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A Novel Model to Characterize Structure and Function of BRCA1

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Abstract

BRCA1 plays a central role in DNA repair. Although N-terminal RING and C-terminal BRCT domains are studied, the functions of the central region of BRCA1 is poorly characterized. Here we report a structural and functional analysis of BRCA1 alleles and functional human BRCA1 in chicken B-lymphocyte cell line DT40. The combination of “Homologous Recombineering” and “RT-cassette” enables modifications of chicken BRCA1 gene in *E.coli*. Mutant BRCA1 knock-in DT40 cell lines were generated using BRCA1 mutation constructs by homologous recombination with a targeting efficiency of up to 100%. Our study demonstrated that deletion of motif 2 to 9 BRCA1 / 181–1415 (*C.elegans* BRCA1 mimic) or deletion of motif 1 BRCA1 / 126–136 decreased cell viability following cisplatin treatment. Furthermore, deletion of motif 5 and motif 6 BRCA1 / 525–881 within DNA binding region, even the conserved 7-amino acid deletion BRCA1 / 872–878 within motif 6, caused a decreased cell viability upon cisplatin treatment. Surprisingly, human BRCA1 is functional in DT40 cells as indicated by DNA damage-induced Rad 51 foci formation in human BRCA1 knock-in DT40 cells. These results demonstrate that those conserved motifs within the central region are essential for DNA repair functions of BRCA1. These findings provide a valuable tool for the development of new therapeutic modalities of breast cancer linked to BRCA1.

Keywords

BRCA1; Conserved Motif; DT40 Cell; Homologous Recombineering; Mutations; RT-Cassette

1. Introduction

The BRCA1 gene encodes a large phosphoprotein involved in DNA repair. Despite the large size of BRCA1, only two small conserved domains have been characterized: N-terminal RING domain and C-terminal BRCT domain. The RING domain forms a heterodimer with

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BARD1, resulting in an active E3 ubiquitin ligase complex (Brzovic et al., 2003). Two tandem copies of the BRCT domain at the C-terminus is phospho-protein binding domain (Manke et al., 2003, Williams et al., 2004). Breast cancer-associated mutations have been found in both the Ring and BRCT domains of BRCA1, indicating that both domains are important to suppress breast and ovarian cancer formation (Brzovic et al., 2001, Monteiro et al., 1996).

The central region of BRCA1 is between the RING and BRCT domains. The central region of BRCA1 is thought to be largely unstructured (Mark et al., 2005) and thus possesses the structural flexibility that is required for the numerous interactions including the binding of DNA (Mark, Liao, 2005, Naseem et al., 2006, Paull et al., 2001) and a number of other proteins involved in DNA damage response and repair, such as c-Myc (Wang et al., 1998), RB (Aprelikova et al., 1999), p53 (Zhang et al., 1998), FANCA (Folias et al., 2002), Rad50 (Zhong et al., 1999), Rad51 (Scully et al., 1997), JunB (Hu and Li, 2002) and BRCA2 (Chen et al., 1998). Chicken BRCA1 gene was cloned and sequenced in 2001 (Orelli et al., 2001). The primary sequence in the chicken BRCA1 has diverged considerably from its mammalian orthologues. Comparison of this divergent chicken BRCA1 protein to mammalian orthologues not only reveals the presence of the conserved RING and BRCT repeat domains but also identifies nine additional highly-conserved motifs (named from motif 1 to motif 9) in the central region. Interestingly, the *C. elegans* orthologue of BRCA1 contains only a RING domain, motif 1 and two BRCT domains compared with human and chicken one (Boulton et al., 2004). However, the function of these motifs in the central region of BRCA1 is poorly studied.

The λ Red-mediated recombineering is an *in vivo* method of genetic engineering to make precisely defined insertions, deletions, and point mutations in *E. coli*, requiring as few as 35 bp of homology on each side of the desired alteration (Mosberg et al., 2010, Sharan et al., 2009). Linear DNA, either in form of single-stranded DNA (ssDNA) oligonucleotides (Costantino and Court, 2003, Ellis et al., 2001) or double-stranded DNA (dsDNA) (Murphy et al., 2000, Swaminathan et al., 2001, Yu et al., 2000) have been introduced to cells by electroporation and provide the homologous substrates to create genetic changes. For example, using recombineering technology ssDNA oligonucleotides have been applied to efficiently modify *E. coli* chromosomal targets (Costantino and Court, 2003, Ellis, Yu, 2001), bacterial artificial chromosome (Swaminathan, Ellis, 2001) and plasmids (Thomason et al., 2007). Whereas, linear dsDNA recombineering technique has been used to replace chromosomal genes (Murphy, 1998, Murphy, Campellone, 2000), to disrupt gene function (Datsenko and Wanner, 2000), to create a library of single-gene knockout *E. coli* strains (Baba et al., 2006), to remove 15% of the genomic material from a single *E. coli* strain (Posfai et al., 2006), to insert heterologous genes and entire pathways into the *E. coli* chromosome (Bouvier and Cheng, 2009).

The DT40 cells are originated from chicken B-lymphocyte derived from an avian leucosis virus induced bursal lymphoma (Baba et al., 1985). The DT40 cell line is rather unique among higher eukaryotic cells which exhibits a high ratio of targeted integration of transfected DNA occurring at essentially all loci with efficiencies that are orders of magnitude higher than those observed in mammalian cells (Buerstedde and Takeda, 1991).

DT40 cell line has been selected in for this study since BRCA1 is not essential for survival of DT40 cells (Martin et al., 2007), due to p53 deficiency in DT40 cells and that loss of p53 is known to rescue BRCA1^{-/-} viability (Xu et al., 2001). In addition, DT40 has a number of additional advantages as a model system (Ishiai et al., 2012), such as growing very rapidly, well-established targeting procedure, relatively invariant character in both karyotype and phenotype even during extended period of cell culture and a culture on a large scale with the stable characters under the same genetic background.

Given the advantage of λ Red-mediated recombineering method for genetic engineering and DT40 cells with high efficiency of targeted integration of transfected DNA and BRCA1-dispensable survival, here we developed a rapid method for construction of targeting vector containing BRCA1 mutation through a DNA cassette called RT-cassette which carries *rpsL* and *tetA* genes (R stands for *rpsL*, T for *tetA*) and generation of isogenic DT40 cell lines that carried specified alleles of BRCA1 by homologous recombination (HR). The analysis of chicken BRCA1 mutants showed that those conserved motifs within the central region are essential for DNA repair functions of BRCA1. Furthermore, our results showed that Human BRCA1 can functionally replace chicken BRCA1 in DT40 cells, suggesting that human BRCA1 may exert its biological functions in DT40 cells.

2. Materials and Methods

2.1. Cell lines, plasmids and bacterial strain

The chicken lymphoma B-cell line DT40 was cultured at 39°C, 5% CO₂ in RPMI medium supplemented with 10% fetal bovine serum (Atlas), 1% chicken serum (Sigma), and 50 μ mol/L β -mercaptoethanol (Sigma). Chicken wild-type BRCA1 targeting vector pNRB436 and full-length human BRCA1 cDNA targeting vector phBRCA1-cDNA were provided by Dr. Douglas Bishop's laboratory. BRCA1^{-/-} cell line in which two wild-type alleles of BRCA1 are replaced by puromycin and neomycin resistance genes was provided by Douglas Bishop's laboratory and was described as previous (Martin, Orelli, 2007). *E.coli* EL350 cells were generous gift from the laboratory of Dr. Neal G. Copeland at the Mouse Cancer Genetics Program, National Cancer Institute, Frederick, Maryland. RT-cassette was generous gift from the laboratory of Dr. Craig A. Strathdee at The John P. Robarts Research Institute, 100 Perth Drive, London, Ontario, Canada.

2.2. Plasmid recombineering

2.2.1. Generation of RT-cassette targeting fragment by PCR—The RT-cassette carries *rpsL* gene from *E.coli* and *tetA* gene from transposon Tn10 with positive and negative selection because *tetA* confers resistance to tetracycline and sensitivity to kanamycin, streptomycin and osmotic pressure and *rpsL* confers additional sensitivity to streptomycin (Stavropoulos and Strathdee, 2001). RT-cassette targeting fragments were obtained by PCR using primer P1 whose 3' end anneals to the 5' end of the RT-cassette and P2 whose 3' end anneals to 3' end of RT-cassette (Figure 1A). The 5' ends of both the primer P1 and P2 have 45 bases of homology to the target site of chicken BRCA1 targeting vector pNRB436 (Supplementary Figure S1D). The resulting PCR products were purified with a QIAGEN Gel Extraction Kit. The recovered DNA concentration was determined by

spectrophotometer. Primer sequences are: P1, 5'-upstream homology of target site-GTCGAGATATGACGGTGTTCAC-3' P2, 5'-downstream homology of target site-GTCGAG ATGGCGGACGCGATGG-3' (Supporting information Table S1).

2.2.2. Positive selection of RT containing plasmids after electroporation—

Given that it carries temperature inducible λ Red functions, *E.coli* EL350 harboring the targeting vector pNRB436 was shaken at 30°C overnight. 0.5 ml of the overnight culture was added to 35 ml of LB medium in a 250-ml flask at 30°C with shaking. When the A_{600} is between 0.4 and 0.6, the culture was transferred to the 42°C water bath and shaken for 15 min. The flask containing the bacteria was cooled down in ice-water slurry for 10 min. The cells were spun down in a pre-chilled 50-ml tube at 5000rpm for 5min. The cell pellet was suspended in 30 ml ice-cold distilled water. Centrifuge again as above and resuspend the cell pellet in 1 ml ice-cold distilled water. The cells were transferred to a microcentrifuge tube, spun down and resuspended in 200 μ l ice cold distilled water. 70 μ l of cell suspension was mixed with 100 ng of RT-cassette targeting DNA fragment with flanking homology (PCR products) to target site in microcentrifuge tube on ice. The mixture was rapidly transferred to a pre-chilled 0.1-cm electroporation cuvette. Electroporation with 1.80 kV was carried out to introduce the DNA into cells. 1 ml of LB medium was immediately added to the cuvette after electroporation. The electroporation mixture was transferred to sterile culture tube and incubated with shaking at 30°C for 1 to 2 hr. The cells were spread on an LB plate with ampicillin and tetracycline, following incubation at 30°C. Positive clones are confirmed by analysis of Sal I digestion of mini-prep plasmid DNA isolated from single colonies because there exists a Sal I enzyme site within RT-cassette.

2.2.3. Negative selection of plasmids without RT-cassette after electroporation

—Pick a single colony harboring RT-cassette containing plasmid and inoculate LB medium containing ampicillin and tetracycline with shaking at 30°C. Electroporation was done as above. The differences are (1) the cells harboring RT-cassette containing plasmid are induced at 42°C for only 8 min, (2) The tailed RT-cassette (PCR products) is replaced with DNA oligos with homology spanning the site of the RT-cassette insertion (Figure 1B and 1C), (3) the transformation mixture was spread on the NSLB (non sodium chloride LB) plate with ampicillin, streptomycin and kanamycin, following incubation at 30°C. Positive clones are determined by analysis of Sal I digestion of mini-prep plasmid DNA isolated from single colonies because the loss of the unique Sal I site within RT-cassette indicates the loss of RT-cassette. Antibiotic concentrations were used: ampicillin (100 μ g/ml), tetracycline (2 μ g/ml), streptomycin (500 μ g/ml), kanamycin (1 μ g/ml). LB recipe is 0.5% NaCl, 0.5% yeast extract and 1.0% drystone. NSLB plate recipe is 0.5% yeast extract, 1.0% tryptone and 0.75% agar.

2.3. Transfection of DT40 cells

The chicken lymphoma B-cell line DT40 was cultured and transfected as described (Martin, Orelli, 2007). The targeting plasmids were linearized with NotI digestion and purified. For transfection, 10^7 cells in logarithmic phase growth at 80×10^4 per ml was spun down and washed once in PBS, then suspended in 500 μ l of ice-cold PBS with 20 μ g of linearized targeting plasmid and transfer to a pre-chilled 0.4-cm electroporation cuvette. After 10 min on ice, samples were electroporated with the Gene Pulser Apparatus (Bio-Rad) at the

condition of 550V, 25 μ FD, left on ice for a further 5 min, and diluted into 20 ml of fresh media in T75 flask. After 12~24 hours of incubation at 39°C, 5 % CO₂, 80~120 ml of selection media (plasmid resistance marker histidinol, 1mg/ml) was added to the cell, and plate out into four sets of 96-well plates with 200 μ l in each well. In 5~7 days, clones can be visible on the bottom of wells. The single colonies were transferred to media containing either neomycin (1.5mg/ml) or puromycin (0.5mg/ml). 3~5 days after incubation, positive candidates can be determined which grow only in one of the selection media containing either neomycin or puromycin. PCR resulting from primer 1 (forward primer) that is in the upstream of homologous region before BRCA1 promoter in DT 40 cells and primer 2 (reverse primer) that is in the exon 1 of BRCA1 confirms the positive clones (Figure 2B). The forward primer 1 sequences are 5'-GCTCAGAGTGCTCAGCAATCATGTTC-3'. The reverse primer 2 sequences are 5'-CTGGACACTCCAAGTTCTTCTGC-3'.

2.4. MTS assay

Cells were suspended at 60,000 cells/ml. Put 50 μ l of cells into 96 well plates (3000 cells/well). 50 μ l of media containing twice the desired dose of cisplatin at 0nM, 25nM, 50nM, 125nM, and 500nM was added to the wells. Plates were incubated for 5 doubling times (40h for WT, 60h for BRCA1^{-/-} and mutants). After incubation, 20 μ l of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) with PMS (phenazine methosulfate) was added to each wells. The plates were mixed and incubated at 37°C for 3 hours. Colorimetric change of the MTS compound was determined on a Synergy HT plate reader (Bio-Tek) at 490 nm. The background reading taken from wells with no cells were subtracted from the samples before killing was determined.

2.5. Western blot analysis

Whole cell lysates from BRCA1 knock-in and BRCA1^{-/-} DT40 cells were subjected to electrophoresis on 7.5% Tris-Glycine extended gels. After electrophoresis, proteins were transferred to PVDF membranes, and the membranes were incubated with anti-chicken BRCA1 antibody, followed by incubation with a secondary antibody, and visualized by the ECL Western blotting detection system.

2.6. Immunofluorescence

DT40 cells were treated with 6Gy of ionizing radiation. After 4 hours, 6 $\times$ 10⁶ cells were dried onto a polylysine-coated slide. For analysis of Rad 51 foci formation, cells were fixed in 4% paraformaldehyde/PBS for 10 min, and then blocked in 0.1% Triton X-100 / 0.02% SDS in PBS. Primary and secondary antibodies were diluted in the same buffer. The primary antibodies were anti-RAD51 (rabbit polyclonal H-92; Santa Cruz). Fluorescence-labeled secondary antibodies were obtained from Molecular Probes.

3. Results

3.1. A rapid method to generate isogenic cell lines that carried specified alleles of BRCA1

3.1.1. Using E.coli recombineering to generate fragment deletion and point mutations in BRCA1: RT-cassette-based positive and negative selection method

—This system uses a EL350 bacterial strain that contains a replication defective λ

prophage where the phage recombination systems are under control of temperature sensitive $\text{cI}857$ repressor because p_L operon encoding *gam* and the red recombination genes *exo* and *bet* is under its control. At low temperatures (30–34°C), the recombination genes are not expressed. However, after shifting the bacterial cultures to 42°C, the recombination genes are expressed at high levels from the λ p_L promoter (Westenberg et al., 2010).

The RT-cassette-based positive-negative selection method is a two-step system which allows the modification of BRCA1 DNA in chicken wild-type BRCA1 targeting vector pNRB436 (Supplementary Figure S1D) without introducing a selectable marker at the modification site. The RT-cassette carries *rpsL* gene from *E. coli* and *tetA* gene from transposon Tn10 with positive and negative selection because *tetA* confers resistance to tetracycline and sensitivity to kanamycin, streptomycin and osmotic pressure and *rpsL* confers additional sensitivity to streptomycin (Stavropoulos and Strathdee, 2001). First step is positive selection which involves targeting the region of interest with the RT-cassette containing BRCA1 homology (PCR products) (Figure 1A and Supplementary Table S1) to a selected position of BRCA1. The bacteria containing recombinant plasmid are selected on the plate containing tetracycline for RT-cassette positive selection because *tetA* confers resistance to tetracycline. As shown in Table 1, the results in positive selection step indicated that all tetracycline-resistant transformants contained the RT-cassette targeted to the correct site of BRCA1. The second step, a negative selection step, involves the replacement of RT-cassette with a DNA oligo containing the particular mutation of interest with homology spanning the site of the RT-cassette insertion. BRCA1 mutations include fragment deletion (Figure 1B) and point mutation (Figure 1C). The DNA oligos used in this study are listed in Supplemental Table S1. Since EL350 is *rpsL*[−], when expressed in a *rpsL*[−] host, the wild-type *rpsL*⁺ gene combining with *tetA* provide the negative selection marker. Therefore, cells are selected for the loss of RT-cassette on NSLB plate in the presence of 500 ug/ml of streptomycin and 1 ug/ml of kanamycin because *tetA* in RT-cassette confers sensitivity to kanamycin, streptomycin and osmotic pressure and *rpsL* confers additional sensitivity to streptomycin. The results in the negative selection step showed that the right BRCA1 mutant without RT-cassette is about 5% of the colonies. Summary of chicken BRCA1 mutations generated by recombineering in *E. coli* and their human BRCA1 counterparts is shown in Table 2. Combining *rpsL* with *tetA* makes possible both positive and negative selection with plating efficiencies on the order of 10^{−9} even with 45 bases of homology on both sides.

3.1.2. Mutant BRCA1 allele knock-in in DT40 cells—Mutant BRCA1 knock-in cell line generation strategy is illustrated in Figure 2A. The targeting BRCA1 DNA fragment is 17 kb in size with 4.4 kb of 5' homology arm (upstream homology) and 1.1 kb of 3' homology arm (downstream homology) identical to the genomic sequences surrounding the intended integration site (Supplementary Figure S1D), which enable HR events to occur. DT40 is the host cell line that carry a homozygous complete BRCA1 deletion BRCA1^{−/−} (Figure 2A), in which two wild-type alleles are replaced by puromycin and neomycin resistance genes, respectively. The linearized targeting BRCA1 DNA digested with NotI is delivered into host cells by electroporation. PCR analysis determined positive clones using forward primer 1 which is located outside of the targeted region (outside of the upstream homology) and the reverse primer 2 which is in the exon 1 of BRCA1 on genomic DNA

isolated from single cell clones (Figure 2B). This analysis indicates that the efficiency of targeting among the clones with puromycin+ and neomycin- is between 25% to 100%. One possible reason for the high homologous recombination frequency might be that BRCA1 is not essential for survival of DT40 cells due to p53 deficiency in DT40 cells although it is critical in mammalian cells. Moreover, it has been reported that elimination of one p53 allele completely rescues the embryonic lethality and restores normal mammary gland development in mouse model although BRCA1^{-/-} embryos die late in gestation because of widespread apoptosis (Xu, Qiao, 2001). It is worth noting that not any targeting was found with neomycin+ and puromycin-. The targeted homologous recombination took place in neomycin allele preferences in an unknown mechanism. Western blot analysis showed the mutant BRCA1 expressions in the BRCA1 knock-in cell lines (Figure 2C).

3.2. cell viability analysis of Mutant BRCA1 allele knock-in in cells following cisplatin treatment

3.2.1. Either deletion of motif 2 through 9 or deletion of motif 1 of BRCA1 impaired DNA repair—In addition to an N-terminal RING finger and two C-terminal BRCT repeat domains, the *C. elegans* BRCA1 contains only motif 1 domain in the central region (Figure 3A). However, evidence shows that *C. elegans* BRCA1 is required for DNA repair in *C. elegans* (Adamo et al., 2008, Boulton, Martin, 2004). To determine whether the deletion mutation of central region from motif 2 to 9 in chicken BRCA1 mimicking *C. elegans* BRCA1 still functions in DNA repair, we generated a DT40 cell line that carries chicken BRCA1^{+/181-1415} (*C. elegans* BRCA1 mimic). Although poly [ADP ribose] polymerase (PARP) inhibitors target the same HR-based DNA repair defect as cisplatin chemotherapy, evidence showed the efficacy for the PARP inhibitor olaparib in *BRCA1* mutation breast cancer with substantial prior chemotherapy exposure (Audeh et al., 2010). The requirement for BRCA1 in DNA repair may explain the increased cisplatin sensitivity that has been observed in BRCA1-deficient cells (Quinn et al., 2003, Tassone et al., 2003). Therefore, we treated the cells with cisplatin, and cell viability was measured using MTS assay. As shown in Figure 3B, our results indicated that, comparison with the control wild-type BRCA1^{+/+}, DT40 cells carrying chicken BRCA1^{+/181-1415} decreased significantly in cell viability upon cisplatin-induced DNA damage, whose phenotype is similar to BRCA1 knockout cells BRCA1^{-/-}, suggesting that motif 2-9 in the central region is required for chicken BRCA1 in the function of DNA repair although it is missing in the *C. elegans* orthologue of BRCA1 which still possesses DNA repair function. Further investigations revealed that DT40 cells carrying motif 1 deletion mutation BRCA1^{+/126-136} decreased in comparison with BRCA1 wildtype but are more resistant than BRCA1 null mutant in cell viability upon cisplatin treatment (Figure 3C), suggesting that motif 1 partially affects the DNA repair function of chicken BRCA1. Taken together, these results indicate that the central region of chicken BRCA1 is important for its role of DNA damage response. This may be because BRCA1 is believed to act as a scaffold that juxtaposes members and integrate various signals in the DNA damage response pathway although a ~1500 residue central region of human or chicken BRCA1 is mostly intrinsically disordered (Mark, Liao, 2005).

3.2.2. Either deletion of motif 5–6 or deletion of conserved 7-amino acids

within motif 6 in the DNA binding domain impaired DNA repair—Although BRCA1 is involved in many important biological processes, the function of BRCA1 in homologous recombination of DNA double-strand break repair is considered one of the major mechanisms contributing to its tumor suppression activity. It is demonstrated that BRCA1 is recruited to DNA damage sites (Li and Yu, 2013) and directly binds DNA in a nonspecific manner (Paull, Cortez, 2001) through DNA binding domain which covers motif 5 and 6 (Orelli, Logsdon Jr, 2001, Paull, Cortez, 2001) (Figure 4A). We attempted to determine whether the conserved motif deletion of BRCA1 within DNA binding domain influenced its function of DNA repair. For this reason, we generated DT40 cell lines carrying either both motif 5 and motif 6 deletion mutation BRCA1^{Δ525–881} or the conserved 7-amino acid deletion mutation BRCA1^{Δ872–878} in motif 6. Our experimental results showed that DT40 cells that carry either both motif 5 and motif 6 deletion mutation BRCA1^{Δ525–881} (Figure 4B) or the conserved 7-amino acid deletion mutation BRCA1^{Δ872–878} (Figure 4C) decreased cell viability upon cisplatin treatment, indicating that those conserved motifs within DNA binding domain are required for the function of BRCA1 in DNA repair.

3.3. Functional human BRCA1 in DT40 cells

The fact that BRCA1 is not essential for survival of DT40 cells (Martin, Orelli, 2007) make it possible to study the specific aspect of human BRCA1 functions by knock-in a mutant allele in DT40 cells. To this end, chicken BRCA1 was replaced with the human BRCA1 cDNA in the chicken BRCA1 targeting vector (Supplementary Figure S1D) to generate human BRCA1 cDNA targeting vector phBRCA1-cDNA (Figure 5A). The phBRCA1-cDNA incorporated into the genome of DT40 (BRCA1^Δ) at the target locus by homologous recombination resulting in generation of a DT40 derivative expressing human BRCA1 (BRCA1^Δ/human BRCA1 cDNA). To evaluate functional activity of human BRCA1 in DT40 cells, we induced DNA damage with ionizing radiation and evaluated Rad51 foci by immunohistochemistry. The results showed that the pattern of radiation-induced formation of Rad51 foci in cells with BRCA1^Δ/human BRCA1 cDNA was similar to that in cells with chicken BRCA1^{+/+} (Figure 5B and 5C). Moreover, it is well known that Rad51 complexes function downstream of BRCA1 recruitment to damage foci. Therefore, human BRCA1 expression in DT40 was confirmed indirectly by radiation-induced Rad51 foci formation. These results imply the possibility of characterization of human BRCA1 in DT40 cells where BRCA1 is not essential for cell survival due to p53 deficiency.

4. Discussion

BRCA1 is a large tumor suppressor protein. The BRCA1 function is highly dependent on its discrete folded domains. Investigations to define the structure of BRCA1 domains have been restricted largely by the lack of similarity to any other protein and its immense size. Here we developed an efficient system to create BRCA1 mutations in *E.coli* by the combination of λ Red-mediated recombineering and RT-cassette and generate DT40 cell lines with specified BRCA1 alleles by homologous recombineering. This new method overcomes the lengthy and labor-intensive methods including PCR amplification and subcloning into expression

vector (Mark, Liao, 2005). This study utilizes an efficient and powerful system even for large plasmids such as 22 kb BRCA1 knock-in fragment, which is very difficult using standard mutagenesis protocols (for example, QuickChange/Site-directed mutagenesis).

A number of genomic editing technologies have been emerged in recent years, including zinc-finger nucleases, transcription activator-like effector nucleases and the RNA-guided CRISPR-Cas9 nuclease system (Harrison et al., 2014). CRISPR-Cas9 nuclease system provides a simple and an efficient method to precisely manipulate the genome of cells or whole organisms compared to other technologies. However, it has been a major problem for genomic editing in large DNA fragment deletion, insertion or replacement of 1kb or larger fragments. It has been shown that recombination efficiency decreases as the fragment size increases (Canver et al., 2014). In this study, using homologous recombineering technology with RT-cassette-based positive-negative selection leads to deletion of up to 3.708 kb DNA fragment (Table 2, BRCA1- 181–1415, $(1.236 \times 3 = 3.708)$). Moreover, off-target effects are a critical issue for the applications of CRISPR-Cas9 nuclease system to mutate specific genes (Wu et al., 2014). Off-targets could generate spurious phenotypes and mistaken interpretations. The only really valid assay for off-targets is the whole-genome deep sequencing of the cloned mutated cell which is time consuming and expensive. Whereas the homologous recombineering technology is considered to be error-free (Moynahan and Jasin, 2010). Taken together, these advantages of homologous recombineering technology with RT-cassette benefit gene modifications.

One of the most important findings of this study is that cells missing middle region from motif 2 to 9 of chicken orthologue (*C.elegans* BRCA1 mimic, BRCA1 / 181–1415), lose their DNA repair function and consequently increase sensitivity against cisplatin treatment (Figure 3B). Further studies showed that even deletion of motif 1 (BRCA1 / 126–136), the only conserved motif left of central region in *C.elegans* orthologue, also decreases cell viability with cisplatin treatment (Figure 3C). This suggests that motif 1 partially affects the DNA repair function. It is known that central region residues 452–1079, which covers motif 5 and 6, within human BRCA1 were able to directly bind DNA in a non-specific manner (Paull, Cortez, 2001). We observed that DT40 cells carrying mutant BRCA1 / 525–881, deletion of both motif 5 and motif 6, even BRCA1 / 872–878, a deletion of conserved 7-amino acids within motif 6, decrease cell viability with cisplatin treatment (Figure 4B and 4C), suggesting the DNA binding domain of BRCA1 is important for DNA damage response. Our other important finding is that ectopic expression of human BRCA1 cDNA in DT40 cells caused damage-dependent Rad51 foci formation upon irradiation (Figure 5), suggesting that human BRCA1 is functional in engineered DT40 cells. This makes it possible to easily manipulate the sequence of the human BRCA1 and investigate its functions in DT40 cells.

Although we measured the cisplatin sensitivity only in four mutants out of the seven isogenic mutants (Figure 3 and Figure 4), Our data shows that this model works for characterizing the structure and function of BRCA1 as indicated by analysis of one of multiple functions of BRCA1, for example, the repair function of damaged DNA caused by cisplatin.

Due to the facility with which the DT40 cell line can be manipulated genetically, and the fact that DT40 mutants reveal a strong phenotypic resemblance to murine mutants with respect to genes involved in DNA damage response pathway (Sonoda et al., 2001), it has been a steady growth in its use in genetic studies, such as immunoglobulin diversification, DNA repair, chromosome segregation, RNA metabolism and cell signaling (Dhar et al., 2001, Ridpath et al., 2011, Winding and Berchtold, 2001, Yamazoe et al., 2004). Another feature of the DT40 cells is that BRCA1 is not essential for survival of DT40 cells (Martin, Orelli, 2007). This result is likely to depend on DT40 p53 deficiency as loss of p53 is known to rescue BRCA1^{-/-} viability (Xu, Qiao, 2001). Furthermore, the DT40 cell line exhibits a high ratio of targeted integration of transfected DNA with efficiencies that are orders of magnitude higher than those observed in mammalian cells (Buerstedde and Takeda, 1991). Moreover, there are many other advantages of using DT40 as a model system (Ishiai et al., 2012), such as growing rapidly, well-established targeting procedure, relatively invariant character in both karyotype and phenotype even during the extended period of time and on a large scale of cell culture with the stable characters under the same genetic background. In addition, our results validating functional human BRCA1 in chicken DT40 cells provide an important and valuable system to study the structure and function of human BRCA1, thereby supporting the development and validation of new drugs targeting BRCA mutations.

5. Conclusion

Our study demonstrates that those conserved motifs in the central region are important for DNA repair functions of BRCA1. Our results show that this system works for the structural and functional analysis of BRCA1 including human BRCA1. These findings provide a valuable tool for the development of new therapeutic modalities of breast cancer linked to BRCA1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

BRCA1	breast cancer 1
BRCT domain	BRCA1 C Terminus domain
HR	homologous recombination

RING domain	really interesting new gene domain
RT-cassette	<i>rpsL</i> and <i>tetA</i> cassette

References

- Adamo A, Montemauri P, Silva N, Ward JD, Boulton SJ, La Volpe A. BRC-1 acts in the inter-sister pathway of meiotic double-strand break repair. *EMBO Rep.* 2008; 9:287–92. [PubMed: 18219312]
- Aprelikova ON, Fang BS, Meissner EG, Cotter S, Campbell M, Kuthiala A, Bessho M, Jensen RA, Liu ET. BRCA1-associated growth arrest is RB-dependent. *Proc Natl Acad Sci U S A.* 1999; 96:11866–71. [PubMed: 10518542]
- Audeh MW, Carmichael J, Penson RT, Friedlander M, Powell B, Bell-McGuinn KM, Scott C, Weitzel JN, Oaknin A, Loman N, Lu K, Schmutzler RK, Matulonis U, Wickens M, Tutt A. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: a proof-of-concept trial. *Lancet.* 2010; 376:245–51. [PubMed: 20609468]
- Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol.* 2006; 2:2006 0008.
- Baba TW, Giroir BP, Humphries EH. Cell lines derived from avian lymphomas exhibit two distinct phenotypes. *Virology.* 1985; 144:139–51. [PubMed: 2998040]
- Boulton SJ, Martin JS, Polanowska J, Hill DE, Gartner A, Vidal M. BRCA1/BARD1 orthologs required for DNA repair in *Caenorhabditis elegans*. *Curr Biol.* 2004; 14:33–9. [PubMed: 14711411]
- Bouvier J, Cheng JG. Recombineering-based procedure for creating Cre/loxP conditional knockouts in the mouse. *Curr Protoc Mol Biol.* 2009 Chapter 23:Unit 23.13.
- Brzovic PS, Keeffe JR, Nishikawa H, Miyamoto K, Fox D 3rd, Fukuda M, Ohta T, Klevit R. Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-ligase complex. *Proc Natl Acad Sci U S A.* 2003; 100:5646–51. [PubMed: 12732733]
- Brzovic PS, Meza JE, King MC, Klevit RE. BRCA1 RING domain cancer-predisposing mutations. Structural consequences and effects on protein-protein interactions. *J Biol Chem.* 2001; 276:41399–406. [PubMed: 11526114]
- Buerstedde JM, Takeda S. Increased ratio of targeted to random integration after transfection of chicken B cell lines. *Cell.* 1991; 67:179–88. [PubMed: 1913816]
- Canver MC, Bauer DE, Dass A, Yien YY, Chung J, Masuda T, Maeda T, Paw BH, Orkin SH. Characterization of genomic deletion efficiency mediated by clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 nuclease system in mammalian cells. *J Biol Chem.* 2014; 289:21312–24. [PubMed: 24907273]
- Chen J, Silver DP, Walpita D, Cantor SB, Gazdar AF, Tomlinson G, Couch FJ, Weber BL, Ashley T, Livingston DM, Scully R. Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. *Mol Cell.* 1998; 2:317–28. [PubMed: 9774970]
- Costantino N, Court DL. Enhanced levels of lambda Red-mediated recombinants in mismatch repair mutants. *Proc Natl Acad Sci U S A.* 2003; 100:15748–53. [PubMed: 14673109]
- Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A.* 2000; 97:6640–5. [PubMed: 10829079]
- Dhar PK, Sonoda E, Fujimori A, Yamashita YM, Takeda S. DNA repair studies: experimental evidence in support of chicken DT40 cell line as a unique model. *J Environ Pathol Toxicol Oncol.* 2001; 20:273–83. [PubMed: 11797836]
- Ellis HM, Yu D, DiTizio T, Court DL. High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. *Proc Natl Acad Sci U S A.* 2001; 98:6742–6. [PubMed: 11381128]
- Folias A, Matkovic M, Bruun D, Reid S, Hejna J, Grompe M, Andrea A, Moses R. BRCA1 interacts directly with the Fanconi anemia protein FANCA. *Hum Mol Genet.* 2002; 11:2591–7. [PubMed: 12354784]

- Harrison MM, Jenkins BV, O'Connor-Giles KM, Wildonger J. A CRISPR view of development. *Genes Dev.* 2014; 28:1859–72. [PubMed: 25184674]
- Hu YF, Li R. JunB potentiates function of BRCA1 activation domain 1 (AD1) through a coiled-coil-mediated interaction. *Genes Dev.* 2002; 16:1509–17. [PubMed: 12080089]
- Ishiai M, Uchida E, Takata M. Establishment of the DNA repair-defective mutants in DT40 cells. *Methods Mol Biol.* 2012; 920:39–49. [PubMed: 22941595]
- Li M, Yu X. Function of BRCA1 in the DNA damage response is mediated by ADP-ribosylation. *Cancer Cell.* 2013; 23:693–704. [PubMed: 23680151]
- Manke IA, Lowery DM, Nguyen A, Yaffe MB. BRCT repeats as phosphopeptide-binding modules involved in protein targeting. *Science.* 2003; 302:636–9. [PubMed: 14576432]
- Mark WY, Liao JC, Lu Y, Ayed A, Laister R, Szymczyna B, Chakrabarty A, Arrowsmith CH. Characterization of segments from the central region of BRCA1: an intrinsically disordered scaffold for multiple protein-protein and protein-DNA interactions? *J Mol Biol.* 2005; 345:275–87. [PubMed: 15571721]
- Martin RW, Orelli BJ, Yamazoe M, Minn AJ, Takeda S, Bishop DK. RAD51 up-regulation bypasses BRCA1 function and is a common feature of BRCA1-deficient breast tumors. *Cancer Res.* 2007; 67:9658–65. [PubMed: 17942895]
- Monteiro AN, August A, Hanafusa H. Evidence for a transcriptional activation function of BRCA1 C-terminal region. *Proc Natl Acad Sci U S A.* 1996; 93:13595–9. [PubMed: 8942979]
- Mosberg JA, Lajoie MJ, Church GM. Lambda red recombineering in *Escherichia coli* occurs through a fully single-stranded intermediate. *Genetics.* 2010; 186:791–9. [PubMed: 20813883]
- Moynahan ME, Jasin M. Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. *Nat Rev Mol Cell Biol.* 2010; 11:196–207. [PubMed: 20177395]
- Murphy KC. Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*. *J Bacteriol.* 1998; 180:2063–71. [PubMed: 9555887]
- Murphy KC, Campellone KG, Poteete AR. PCR-mediated gene replacement in *Escherichia coli*. *Gene.* 2000; 246:321–30. [PubMed: 10767554]
- Naseem R, Sturdy A, Finch D, Jowitt T, Webb M. Mapping and conformational characterization of the DNA-binding region of the breast cancer susceptibility protein BRCA1. *Biochem J.* 2006; 395:529–35. [PubMed: 16460311]
- Orelli BJ, Logsdon JM Jr, Bishop DK Jr. Nine novel conserved motifs in BRCA1 identified by the chicken orthologue. *Oncogene.* 2001; 20:4433–8. [PubMed: 11466627]
- Paull TT, Cortez D, Bowers B, Elledge SJ, Gellert M. Direct DNA binding by Brca1. *Proc Natl Acad Sci U S A.* 2001; 98:6086–91. [PubMed: 11353843]
- Posfai G, Plunkett G 3rd, Feher T, Frisch D, Keil GM, Umenhoffer K, Kolisnychenko V, Stahl B, Sharma SS, de Arruda M, Burland V, Harcum SW, Blattner FR. Emergent properties of reduced-genome *Escherichia coli*. *Science.* 2006; 312:1044–6. [PubMed: 16645050]
- Quinn JE, Kennedy RD, Mullan PB, Gilmore PM, Carty M, Johnston PG, Harkin DP. BRCA1 functions as a differential modulator of chemotherapy-induced apoptosis. *Cancer Res.* 2003; 63:6221–8. [PubMed: 14559807]
- Ridpath JR, Takeda S, Swenberg JA, Nakamura J. Convenient, multi-well plate-based DNA damage response analysis using DT40 mutants is applicable to a high-throughput genotoxicity assay with characterization of modes of action. *Environ Mol Mutagen.* 2011; 52:153–60. [PubMed: 20839229]
- Scully R, Chen J, Plug A, Xiao Y, Weaver D, Feunteun J, Ashley T, Livingston DM. Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell.* 1997; 88:265–75. [PubMed: 9008167]
- Sharan SK, Thomason LC, Kuznetsov SG, Court DL. Recombineering: a homologous recombination-based method of genetic engineering. *Nat Protoc.* 2009; 4:206–23. [PubMed: 19180090]
- Sonoda E, Morrison C, Yamashita YM, Takata M, Takeda S. Reverse genetic studies of homologous DNA recombination using the chicken B-lymphocyte line, DT40. *Philos Trans R Soc Lond B Biol Sci.* 2001; 356:111–7. [PubMed: 11205323]
- Stavropoulos TA, Strathdee CA. Synergy between tetA and rpsL provides high-stringency positive and negative selection in bacterial artificial chromosome vectors. *Genomics.* 2001; 72:99–104. [PubMed: 11247671]

- Swaminathan S, Ellis HM, Waters LS, Yu D, Lee EC, Court DL, Sharan SK. Rapid engineering of bacterial artificial chromosomes using oligonucleotides. *Genesis*. 2001; 29:14–21. [PubMed: 11135458]
- Tassone P, Tagliaferri P, Perricelli A, Blotta S, Quaresima B, Martelli ML, Goel A, Barbieri V, Costanzo F, Boland CR, Venuta S. BRCA1 expression modulates chemosensitivity of BRCA1-defective HCC1937 human breast cancer cells. *Br J Cancer*. 2003; 88:1285–91. [PubMed: 12698198]
- Thomason LC, Costantino N, Shaw DV, Court DL. Multicopy plasmid modification with phage lambda Red recombineering. *Plasmid*. 2007; 58:148–58. [PubMed: 17434584]
- Wang Q, Zhang H, Kajino K, Greene MI. BRCA1 binds c-Myc and inhibits its transcriptional and transforming activity in cells. *Oncogene*. 1998; 17:1939–48. [PubMed: 9788437]
- Westenberg M, Bamps S, Soedling H, Hope IA, Dolphin CT. Escherichia coli MW005: lambda Red-mediated recombineering and copy-number induction of oriV-equipped constructs in a single host. *BMC Biotechnol*. 2010; 10:27. [PubMed: 20350301]
- Williams RS, Lee MS, Hau DD, Glover JN. Structural basis of phosphopeptide recognition by the BRCT domain of BRCA1. *Nat Struct Mol Biol*. 2004. 2004; 11:519–25.
- Winding P, Berchtold MW. The chicken B cell line DT40: a novel tool for gene disruption experiments. *J Immunol Methods*. 2001; 249:1–16. [PubMed: 11226459]
- Wu X, Kriz AJ, Sharp PA. Target specificity of the CRISPR-Cas9 system. *Quant Biol*. 2014; 2:59–70. [PubMed: 25722925]
- Xu X, Qiao W, Linke SP, Cao L, Li WM, Furth PA, Harris CC, Deng CX. Genetic interactions between tumor suppressors Brca1 and p53 in apoptosis, cell cycle and tumorigenesis. *Nat Genet*. 2001; 28:266–71. [PubMed: 11431698]
- Yamazoe M, Sonoda E, Hohegger H, Takeda S. Reverse genetic studies of the DNA damage response in the chicken B lymphocyte line DT40. *DNA Repair (Amst)*. 2004; 3:1175–85. [PubMed: 15279806]
- Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL. An efficient recombination system for chromosome engineering in Escherichia coli. *Proc Natl Acad Sci U S A*. 2000; 97:5978–83. [PubMed: 10811905]
- Zhang H, Somasundaram K, Peng Y, Tian H, Bi D, Weber BL, El-Deiry WS. BRCA1 physically associates with p53 and stimulates its transcriptional activity. *Oncogene*. 1998; 16:1713–21. [PubMed: 9582019]
- Zhong Q, Chen CF, Li S, Chen Y, Wang CC, Xiao J, Chen PL, Sharp ZD, Lee WH. Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. *Science*. 1999; 285:747–50. [PubMed: 10426999]

Figure 1. Schematic representation of RT-cassette-based recombineering to generate fragment deletion and point mutations

(A) Generation of RT-cassette targeting fragment by PCR using primers P1 and P2. 3' end of P1 and P2 anneals to the 5' and 3' ends of the RT-cassette, respectively. The 5' ends of the primers have 45 bases of homology to the target site. HA, homology arm. (B) The two steps depicting the recombineering procedure to generate fragment deletion mutation. RT-cassette targeting fragment is designed to replace the fragment to be deleted in mutant BRCA1. The first step of recombineering, the fragment to be deleted is replaced with RT-cassette under tetracycline selection. The second step, the RT-cassette is driven out under streptomycin and kanamycin selection. (C) The two steps depicting the recombineering procedure to generate point mutation. The base to be replaced in the chicken wild-type BRCA1 and the mutated base in mutant BRCA1 are shown in red and green, respectively. RT-cassette targeting fragment with addition of the mutated base in front of RT-cassette (between upstream homology and RT-cassette) is designed to insert to the site of mutated base in mutant BRCA1. The first step of recombineering, the base to be substituted is replaced with RT-cassette with the mutated base under tetracycline selection. The second step, the RT-cassette is driven out under streptomycin and kanamycin selection.

Figure 2. Schematic representation of homologous recombineering to generate mutant BRCA1 knock-in DT40 cell lines from isogenic BRCA1 knockout cell line

(A) Schematic representation to generate BRCA1 knock-in DT40 cell lines. Linear plasmid with mutant BRCA1 is targeted to target site by homologous recombination in BRCA1 knockout DT40 cells. (B) Schematic representation to verify right targeting. Primer 1 which is located outside the targeted region and primer 2 which is located in the exon 1 of BRCA1 mark the two PCR primers that can be used for screening the correctly targeted clones. UH, upstream homology; DH, downstream homology. (C) Western blot analysis for knock-in BRCA1 mutant expression.

Figure 3. The impaired DNA repair function of chicken BRCA1 in DT 40 cells that carry the deletion of either central region from motif 2 to 9 (*C.elegans* BRCA1 mimic) or motif 1
(A) Schematic representation of the orthologs of the BRCA1 in *C. elegans*. In addition to an N-terminal RING finger and two C-terminal BRCT repeat domains, the *C. elegans* BRCA1 contains only motif 1 domain in the central region compared with human or chicken BRCA1. **(B)** Cell viability assay in DT40 cells that carry BRCA1 Δ / Δ ¹⁸¹⁻¹⁴¹⁵ (*C.elegans* BRCA1 mimic) with cisplatin treatment. DT40 cells carrying chicken BRCA1 Δ / Δ ¹⁸¹⁻¹⁴¹⁵ decreased significantly in cell viability upon cisplatin-induced DNA damage compared to the control wild-type BRCA1 Δ / +. **(C)** Cell viability assay in DT40 cells carrying carrying motif 1 deletion mutation BRCA1 Δ / Δ ¹²⁶⁻¹³⁶ upon cisplatin treatment. DT40 cells carrying

chicken BRCA1 / 126–136 decreased significantly in cell viability upon cisplatin-induced DNA damage compared with the control wild-type BRCA1 /+.

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Figure 4. The impaired DNA repair function of chicken BRCA1 in DT40 cells that carry the DNA binding domain deletion mutations

(A) Schematic representation of DNA binding domain of BRCA1. The DNA binding domain of BRCA1 encompasses motif 5 and 6. (B) Cell viability assay in DT40 cells that carry both motif 5 and motif 6 deletion mutation $BRCA1^{\Delta/\Delta 525-881}$ with cisplatin treatment. DT40 cells carrying chicken $BRCA1^{\Delta/\Delta 525-881}$ decreased significantly in cell viability upon cisplatin-induced DNA damage compared with the control wild-type $BRCA1^{\Delta/+}$. (C) Cell viability assay in DT40 cells carrying the conserved 7-amino acid deletion mutation within motif 6 $BRCA1^{\Delta/\Delta 872-878}$ upon cisplatin treatment. DT40 cells carrying chicken

BRCA1^{-/-872-878} decreased significantly in cell viability upon cisplatin-induced DNA damage compared with the control wild-type BRCA1^{+/+}.

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Figure 5. Schematic map of vector phBRCA1-cDNA used for human BRCA1 cDNA targeting in DT40 cells and Rad51 immunofluorescence assay in DT40 cells

(A) Schematic map of vector phBRCA1-cDNA used for human BRCA1 cDNA targeting in DT40 cells. phBRCA1-cDNA is identical to pNRB436 except that the transcription region of chicken BRCA1 is replaced with full-length human BRCA1 cDNA. (B) Rad51 immunofluorescence assay in DT40 cells. Cells were exposed to 6 Gy X-rays, fixed at 4 h post-ionizing radiation, and stained with anti-Rad51 antibodies followed by FITC-conjugated secondary antibody and compared to unexposed control cells. Scale bar, 10 μ m.

(C) Quantification of Rad51 foci. Rad51 foci was obtained by counting at least 100 nuclei. Data represent the means and SD of the results of three independent experiments.

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Table 1

Summary of transformants with right RT cassette targeting

Target site	Number with tetracycline resistance checked	Number with correct targeting
BRCA1- 181–1415	10	10
BRCA1- 126–136	10	10
BRCA1-C62G	8	8
BRCA1-M1666R	10	10
BRCA1-S1414A	5	5
BRCA1- 525–881	10	10
BRCA1- 872–878	7	7

Table 2Summary of BRCA1 mutations generated by recombineering in *E.coli*

mutations in chicken BRCA1	deletion/single base mutation	counterpart in human BRCA1	expected functions
BRCA1- 181–1415	deletion of motif 2 to 9	BRCA1- 178–1424	C.elegans BRCA1 mimic
BRCA1- 126–136	deletion of motif 1	BRCA1- 123–133	impaired C.elegans BRCA1 mimic
BRCA1-C62G	single base mutation	BRCA1-C61G	defect of E3 ubiquitin ligase
BRCA1-M1666R	single base mutation	BRCA1-M1775R	defect of BRCT repeat
BRCA1-S1414A	single base mutation	BRCA1-S1423A	defect of damage and ATM-dependent phosphorylation
BRCA1- 525–881	deletion of motif 5 to 6	BRCA1- 512–871	loss of DNA binding domain
BRCA1- 872–878	deletion of 7 amino acids within motif 6	BRCA1- 862–868	impaired DNA binding