ects in pro teins assoc iated with th e BRCA1 pathways are believed to be m ore se nsitive to DNA da mage-based therapies. BRCA1 can interact directly or indire ctly with other tum or suppressors, DNA da mage sensors, ubiquitin ligase partn ers, and s ignal transducers to form multi-subun it protein co mplexes. These pro tein complexes are involved in a broad range of biological processes including DNA repair, cell cycle control, ubiquitin ation, and chrom atin rem odeling. Growing evidence suggests that m utation and/or aberrant expression of one or more key members of the BRCA1- associated multi-protein complexes may result in loss of norm al BRCA1 activity and disruption of the BRCA1 pathways. These BRCA1-associated proteins are potential m odifiers o f BR CA1 functions and, therefore, potential targets for sensitizing breast cancer cells to radiation therapy.

Keywords: *BRCA1, breast cancer, radiation resistance, DNA repair, cell cycle, ubiquitination, chromatin remodeling.*

Introduction

Breast cancer is the most common cancer affecting women, with an estimated lifetime risk of approximately 10% by 80 years of age. In the United States, it is estimated that approximately 182,450 new cases of female breast cancer will be diagnosed and greater than 40,000 breast cancer-related deaths will occur in 2008 (Jemal *et al.*, 2008). Approximately 13.2% of all American women (1 in 8) are expected to develop breast cancer sometime during their lifetime and 3.0% will subsequently die from the disease (Ries *et al.*, 2008). Despite advances in treatment and early detection, the breast cancer mortality rate among women in the United States decreased by only 2.2% per year between 1990 and 2002 (Jemal *et al.*, 2008).

Importantly, estimates from previous studies (Collaborative Group on Hormonal Factors in Breast Cancer, 2001; Margolin *et al.*, 2006) indicate that family history is associated with 15% to 20% of breast cancer cases in the United States. The *BRCA1* gene (OMIM: 113705) is one of the most intensively studied breast cancer susceptibility genes and has a profound role in breast cancer etiology owing to its involvement in several important cellular processes. Deleterious mutations in *BRCA1* are thought to account for approximately 10% to 20 % of hereditary breast cancers (Bove *et al.*, 2002; King *et al.*, 2003; Walsh *et al.*, 2006). Among its many biological functions, the BRCA1 protein is involved in DNA repair. Because DNA repair pathways and associated proteins are targeted by radiation therapy, there is considerable interest in the development of novel therapeutic strategies to sensitize breast cancer patients with mutations in *BRCA1* to radiation therapy. This article will provide an overview of BRCA1 and its associated proteins with a particular emphasis on their role in DNA repair, as well as summarize current paradigms for breast cancer treatment with a focus on the development of new strategies to exploit the role of BRCA1 associated proteins and improve the efficacy of breast cancer radiation therapy.

Radiation Therapy for Breast Cancer

Current treatment paradigms for breast cancer are complex and reflect the considerable heterogeneity of the disease (NCCN breast cancer treatment guidelines 2008). Treatment options for noninvasive breast cancers range from observation alone, to breast-conserving lumpectomy with or without breast radiation, to total mastectomy depending on the tumor biology and individual patient risk. Radiation therapy is also used in the adjuvant setting and in combination with lumpectomy for locoregional treatment of early stage invasive breast cancers. Systemic treatment for advanced or metastatic breast cancer includes chemotherapy, endocrine therapy, and newer types of targeted therapeutic agents (e.g., targeted monoclonal antibodies and tyrosine kinase inhibitors). Radiation therapy has been a treatment modality for breast cancer patients for more than 100 years and, over the last 3 decades, has become a critical component of successful treatment strategies for breast cancer. An increasing role for radiation therapy developed in the early 1970s, when Fletcher documented that radiation therapy was instrumental in decreasing local recurrences (Fletcher, 1972). In particular, supraclavicular metastases were reduced from 20% to 25% to only 1.3% to 3% with the addition of ionizing radiation (IR). Radiation therapy has also been utilized to treat patients with tumors that have

undergone total mastectomy resulting in a reduction in local recurrences by greater than two-thirds (Fletcher, 1972). This early work led to an expanded role for radiation therapy in breast cancer.

The emergence of radiation therapy to the forefront of modern breast cancer treatment lies in its application in breast conservation therapy. Current NCCN treatment guidelines support the preferred use of breast conservation therapy (i.e., lumpectomy with or without breast radiation) as a breast treatment for the majority of women with early stage breast cancers (i.e., ductal carcinoma in situ, stage I and II breast cancers). Evidence suggests that the addition of radiation therapy may significantly reduce recurrence in this patient population. Landmark studies on the necessity of radiation therapy in breast conservation therapy came from Fisher and colleagues, as a part of a clinical trial conducted by the National Surgical Adjuvant Breast and Bowel Project (NSABP; Protocol B-06) that showed lumpectomy with radiation therapy had much lower recurrence rates than lumpectomy alone (10% versus 35%; $P < 0.001$) after 12 years of follow-up (Fisher *et al.*, 1995). This observation has been further validated by an extensive meta-analysis that supported improved local control with the addition of radiation therapy (Fisher *et al.*, 2002; Veronesi *et al.*, 2002). Recently, clinical research has examined the possible survival benefits of radiation therapy in breast cancer. The Early Breast Cancer Trialists' Collaborative Group (EBCTG) examined 78 trials involving more than 42,000 patients with breast cancer (Clarke *et al.*, 2006). In the analyses of trials directly comparing patients receiving radiation therapy versus those not receiving radiation therapy, a clear reduction in local recurrences occurred in the radiotherapy group, including patients undergoing mastectomy or breast conservation therapy (Clarke *et al.*, 2006). Interestingly, there was also a notable improvement in survival among patients treated with radiotherapy. In fact, patients receiving radiotherapy for their breast cancer had a nearly 6% reduction in their 15-year breast cancer mortality risk and a 4% to 5% reduction in overall mortality (Clarke *et al.*, 2006). These findings support the contribution of radiotherapy to both the reduction of local recurrences and in 15-year overall mortality rates. Researchers have noted that breast cancer recurrences in the non-irradiated breast often occur within 3 years of initial diagnosis (Kurtz *et al.*, 1989). In comparison, local recurrences in irradiated breast tissue occur much later, with the risk increasing with time (7% risk at 5 years, 14% risk at 10 years, and 20% risk at 20 years) (Smith *et al.*, 2000). Thus, despite the benefits of radiation therapy in the treatment of breast cancer, patients continue to develop local recurrences in the targeted breast. The persistent recurrence of breast cancers following radiation therapy in multiple patient settings has prompted significant research efforts, particularly in understanding the etiology of radioresistant breast tumors and subsequent development of novel treatment paradigms to overcome this resistance.

Despite the benefits of radiation therapy in the treatment of breast cancer, patients continue to develop local recurrences in the targeted breast. Researchers have noted that breast cancer recurrences in the non-irradiated breast often occur within 3 years of initial diagnosis (Kurtz et al., 1989). In comparison, local recurrences in irradiated breast tissue occur much later, with the risk increasing with time (7% risk at 5 years, 14% risk at 10 years, and 20% risk at 20 years) (Smith et al., 2000). It is these recurrences that have spurred research into both breast cancer recurrences and the

possibility of radio-resistant breast tumors. One of the major radio-resistance mechanisms is related to a score of genes which are involved in the repair of DNA damage by radiation.

BRCA1 in Resistance to Breast Cancer Radiation Therapy

The clinical benefit of radiation therapy can be attributed to its mechanism of DNA damage and subsequent activation of apoptosis pathways. The damage caused by IR activates specific DNA damage cell cycle checkpoints, which leads to induction of various DNA repair pathways. The central component of these pathways is the ATM/CHEK2 kinase, which is activated upon DNA damage and subsequently phosphorylates multiple proteins, including BRCA1 (Canman *et al.*, 1998; Cortez *et al.*, 1999; Lee *et al.*, 2000). In response to DNA damage induced by IR, BRCA1 is phosphorylated at specific tyrosine residues by ATM (the gene mutated in ataxia telangiectasia), CHEK2 (the human homologue of yeast checkpoint protein kinase [hCds1]), or by the ATM-related kinase, ATR (Cortez *et al.*, 1999; Lee *et al.*, 2000; Tibbetts *et al.*, 1999). This phosphorylation, which occurs in a region containing clusters of serine-glutamine residues, has been shown to be functionally important using mouse models. In these studies, a mutated form of *BRCA1* lacking these phosphorylation sites failed to rescue radiation hypersensitivity when introduced into *BRCA1*-deficient cells (Cortez *et al.*, 1999). In addition, phosphorylation by ATM/CHEK2 following DNA damage is critical for the recruitment of BRCA1 to both DNA repair and chromatin remodeling protein complexes (Zhong *et al.*, 1999).

BRCA1 has been implicated in normal cellular processes, including DNA fidelity and damage repair, and has therefore been examined as having a possible role in the radioresistance of breast tumors. However, the specific role of BRCA1 in radioresistant breast cancer remains somewhat unclear. In vitro studies (Abbott *et al.*, 1999; Foray *et al.*, 1999; Mamon *et al.*, 2003; Ruffner *et al.*, 2001; Shen *et al.*, 1998) have demonstrated an increased sensitivity to IR when *BRCA1* is mutated in human breast cancer cell lines. However, clinical observations in breast cancer patients fail to reliably support these in vitro findings (Baeyens *et al.*, 2004; Garcia-Higuera *et al.*, 2001; Leong *et al.*, 2000). One study (Kirova *et al.*, 2005) found that *BRCA1* mutation carriers exhibited increased sensitivity to radiation therapy as assessed by the reduced rate of breast cancer recurrence following breast conserving treatment; however, Pierce and colleagues (Pierce *et al.*, 2000) noted no significant differences in local recurrences between *BRCA1* mutation carriers and patients with sporadic forms of breast cancer in a multicenter study. Two additional human studies (Baeyens *et al.*, 2004; Leong *et al.*, 2000) indicated that mutations in *BRCA1* may not account for clinical radiation hypersensitivity. These conflicting findings pose the question of whether *BRCA1* mutations will indeed increase the sensitivities of tumor cells to the radiation-based therapies. Therefore, the role of BRCA1 and its influence on tumor cell sensitivity to radiation in vitro and in vivo will require further investigation.

Role of BRCA1 and Associated Proteins in Breast Cancer Etiology

Since its cloning and characterization in the mid-1990s (Miki *et al.*, 1994), BRCA1 has been implicated in many cellular processes including DNA repair, cell-cycle-checkpoint control, protein ubiquitination, and chromatin remodeling. Although mutations in *BRCA1* are known to contribute to the development of hereditary breast and

ovarian cancers, *BRCA1* mutations in sporadic breast cancers, which account for approximately 90% of all breast cancers, are surprisingly rare (Futreal *et al.*, 1994). In this aspect, various studies have indicated that loss of *BRCA1* expression through epigenetic mechanisms may contribute about 10% of sporadic breast cancer (Esteller *et al.*, 2000; Rio *et al.*, 1999; Yang *et al.*, 2001). In addition, accumulating evidence suggests that dysfunction of other genes, coding for proteins in pathways complementary to BRCA1, may be important in the pathogenesis of a significant proportion of sporadic, non-hereditary cancers. This hypothesis is supported by several lines of evidence, including phenotypic analyses of breast and ovarian tumors, as well as mechanistic studies of BRCA1-associated pathways (Farmer *et al.*, 2005; Jazaeri *et al.*, 2002).

BRCA1-Associated Proteins: Functional Modifiers of BRCA1 Activity

Due to its clinical significance, the *BRCA1* gene is one of the most intensively studied breast cancer susceptibility genes. The *BRCA1* gene encodes for a 220 kDa nuclear phosphoprotein that has been suggested to play a role in maintaining genomic stability and to act as a tumor suppressor. The BRCA1 protein interacts directly or indirectly with other tumor suppressor proteins (e.g., p53 and BRCA2), DNA damage sensors (e.g., RAD51, RAD50, MRE11 and NBS1), signal transducers (e.g., p21 and cyclin B), and ubiquitination proteins (e.g., BARD1, BRCC36, and RAP80) to form multi-subunit protein complexes (**Figure 1**), such as the BRCA1-associated genome surveillance complex (BASC) and the BRCA1 and BRCA2 containing complex (BRCC). Importantly, the proper formation of these multi-subunit protein complexes is critical in carrying out the multiple biological processes associated with BRCA1, including DNA repair, cell cycle control, chromatin remodeling, and ubiquitination.

The majority of BRCA1 functional studies have focused on its potential role in DNA damage responses. The implication that BRCA1 is a direct component of DNA damage response pathways comes from evidence of its interactions with BRCA2 and RAD51. The protein complex comprised of BRCA1, BRCA2, and RAD51 has been shown to activate DNA double-strand break (DSB) repair and to initiate homologous recombination, an observation which links the maintenance of genomic integrity to tumor suppression (Chen *et al.*, 1999). In addition, the BRCA1-associated MRE11-RAD50-NBS1 (MRN) complex has recently been demonstrated to activate CHEK2 downstream from ATM in response to replication-mediated DSBs (Takemura *et al.*, 2006). Disruption of any of these pathways may contribute to increased genomic instability and potentially sensitize cells to the effects of IR, specifically through the induction of cellular apoptosis.

The involvement of BRCA1 and its associated partners in normal DNA repair processes suggests that mutations in these tumor suppressor proteins would hinder DNA damage responses, predispose cells to additional accumulated mutations, and potentially contribute to subsequent malignant transformation. Importantly, compromised DNA repair mechanisms would also be expected to sensitize cells to the lethal effects of IR. Thus, while *BRCA1* mutations may play a profound role in breast cancer etiology, consequent disruption of normal DNA repair may actually be therapeutically exploited to increase clinical radiation hypersensitivity in breast cancer patients who are *BRCA1* mutation carriers.

BRCA1 has also been shown to play a role in cell cycle control. For example, BRCA1 stimulates expression of the cyclin–dependent kinase (CDK) inhibitor, p21, and to inhibit cell-cycle progression into the S-phase (Somasundaram *et al.*, 1997). In addition, research has shown that BRCA1 is not only essential for activating the CHEK1 kinase that regulates G2/M arrest induced by DNA damage, but also controls the expression, phosphorylation, and cellular localization of Cdc25C and Cdc2/cyclin B kinases (Yarden *et al.*, 2002). Therefore, BRCA1 appears to be involved in regulating the onset of mitosis. Furthermore, a mouse study demonstrated that *BRCA1* knockout mice, generated by removal of exon 11, have a defective G_2/M cell cycle checkpoint and extensive chromosomal abnormalities (Xu *et al.*, 1999). It is also reported that elimination of one *Tp53* allele (*BRCA1 exon11*-/-;*Tp53*+/-) rescued the embryonic lethality caused by the deletion of *BRCA1* exon 11 and restored normal mammary gland development (Xu *et al.*, 2001). However, most female mice homozygous for the *Brca1* exon 11 deletion and heterozygous for loss of the *Tp53* gene developed mammary tumors within 6 to 12 months. Importantly, the resulting tumors lose the remaining *Tp53* allele (Xu *et al.*, 2001). These findings indicated that the genetic interactions between Brca1 and p53 are associated with breast carcinogenesis.

BRCA1 and its associated protein have also been found to be involved in the process of chromatin remodeling. Wang and colleagues (Wang *et al.*, 2000) used immunoprecipitation and mass spectrometry to identify a large multi-subunit protein complex referred to as BASC (BRCA1-associated genome surveillance complex), which is comprised of ATM, BLM, MSH2, MSH6, MLH1, the RAD50-MRE11-NBS1 complex, and the RFC1-RFC2-RFC4 complex. Confocal microscopy demonstrated that BRCA1, BLM, and the RAD50-MRE11-NBS1 complex co-localized to large nuclear foci, and BASC has subsequently been shown to be involved in chromatin remodeling at sites of double-strand DNA breaks (Wang *et al.*, 2000). In addition, BRCA1 directly interacts with the brahma-related gene 1 (BRG1) subunit of SW1/SNF-associated complex which has been demonstrated to be involved in chromatin-remodeling (Bochar *et al.*, 2000). This finding links chromatin remodeling processes to breast cancer. Furthermore, the BRCT domain of BRCA1 has been reported to be associated with the histone deacetylases, HDAC1 and HDAC2 (Yarden and Brody, 1999). Collectively, these findings may help explain the involvement of BRCA1 in multiple, seemingly unrelated processes such as transcription and DNA repair.

BRCA1 also interacts with a number of proteins and displays significant ubiquitin ligase activities. Importantly, deleterious mutations affecting the BRCA1 RING-finger domain, found in clinical specimens, abolish the ubiquitin ligase activity of BRCA1 (Ruffner *et al.*, 2001; Wu *et al.*, 1996). These findings support a relationship between the ligase activity of BRCA1 and the predisposition to breast cancer. Using a combination of affinity purification of anti-FLAG and mass spectrometric sequencing, a multiprotein protein complex, termed BRCC (BRCA1/2 Containing Complex), which contains seven polypeptides including BRCA1, BRCA2, BARD1 and RAD51, has been identified (Dong *et al.*, 2003). BRCC is an E3 ubiquitin ligase complex exhibiting activities in the E2-dependent ubiquitination of the tumor suppressor p53 (Dong *et al.*, 2003). In this multiprotein complex, one protein, referred to as BRCC36, has been found to be directly interacted with BRCA1.

Cancer-causing truncations of BRCA1 have been found to abrogate the association of BRCC36 with BRCC (Dong *et al.*, 2003). We have also demonstrated that depletion of BRCC36 resulted in increased sensitivity in breast cancer cells to ionizing radiation (IR) and disruption of IR-induced BRCA1 phosphorylation and nuclear foci formation (Chen *et al.*, 2006). Previous study has shown that a recombinant four-subunit BRCC complex containing BRCA1-BARD1-BRCC45-BRCC36 revealed an enhanced E3 ubiquitin ligase activity compared to that of BRCA1-BARD1 heterodimer (Dong *et al.*, 2003). Furthermore, BRCC36 has recently been reported to also be present in a novel BRCA1-associated complex, BRCA1-BARD1-RAP80-Abraxas-BRCC36, which plays a role in recognizing DNA damage site (Wang *et al.*, 2007). These findings may suggest that the role of BRCC36 in DNA damage response could be dynamic and mediated by other protein partners (e.g., BRCC45, BRCC120, RAP80 or Abraxas) in the same complexes (**Figure 2**). In addition, BRCA1 has also been reported to interact with the RNA Pol II holoenzyme (Scully *et al.*, 1997). Two recent reports have suggested that BRCA1a nd BARD1 may be involved in the degradation of RNA polymerase II complex and siRNA-mediated knockdown of BRCA1 and BARD1 results in stabilization of RNAP II in the cells following UV exposure (Kleiman *et al.*, 2005; Starita *et al.*, 2005). These studies reported that BRCA1/BARD1 appears to initiate the degradation of stalled RNAP II and thus disrupts the coupled transcription by inhibiting RNA processing machinery in cells exposed to DNA damage. At present, the known substrates that are polyubiquitinated by the BRCA1-BARD1 ubiquitin ligase are very limited and include RNA polymerase II, nucleophosmin/B23, and p53 (Dong *et al.*, 2003; Kleiman *et al.*, 2005; Sato *et al.*, 2004; Starita *et al.*, 2005).

BRCA1-associated Proteins as Potential Targets of Breast Cancer Therapies

In the last several decades, efforts have been made toward understanding the mechanism of response to both cytotoxic chemotherapy and radiation therapy in the treatment of breast cancer. Because tumor cells are typically genomically unstable with dysfunctional DNA damage responses, it has been proposed that targeting DNA repair pathways may lead to an increased therapeutic index in tumor cells versus normal cells. The involvement of BRCA1 and its associated partners in normal DNA repair processes suggests that mutations in these tumor suppressor proteins would hinder DNA damage responses, predispose cells to additional accumulated mutations, and potentially contribute to subsequent malignant transformation. Importantly, compromised DNA repair mechanisms would also be expected to sensitize cells to the lethal effects of IR. Thus, while *BRCA1* mutations may play a profound role in breast cancer etiology, consequent disruption of normal DNA repair may actually be therapeutically exploited to increase clinical radiation hypersensitivity in breast cancer patients who are *BRCA1* mutation carriers.

This speculation is supported by the recent development of the inhibitors of poly (ADP-ribose)-polymerase-1 (PARP). The PARP enzyme is involved in base excision repair which is critical pathway in the repair of DNA single-strand breaks (Ratnam and Low, 2007; Schreiber *et al.*, 2002). Farmer and colleagues have shown that defects in BRCA1 or BRCA2 profoundly sensitize cells to the inhibition of PARP enzymatic activity, resulting in chromosomal instability, cell cycle arrest, and subsequent apoptosis (Farmer *et al.*, 2005). PARP inhibitors are

currently in clinical trials of patients with breast cancer or other malignancies who are *BRCA1* or *BRCA2* mutation carriers. Two phase I studies have shown that AZD2281 (AstraZeneca, UK), a potent orally active PARP inhibitor, is well tolerated and leads to significant PARP inhibition in patients carrying *BRCA1* and *BRCA2* mutations with breast or ovarian cancer (Fong *et al.*, 2008; Yap *et al.*, 2007). Importantly, clinical responses have been observed in all cohorts evaluated thus far, and future phase II studies are planned (Fong *et al.*, 2008; Yap *et al.*, 2007). Findings from these recent studies further suggest that the design of novel therapies, which inhibit components of particular DNA repair pathways, may provide effective and more tolerable therapeutic options for breast cancer patients with BRCA1 defects.

In vitro studies have demonstrated that breast cancer cells expressing mutated *BRCA1* have increased sensitivity to IR (Kennedy *et al.*, 2004; Powell, 2005). Notably, mutations in *BRCA1* itself may not be the only reason for loss of the encoded protein's activity. There is growing evidence that disruption of the BRCA1-associated multi-protein complexes, either through mutations or the aberrant expression of a key member(s) of these complexes, may result in loss of normal BRCA1 activity (Chen *et al.*, 2006; McCarthy *et al.*, 2003; Wang *et al.*, 2007; Wu *et al.*, 2007). In our own studies, we have tested the hypothesis that dysregulated expression (e.g., gain or loss) of protein(s) in BRCA1-associated pathways leads to a BRCA1 "null-like" phenotype and subsequent DNA damage hypersensitivity in breast cancer cells (Chen *et al.*, 2006). As shown in **Figure 3**, BRCA1 and p53 are phosphorylated by the ATM kinase following IR. Depletion of the BRCA1-associated protein, BRCC36, prevents the phosphorylation of BRCA1 and disrupts BRCA1 nuclear foci formation following IR, an event that is associated with the induction of DNA repair. The proposed model illustrates that disruption of BRCA1 activation through depletion of BRCC36 may create an imbalance between the DNA repair and cell survival pathways and the apoptosis/cell death pathways following IR exposure. As a result, abrogation of BRCC36 sensitizes breast cancer cells to IR-induced apoptosis (Chen *et al.*, 2006).

This proposed mechanism is also supported by a number of studies that have demonstrated the impact of cellular resistance to IR upon manipulation of BRCA1-associated proteins, such as RAD51, MRE11, and NBS1 (**Table 1**) (Billecke *et al.*, 2002; Boulton *et al.*, 2004; Chinnaiyan *et al.*, 2005; Digweed *et al.*, 2002; Garcia-Higuera *et al.*, 2001; Houghtaling *et al.*, 2005; Kim *et al.*, 2007; Lio *et al.*, 2004; Liu *et al.*, 2007; Nakanishi *et al.*, 2002; Russell *et al.*, 2003; Sobhian *et al.*, 2007; Wang *et al.*, 2007; Yan *et al.*, 2008). In addition, because multiple genetic hits are necessary for tumorigenesis, individuals that carry defects in DNA damage repair/response genes are particularly cancer prone, due to the genetic instability and hypermutability of their cells (Deng, 2006; Jasin, 2002). Therefore, these BRCA1-associated proteins are likely to be involved in tumorigenesis and are potential therapeutic targets.

Summary

Since BRCA1 was cloned a decade ago, significant progress has been made in defining its biochemical and biological functions, as well as its role in breast and ovarian cancers. BRCA1 has been implicated in many cellular processes, including DNA repair, and protein ubiquitination. Because of the important role

of BRCA1 in DNA repair, breast tumors with defective BRCA1 are believed to be more sensitive to DNA damage-based therapies. Nevertheless, defects in BRCA1 itself may not be the only reason for the loss of its activity nor the increased sensitivity of tumor cells to DNA damage-based agents. A number of studies have demonstrated that manipulation of BRCA1-associated proteins, such as RAD51, MRE11, and NBS1, can impact cellular sensitivity to IR. BRCA1-associated proteins may, therefore, be considered as potential targets for breast cancer therapies. Despite a potentially significant role for BRCA1-associated protein complexes in modifying the activities of BRCA1, the total number of complexes and the identity and function of component proteins has yet to be fully elucidated. Thus, much of the scientific effort related to BRCA1 is currently directed at defining the biochemical functions of BRCA1 in association with these protein complexes.

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Table 1. Radiation Sensitivity Studies Related to BRCA1-associated Proteins

Protein name	BRCA1 pathway affected	*BRCA1* gene manipulation approach	Increased resistance to IR	Increased sensitivity to IR
FANCD2	DNA repair	Defected		Garcia-Higuera et al, 2001 Houghtaling et al, 2005
NBS1	DNA repair	Defected	Nakanishi et al, 2002	
MRE11 DNA	repair	Disrupted		Digweed et al, 2002
RAD51	DNA repair	Deficiency Blocking Overexpression	 Vispe et al, 1998	Lio et al, 2004 Russell et al, 2003
HDAC Chrom	atin remodeling	Blocking		Chinnaivan et al, 2005
RB	Cell cycle checkpoint control	Decreasing Defected		Carlson et al, 2000 Billecke et al, 2002
BARD1	Ubiquitination	Depleted		Boulton et al, 2004
BRCC36	Ubiquitination	Depleted		Dong et al., 2003 Chen et al, 2006
RAP80	Ubiquitination	Depleted		Sobhian, et al, 2007 Yan et al, 2008
CCDC98/Abraxas	Ubiquitination	Deplete		Wang et al, 2007 Kim et al, 2007 Liu et al, 2007

IR, ionizing radiation.

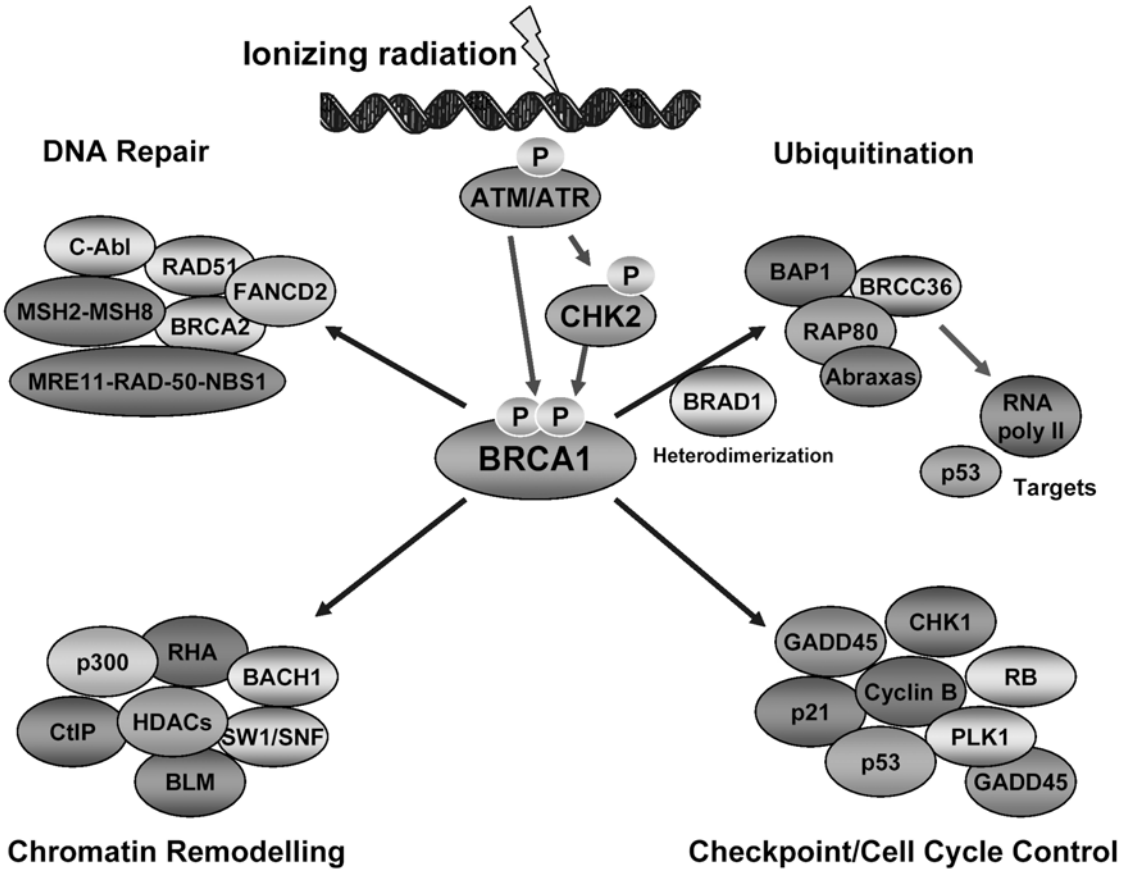

Figure 1. BRCA1-associated protein network. BRCA1 interacts with a number of proteins to form multi-subunit protein complexes, which are involved in DNA repair, cell cycle checkpoint control, protein ubiquitination, and chromatin remodeling.

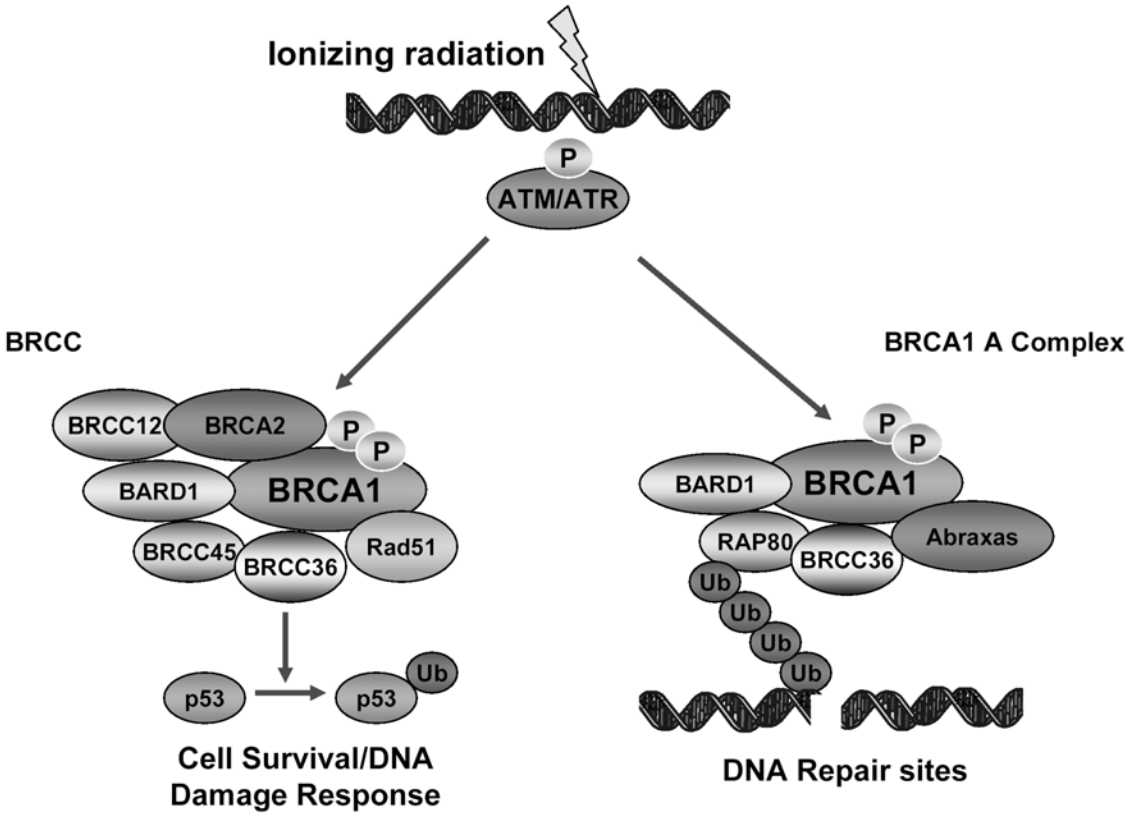

Figure 2. BRCC36 in different BRCA1-associated protein complexes (BRCC or BRCA1 A Complex, respectively). Previous study has shown that BRCC36 potentiates the E3 ubiquitin ligase activity of BRCA1-BARD1 heterodimer. Recently, BRCC36 has been reported to also be present in a novel BRCA1-associated complex, BRCA1-BARD1-RAP80-Abraxas-BRCC36, which plays a role in recognizing DSB site.

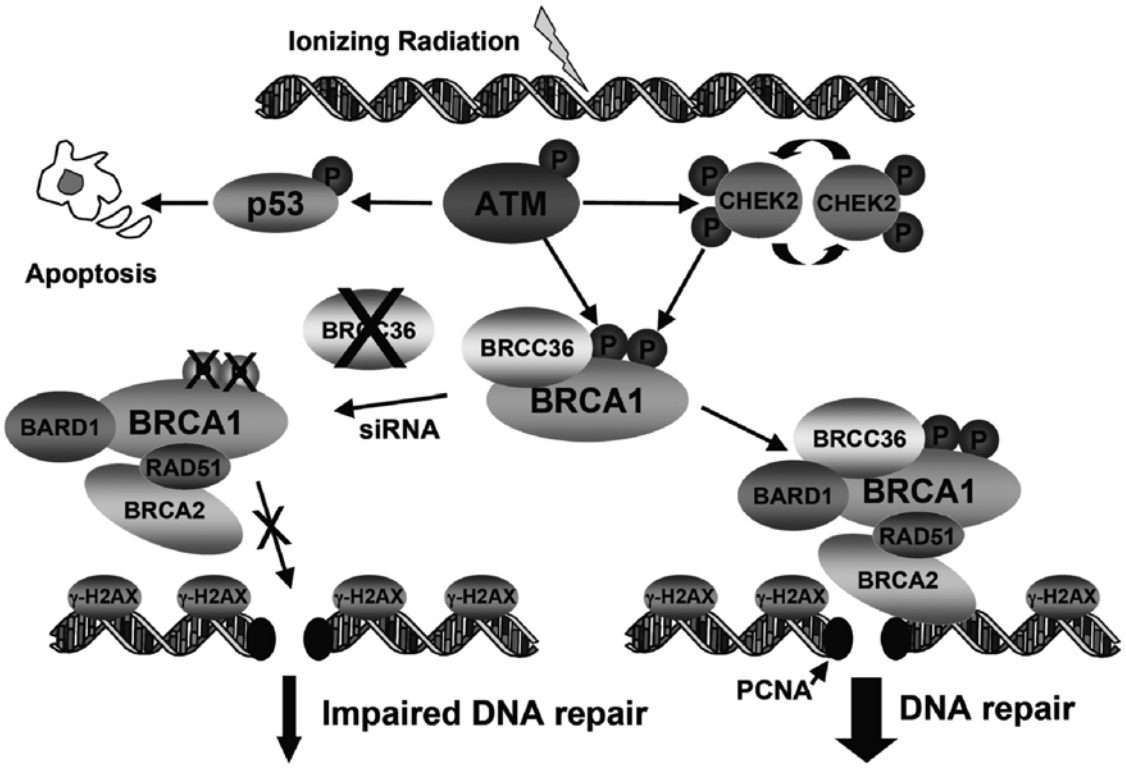

Figure 3. A proposed model illustrating the role of BRCC36 in BRCA1-associated DNA repair pathway in response to ionizing radiation (IR). BRCA1 and p53 are phosphorylated by the ATM kinase following IR. The BRCA1 and p53 proteins are involved in DNA repair and apoptosis pathways, respectively. Depletion of the BRCA1-associated protein, BRCC36, prevents the phosphorylation of BRCA1 and disrupts BRCA1 nuclear foci formation following IR, an event that is associated with the induction of DNA repair. The proposed model illustrates that disruption of BRCA1 activation through depletion of BRCC36 may create an imbalance between the DNA repair and cell survival pathways and the apoptosis/cell death pathways following IR exposure. As a result, abrogation of BRCC36 sensitizes breast cancer cells to IR-induced apoptosis.

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