THE BRCA1 RING DOMAIN IS ESSENTIAL FOR DNA DAMAGE REPAIR AND G2/M CHECKPOINT ACTIVATION

By

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ABSTRACT OF THE THESIS

The Brca1 Ring Domain Is Essential For DNA Damage Repair

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The *BRCA1* (breast cancer 1, early onset) tumor suppressor gene is involved in a variety of cellular pathways, and is an essential factor for normal DNA repair by homologous recombination. The BRCA1 protein has a number of conserved domains, including a coiled-coil domain, two BRCT domains and an N-terminal RING (Really Interesting New Gene) domain. Many cancer-causing mutations have been reported that affect the BRCA1 RING domain, although the exact impact of RING domain mutations on DNA repair is not clear. We characterized a *BRCA1*-mutant mouse model, $BRCA1^{\Delta 2/\Delta 2}$, in which conditional deletion of exon 2 excises a large portion of the RING domain. $BRCA1^{\Delta 2/\Delta 2}$ cells have a defective G2/M cell cycle checkpoint after IR-induced DNA damage. In

addition, *BRCA1*$^{\Delta 2/\Delta 2}$ showed a high rate of genomic instability after treatment with agents that cause DNA double strand breaks or DNA interstrand crosslinks. Co-deletion of the *53BP1* (p53 Binding Protein 1) gene rescues some, but not all, phenotypes of mutation of the BRCA1 RING domain. By clarifying the importance of the RING domain in mediating BRCA1 activity, we aim to better understand why patient mutations in the *BRCA1* gene cause cancer predisposition and identify new targets for therapeutic intervention.

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Dedication

I dedicate my thesis to those who enlightened my life with their unlimited love and support, to those who dedicated their lives and who are the secret of my success, to my parents.

To all my gorgeous family members , my brother and his wife ,my sister and to my wonderful fiancée for their countless kindness, love and devotion.

To my great friends and relative who showed their unlimited support, passion, and care.

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1. Introduction

Breast Cancer 1, early onset (*BRCA1*) is a vertebrate tumor suppressor gene. Its protein product, BRCA1, is a multifunctional protein with important roles in the DNA damage response, cell cycle checkpoint activation, chromatin remodeling, transcriptional regulation (Wu, Lu, & Yu, 2010), and homologous recombination (J. Zhang, 2013).

Breast and ovarian cancer are the second- and fifth-leading causes of death among women in the United States (Siegel, Ma, Zou, & Jemal, 2014). 5-10% of breast and ovarian cancer cases (Malone et al., 1998) involve a germline mutation of either *BRCA1* or *BRCA2*. These mutations cause an increased lifetime breast cancer risk of 40-80%, and of 11-40% for ovarian cancer. To a lesser extent, BRCA1 mutations also cause an increased risk of pancreatic cancer (1-7% increase in lifetime risk), prostate cancer (up to 39%) and male breast cancer (1-10%) (Figure 1) (Petrucelli, Daly, & Feldman, 1993).

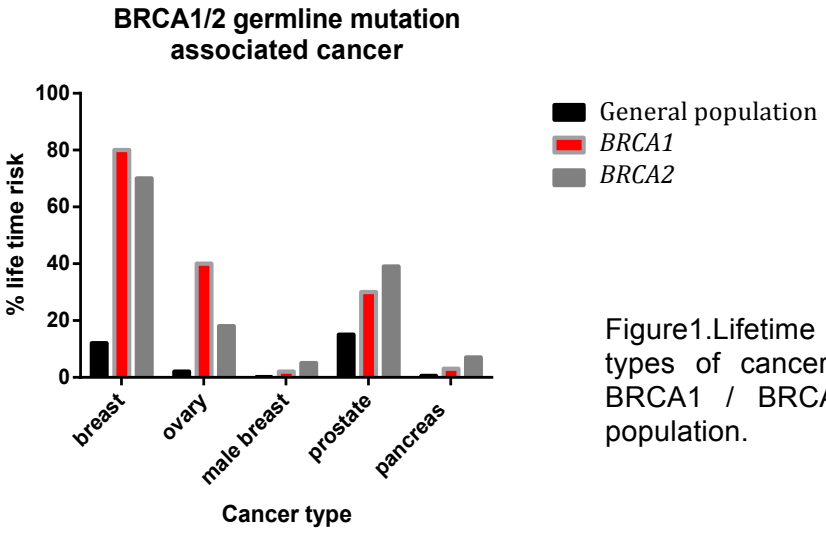


Figure1.Lifetime risk of developing different types of cancers associated with mutant BRCA1 / BRCA2, compared to general population.

Individuals with *BRCA1* mutations carry one mutated copy of the gene. Cancer arises from cells in which the second, functional copy of the *BRCA1* gene becomes mutated. (C. F. Xu & Solomon, 1996). Cancer therefore arises in cells with somatic loss of BRCA1 function. Thus, understanding the functions of BRCA1 in maintaining genomic stability is a major concern for prevention and treatment of human pathology associated with *BRCA1* mutation. In this study we characterize the function of a specific domain of BRCA1 to try to gain insight into BRCA1 function.

1.1 The *BRCA1* gene

Genetic linkage analysis of families with breast cancer in the early 1990s revealed the location of the breast and ovarian cancer susceptibility gene, *BRCA*1, on the long arm of chromosome 17 (17q21) (Hall et al., 1990). The BRCA1 gene spans about 81 kbp within a genomic region that harbors a high density of repetitive Alu DNA sequence (Smith et al., 1996). The *BRCA1* gene consists of 24 exons of which 22 are protein coding exons. The *BRCA1* transcript that encodes full-length protein is a 7.8 kbp messenger RNA (C. F. Xu & Solomon, 1996). In mice, it is normally spliced into four different splice variants.

1.2 BRCA1 Protein Interactions

Full-length BRCA1 protein is a ~220 kDa protein. It contains 1863 amino acids and four conserved protein domains. A RING (Really Interesting New Gene) domain is found at the N-terminus, a coiled coil motif is present in the central part of the protein, and two BRCT (BRCA1 C-terminus) domains are found at the C-terminus (Figure 2). The BRCA1 protein also contains putative nuclear localization signals and a large serine-containing domain (SCD):

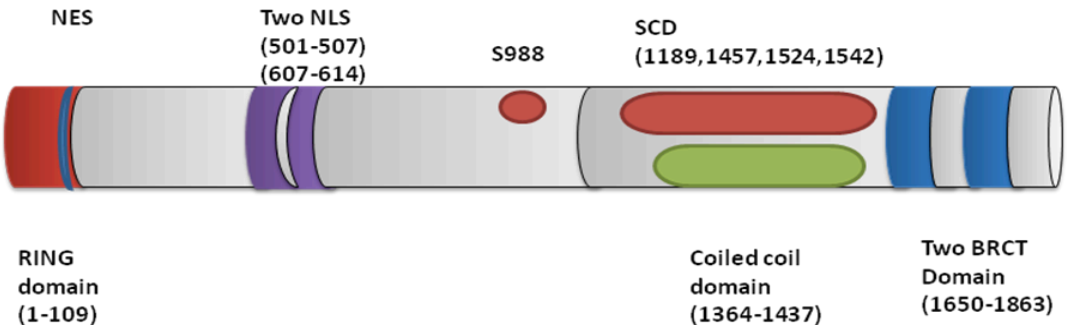


Figure 2. Domains of BRCA1 protein

The BRCA1 RING domain corresponds to residues 24-64. It contains two zinc (Zn^{2+}) ions at two sites: Site 1, in which four cysteine residues (Cys) residues coordinate the Zn metal center; and Site 2, in which the metal center is coordinated by three cysteine residues and one histidine residue (His). The zinc binding residues are flanked by two α-helices (Figure 3a). The RING domain is highly conserved in many proteins, and is often associated with E3 ubiquitin ligase activity. The RING domain of BRCA1 makes a protein-protein interaction

with the RING motif of a second protein, BARD1 (BRCA1-associated RING domain protein 1). These proteins form a heterodimer, with the interaction surface spanning residues 1-109 and 26-119 in BRCA1 and BARD1 respectively.

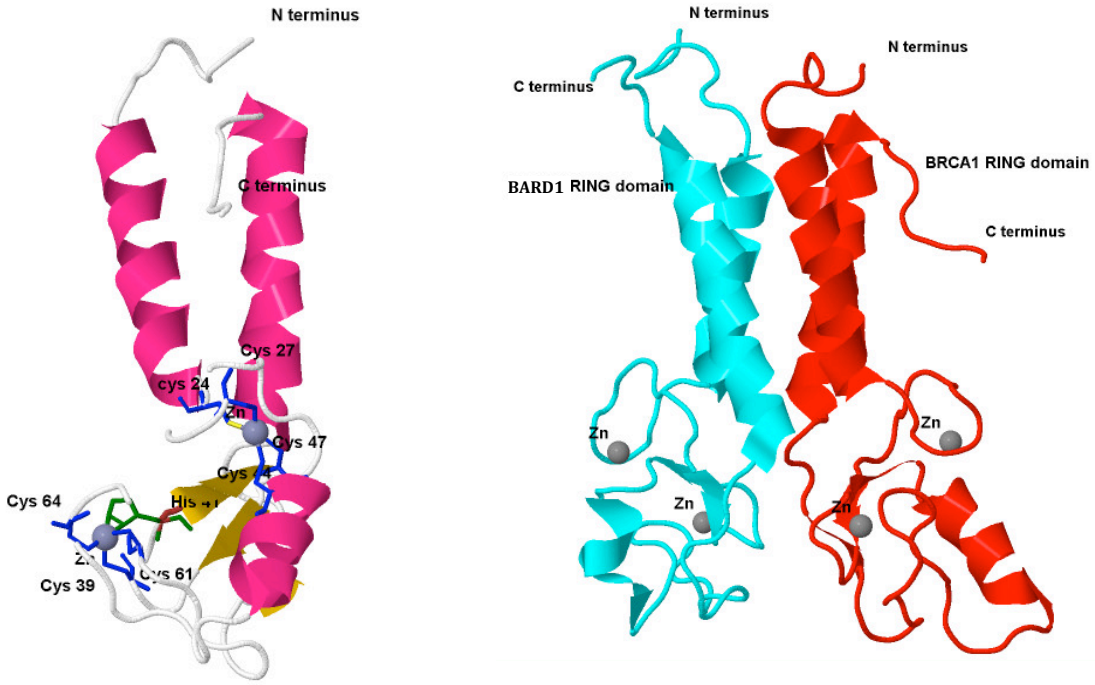


Figure 3.a) BRCA1 RING domain showing two Zn atoms surrounded by four Cys amino acid in Site 1 (Cys 24, Cys 27, Cys 44, Cys 47) and three Cys amino acids (Cys 39, Cys 61, Cys 64) and His 41 at Site 2. There are three anti-parallel β sheets with a central α-helix in the RING finger structure, which is flanked by two α helices for which N and C termini are shown. b) Heterodimeric association of BRCA1/BARD1 RING domains. This model is derived from Protein Data Bank (PDB) number 1JM7 modified by JMOLE .

Although BRCA1 and BARD1 RING domain constructs can form homodimer structures, the proteins preferentially form a stable heterodimer *in vivo* (Figure 3b) (Meza, Brzovic, King, & Klevit, 1999). Heterodimeric BRCA1-BARD1 has significantly greater E3 ubiquitin ligase activity than either protein by itself, although the significance and cellular substrates of the E3 ligase activity are not

clear. The ubiquitylation activity of BRCA1 has been reported to maintain genomic stability by inhibiting the association of ɣ-tubulin with centrosomes (Sankaran, Starita, Groen, Ko, & Parvin, 2005). A reduction in ubiquitylation of histone H2A in cells lacking BRCA1 has also been reported to lead to disruption of heterochromatin integrity, checkpoint defects and genomic instability (Zhu et al., 2011). These reports suggest a mechanistic basis for the strong relationship between mutation of BRCA1 and cancer development (Hashizume et al., 2001).

The BRCA1 coiled coil (CC) domain shows a direct interaction with PALB2 which is essential for RAD51 loading at DNA double-strand breaks. Rad51 loading is essential for homologous recombination, and is promoted by BRCA2 activity. BRCA2 is stabilized at the break site, by interaction with BRCA1-PALB2, demonstrating the importance of the BRCA1 CC domain. (Sy, Huen, & Chen, 2009).

The BRCA1 BRCT domains also form binding sites for protein-protein interactions between BRCA1 and several phospho-proteins. The two domains are linked head to tail forming a hydrophobic pocket between their adjacent helices (Figure 4) (Huen, Sy, & Chen, 2010). Three protein complexes form by association with the BRCA1 BRCT domain (Wang et al., 2007). These are complexes containing either Abraxas protein, BACH1 (BRCA1 –associated c-terminal helicase) or CtIP (CtBP-interacting protein) (Huen et al., 2010). Each of these proteins has been shown to be required for normal DNA repair. The

BRCA1 BRCT domains also bind to chromatin remodeling factors including human histone deacetylase enzymes (HDAC1/HDAC2) (Anderson, Schlegel, Nakajima, Wolpin, & Parvin, 1998; Yarden & Brody, 1999).

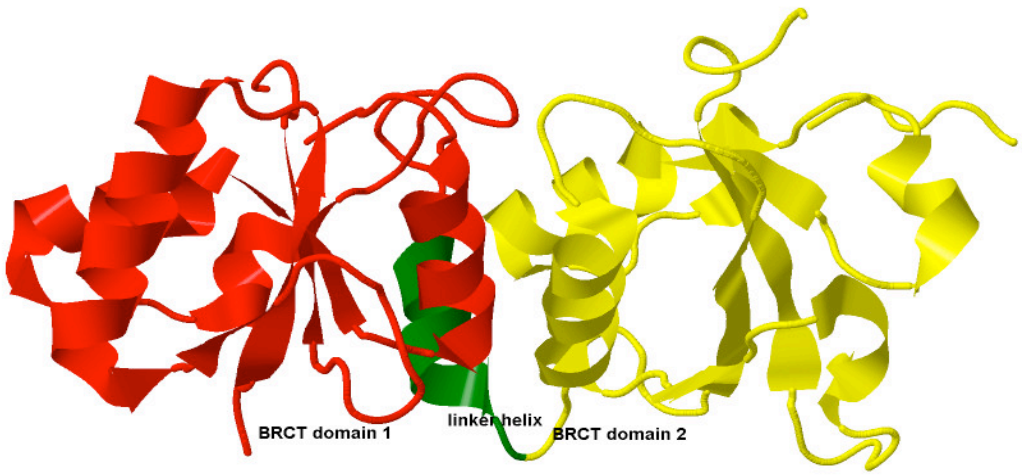


Figure 4. BRCT domains of BRCA1 linked by α-linker helix. Each domain contains four anti-parallel β sheets surrounded by three α-helices.

1.3 Role of BRCA1 in DNA Repair

Many forms of DNA damage affect cells at all times as a consequence of regular cellular metabolism and environmental mutagens. DNA double-strand breaks (DSBs) are among the most dangerous form of DNA damage. DSBs are caused by endogenous factors like reactive metabolites and replication fork collapse, or from exogenous ionizing radiation, UV light or certain chemical agents. Any error in DSB repair could cause loss of chromosomal material,

changes in sequence, or translocations, leading to potential tumor formation (Khanna & Jackson, 2001).

The cellular response to DSBs is orchestrated by DSB sensors and mediators that promote effective repair of DNA damage (Polo & Jackson, 2011). Activation of the DSB response through MRN-ATM or ATRIP-ATR causes phosphorylation of the H2AX histone variant on Ser 139 to form γ-H2AX. A number of protein factors are recruited to DNA sites marked by γ-H2AX, including MDC1 (Mediator of DNA damage check point 1) (Stucki et al., 2005) and the E3 ubiqutin ligases RNF8 and RNF168. Lys63-linked ubiquitin chains formed on histones around break sites by RNF8 and RNF168 recruit BRCA1 to DSB repair sites. (Bin & Elledge, 2007). BRCA1 is recruited to ubiquitylated histones around DSBs as part of a complex involving the proteins Abraxas, BRCC36, MERIT40 and RAP80 (Liu, Wu, & Yu, 2007) (Sobhian et al., 2007).

Repair of DSBs by the homologous recombination (HR) pathway proceeds through several stages (Figure 5). In the first step, the DSB is resected to form a 3’ single-stranded DNA overhang at the break site. RPA, and subsequently Rad51 is recruited to the resected DSB, allowing the broken DNA to pair with homologous sequence and form a Holliday Junction. Sequence is copied across the break site, and subsequent dismantling of the Holliday Junction structure restores the original DNA sequence. BRCA1-deficient cells have defects in HR, as seen by their failure to assemble Rad51 at break sites (Scully et al., 1997) and

their tendency to accumulate chromosome aberrations after treatments that cause DNA damage.

BRCA1 has been reported to act at a minimum of two steps of HR. First, it was reported to interact with CtIP, a protein factor that promotes resection (Yun & Hiom, 2009). This interaction is mediated through the BRCT domain of BRCA1, which binds to phosphorylated CtIP. A failure to recruit CtIP and a subsequent reduction in CtIP-mediated resection would account for the Rad51 recruitment defect seen in BRCA1-deficient cells. However, a specific mutation of the CtIP phosphorylation site that mediates the interaction with BRCA1 was recently reported to cause no effect in terms of increased chromosome aberrations (Reczek, Szabolcs, Stark, Ludwig, & Baer, 2013). Second, BRCA1 binds PALB2, the Partner and Localizer of BRCA2, through its coiled-coil motif (Sy et al., 2009; F. Zhang et al., 2009). PALB2 is essential for normal accumulation of BRCA2 at DSBs, which in turn is essential for appropriate loading of Rad51. Absence of BRCA1, as occurs in patients with biallelic BRCA1 mutations, is therefore likely to seriously undermine normal cellular processes for repair of DSBs by HR. The absence of normal BRCA1 activity is known to lead to chromosome mutations because of the action of nonhomologous end-joining, an alternative, error-prone pathway for DNA repair (Bunting et al., 2010).

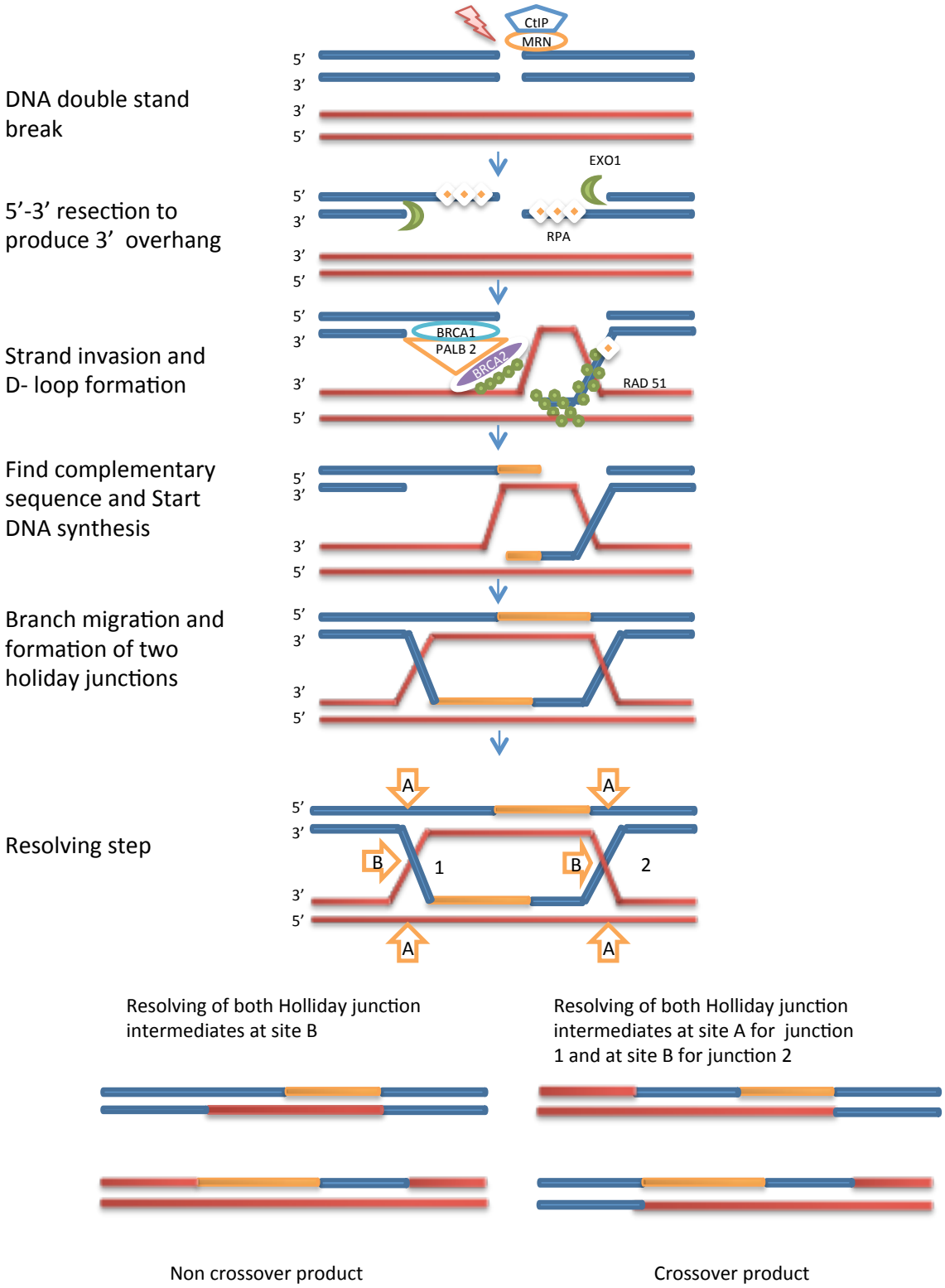


Figure 5: Mechanism of HR, showing two ways to resolve the Holliday Junction intermediates, leading to formation of crossover and non-crossover products.

1.4 BRCA1 role in cell cycle checkpoints

Activation of the kinases ATM and ATR following DNA damage causes a cell cycle checkpoint at the G2/M transition, termed the G2M checkpoint. In the absence of BRCA1, this checkpoint is defective, leading to inappropriate entry of cells containing DNA damage into mitosis. BRCA1 was reported to be necessary for the activation of the kinase Chk1(Yarden & Brody, 1999). Chk1 activates the G2M checkpoint by regulating the activity and nuclear localization of cdc25c and the cdc2/cyclin b kinase, which directly regulate the G2/M transition. The mechanism of BRCA1's action in regulating the G2M checkpoint is unclear. BRCA1 may work as a scaffold protein at the site of DSBs or induce ubiquitylation modifications that enhance ATM signaling and Chk1 activation (Huen et al., 2010).

1.5 Summary

Although several protein-protein interactions formed by BRCA1 have been described, the exact mechanism by which BRA1 mediates homologous recombination remains unclear. In particular, the importance of the RING domain, which has E3 ubiquitin ligase activity and also mediates interaction of BRCA1 and BARD1 is a subject of considerable uncertainty. A recent report showed that the E3 ubiquitin ligase activity of BRCA1 was essential for mediating all the known functions of BRCA1 (Zhu et al., 2011). In this case the E3 activity was reported to be necessary for silencing transcription of satellite DNA repeats.

In the absence of H2A ubiquitylation at satellite repeats, ectopic expression of these sequences was shown to cause all the phenotypes associated with loss of BRCA1 function.

On the other hand, a mouse model featuring a point mutation which disturbs the E3 ubiquitin ligase activity of BRCA1 was shown to cause only minor phenotypes in genetically targeted animals. The authors reported that mutation of the BRCA1 BRCT domains, which mediate interactions with phosophoproteins relevant for DNA repair (Figure 6), is significantly more important for tumor predisposition associated with loss-of-function of BRCA1 (Shakya et al., 2011). There is

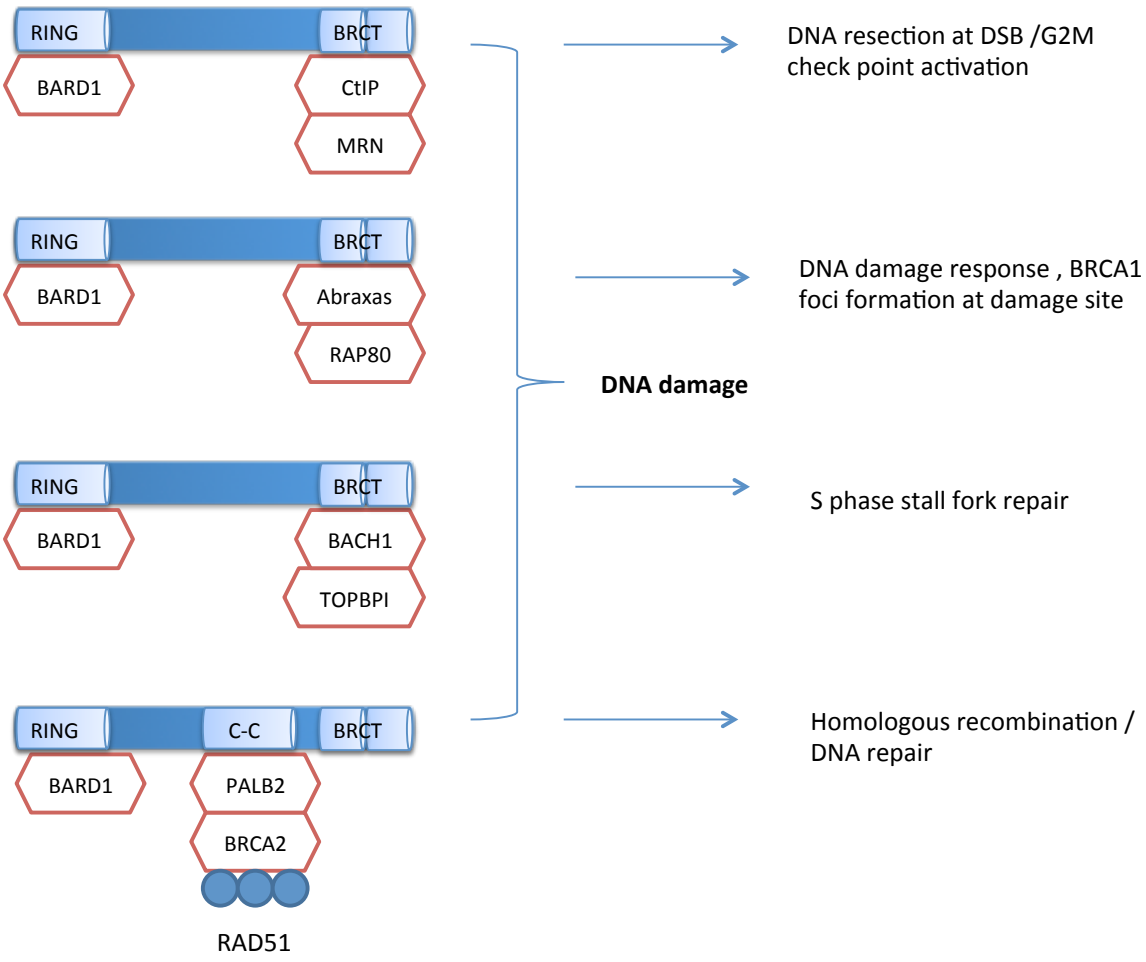


Fig.6 BRCA1 complexes and their role in DNA damage response

therefore a major discrepancy in the field relating to whether the RING domain is essential for BRCA1 activity .

In this study we describe a BRCA mutant mouse in which BRCA1 is expressed at a normal level but in which the RING domain of BRCA1 is absent. Our hypothesis is that the BRCA1 RING domain is essential for BRCA1 activity. We tested this hypothesis using a variety of cellular and molecular techniques as described in the next section.

2. Materials and methods

2.1 B cell isolation and activation :

Spleens were dissected from mice of the appropriate genotypes and broke up in a 35 x 10 mm tissue culture plate (Falcon) containing 2 ml of B cell wash buffer 1x Hank's solution (Sigma H8264), 1% fetal bovine serum (FBS) (Gemini 100-106), and pencillin/streptomycin (Gibco 15070063)). After filtration through a 70μm cell stainer (BD Bioscience), 8 ml wash buffer was added to the cells and samples were spun down using an Eppendorf Centrifuge 5810R, at 1,200 rpm, for 10 min at 4 $^{\circ}$C. Red blood cells (RBC) were lysed by resuspending pellet for 5 min in 3ml of ACK Lysing Buffer (Fisher Scientific 118-156-721). Following one further wash step with wash buffer, the cell pellet was resuspended in 1 ml wash buffer and incubated on ice with anti-CD43 MACS micro-beads (Miltenyi Biotech) for 30 min. Samples were allowed to pass through a Mini-MACS separation column (Miltenyi Biotech) attached to a MACS magnet to remove T cells from the cell suspension. B cells were collected and resuspended in a total volume of 10ml wash buffer. A 10 µl sample volume were stained with 10 µl volume of 0.4% Trypan Blue stain (Gibco) so that dead cells could be excluded during counting. Cells were counted using a hemocytometer (Fisher Scientific) and resuspended at 1 x 10^6 cells/ml in B cell medium containig 500 ml 1x RPMI-1640 (Life Technologies), 10% FBS (Gemini), 1x pencillin /Streptomycin, 2mM L-Glutamine (Gibco 25030-081), 1x MEM Non-essential amino acidd (Gibco), 1mM Sodium Pyruvate (Lonza), 1.8 µl of 14.2M 2- mercaptoethanol (BIO-RAD), and 10mM of

1M HEPES (Sigma-Aldrich). Resting B cells were stimulated by adding LPS (E.coli 0111:B4;Sigma) to a final concentration of 25 μg/ml final concentration, and Interleukin-4 (Sigma I1020, for 50 U/ml final concentraton). For class switch recombination assays, a 1:1000 dilution of purified anti mouse anti-CD180 antibody was also added (BD pharmingen 552128). Cells were plated in 6 well plates (Costar 3516) and incubated at 37 ºC in an incubator supplied with with 5% CO_2 for 48 hrs.

2.2 mRNA and cDNA preperation:

B cells pellets were collected, washed with 1x PBS (Sigma-Aldrich D8537) and transferred to 1.5 ml centrifuge tubes. After a second spin in a bench-top Eppendorf refrigerated centrifuge 5430R, the cells were lysed and total RNA was prepared using the RNeasy Mini Kit (Qiagen 74104). Total RNA concentration was measured using am Eppendorf BioPhotometer plus spectrophotometer. cDNA was prepared by adding 1 µl 10 mM dNTP Mix (10 mM of 100mM each dATP, dGTP, dCTP and dTTP (Invitrogen #1374025, #1345231, #1374026, #1374027 respectively) to 10 pg–5 µg total RNA, 1µl 50 µM Random Hexamers (Invitrgen #N8080127), and RNase-free water to a total volume of 13 µl. The mixture was heated at 65º C for 5 min and incubated on ice for 1 min. 1 µl of 200 units/µl SuperScript III reverse transcriptase, 1µl of 0.1M DTT, 1µL 5x first strand buffer (Invitrogen #18080-093) were added to the

mixture, heated to 50ºC for 30 min, then at 70ºC for 15 min using S1000 Thermal Cycler (BIO-RAD).

2.3 Quantitative RT-PCR and Primers :

A mixture was prepared for each well containing 10 µl SYBR® Green PCR Master Mix (Applied Biosystems 4309155), 0.167µl of each 15µM oligonucleotide primer stock, 1µl cDNA, and RNase-free water to a total volume of 20µl. Each PCR was set up in triplicate in a 96-well Thermal Grid Mini Skirt PCR plate (C18080-10). PCR was performed using an ABI PRISM 7000 Sequence Detection System running SDS 2.3 software.

The sequences of the oligonucleotide primers used are as follows:

Exon1F	5’-GCGCTTGGAAGTACGGATCTT-3’,
Exon3R	5’-ATATGTGGTCACACTTTGTGG-3’ ,
Exon2R	5’-CAGATCGGACACTCTAAGATT-3’,
Exon6F	5’-TT GAATGAGGAGGCGTCGATCA-3’ ,
Exon7R	5’-ATTTCTTTCGAGGTTGGGTC TG-3’,
Exon10F	5’-GGAATTGTTACAGACCGCCCCTCA-3’,
Exon11R	5’-GCT CACACACACATTTGAA-3’,
Cyclophilin F	5’ 5’-GGCCGATGACGAGCCC-3’,
Cyclophilin R	5’-TGTCTTTGGAACTTTGTCTGCAAAT-3’,
Major Satellite F	5’-GGCGAGAAAACTGAAAATCACG-3’,

Major Satellite R 5’-CTTGCCATATTCCACGTCCT-3’,

Minor Satellite F 5’- TTGGAA ACGGGATTTGTAGA-3’,

Minor Satellite R 5’-CGGTTTCCAACATATGTGTTTT-3’.

2.4 Immunoblotting:

B cells were collected 48 hrs after LPS and IL-4 ativation. Cell pellets were lysed in 100 µl 2x Laemmli buffer (prepared by mixing 4ml 10% sodium dodecyl sulphate (Sigma 71736), 2ml glycerol (sigma G2025), 1ml 1M Ultra Pure Tris HCl-pH 7.5 (Invitrogen 15567-027), and 3ml dH_2O. 250µl 1M glycerol-2-phoshate and one protease inhibitor tablet (Roche 11836170001) were added to 5 ml of the 2x Lammli buffer mix. Cells resuspended in lysis buffer were heated at 99ºC for 10 min using a shaking heating block (Fisher Scientific), mixed, heated again at 99 ºC for 5 min, spun down at 13000 rpm, for 15 min. Supernatants were collected and stored at -20ºC. Thermo Scientific™ Pierce™ Bovine Serum Albumin (BSA) Standards (PI-23209) were used to make 9 standard solutions of BSA with dH_2O ranging from 0 µg/µl – 2 µg/µl final concentration. All samples were mixed with 1 ml of a 1:50 mix of BCA™ Protein Assay reagents A and B (Thermo Scientific™ Pierce™ PI-23221) and heated at 60ºC for 30 min. Color development was measured with an Eppendorf Biophotometer spectrophotometer.

Prior to running, a mixture was prepared for each sample with 0.5 µl of 1M DDT, 2.5µl 4xNuPAGE® LDS Sample Buffer (NP0008) and 30 µg protein with dH2O to a 10 µl final volume . Protein samples were run at 100 V using NuPAGE® Novex® 4-12% Bis-Tris Protein Gels, 1.0 mm, 12 well for 4 hrs in a 1x NuPAGE® MOPS SDS Running Buffer. Protein was transferred to a nitrocellulose membrane overnight at 133 mA, 4ºC in 1.5L transfer Buffer containing 75 ml 20 x NuPAGE® Transfer Buffer, 300ml methanol and 1125 ml dH_2O. Membranes were washed in 1X Tris-buffered saline with 1% Tween 20 (TBST) (KPL 15-19-01) and blocked for 1 hr in a 5% ECL prime blocking agent in 1xTBST. Mouse polyclonal anti-BRCA1 primary antibody (Gift of Dr. A. Nussenzweig lab) was used at a 1:500 dilution. Mouse monoclonal anti-α-tubulin primary antibody (Sigma T9026) was used as a loading control at a dilution of 1:50,000. AmershamECL Anti-mouse IgG, peroxidase-linked whole antibody (from sheep, used at 1:2000) (GE Healthcare NXA931) and ECL detection reagent (Life Science) were used for detection of bound immunoglobulin.

2.5 Immunofluorescence

Slides were coated with Celltak reagent (BD 354240) one day before immunostaining using a mixture of 20% 2M sodium carbonate (Sigma 223484) and 2.5% isopropanol. 7µl Celltak coating mixture was added to each slide and allowed to dry for 30 mins. The slides were then washed with water, allowed to dry and kept at 4ºC ready for use. B cells were treated with 5 Gy ionizing

radiation after 48hrs growth in B cell medium with LPS and IL-4. After irradiation, the B cells were incubated at 37º C for 2hrs recovery phase. Where appropriate, 5-ethynyl-2'-deoxyuridine (Click-iT® EdU Alexa Fluor® 555 Imaging Kit; Life technologies C10338) was added for the last 15min of recovery time. To prepare slides for immunofluorescence, B cells were resuspended in 60µl 1x PBS (Sigma D8537) and applied to a CellTak slide. Slides were incubated at 37°C in a humidified chamber for 30 min to allow cells to settle, after which the cells were fixed using 2% paraformaldehyde (Thermo 28908) in a Coplin Jar for 5 min at RT.

After fixation, cells were washed twice with 1xPBS and permeabilized with 0.5% Triton X-100 in PBS for 5 min at RT. After two washes with 1xPBS, 50µl of IF blocking buffer (1% BSA; Sigma #A7906, 5% goat serum; Sigma #G9023, in 1xPBS) containing anti-53BP1 (Novus Biologicals NB100-304) antibody was applied to each slide at 1:2000 dilution. Cells were covered with an 18mm x 18mm cover slip and incubated at 37 °C for 1 hr in a humidified chamber Then cells were washed three times with 1xPBS. For secondary antibody staining, 50µl of IF blocking buffer containing of Alexa Fluor-488 goat anti-rabbit IgG secondary antibody (ab150077) at 1:200 dilution were added and incubated in the same way at 37°C for 1hr. Prior to mounting, the cells were counter-stained with 80 ng/ml final concentration DAPI stain dissolved in 2x SSC (Sigma S6639) for 5 min on an orbital shaker. 40 µl Mowiol mounting medium was added to each

slide and 24x60mm cover slips were applied on the slides and allowed to harden overnight.

2.6 G_2/M cell cycle checkpoint assay

After 48hrs growth at 37ºC, B cells were treated with 5 Gy ionizing radiation and then harvested 1 hr after irradiation, washed in 1xPBS and fixed in 70% ethanol at -20º C for 24 hrs. After fixation, cells were resuspended in 1 ml of 0.25% Triton X-100 in PBS and incubated on ice for 5 min. Cells were washed with 1xPBS, then resuspended in 100µl of PBS containing 1% BSA (Sigma A7906) and 0.75 µg of anti-pSer10-Histone H3 (Millipore 06- 570). Staining was for 3hrs at RT. Cells were washed with 1x PBS/BSA, then resuspended with 10 µl of 1.0 mg/ml Propidium iodide (Sigma P4864) and 0.1mg of 20 mg/mL RNase A (Invitrogen 12091-021) at 37 º C for 30 min in the dark. Mitotic cells were measured using a Becton Dickinson FACSCalibur instrument and data were analysed by FlowJo software.

2.7 Class switch recombination assay

B cells were grown in medium containing LPS, IL-4 and anti-CD180 antibody for 96 hrs Incuabtion period. Cells were spun down and resuspended in 4ml of 1xPBS in polystyrene round bottom 75mm tube. Pellets were blocked with 5µl of purified anti-mouse CD16/CD32 clone 2.4G2 (BD pharmingen 553141) for 5 min

at RT. Biotin-Rat anti-mouse IgG1 (BD Pharmingen 550331) and FITC-Rat anti-mouse CD45R/B220 (BD Pharmingen 553088) were added (10 µl of 1:100 dilution for both) and cells were incubated at RT for 45 min.10 µl of 1:100 streptavidin-Alexa Fluor647 secondary antibody (Life technologies S-21374) was added to each tube with incubation for 15 min at RT. Samples were washed with 4ml of 1x PBS, spun down and resuspended in 1ml PBS. The cells were filtered through 70µm cell strainer (BD Bioscience) and measured with a Becton Dickinson FACSCalibur instrument. Data were analysed by FlowJo software.

2.8 Metaphase fixation

To induce DNA damage to B cells, a final concentration of 2µm Olaparib (Selleck chemicals) or 250 nM Mitomycin C (Sigma) was added overnight prior to metaphase fixation. B cells for metaphase analysis were collected after at total of 48 hrs in culture. 10 µl/ml of 10µg/ml Roche Colcemid solution (295892) was added 1 hr before cell harvesting to arrest cells at metaphase. Cells were collected in 15 ml Falcon tubes, spun down at 4ºC, 1200 rpm for 10 min, and resuspended in ~1ml residual volume. Then, 5 ml of prewarmed 0.075 M KCl (Sigma p9333) was added drop by drop while tapping tube to ensure proper mixing. More KCl was added to a total volume of 15 ml. Cells were incubated in 37ºC water bath for 15 min with the hypotonic KCL solution to swell cells prior to fiaxation. Cells were centrifuged, then resuspended in a 3:1 v/v mix of methanol (J.T.Baker) and acetic acid (J.T.Baker), which was also added drop by drop while

mixing to prevent cell clumping. The initial fixation was at RT for 30 min. Two more rounds of spinning and fixation peformed, without the 30 min incubation step. After the final spin, fixative was add to a 15 ml final volume and caps were sealed with parafilm and stored at -20ºC overnight. The next day, samples were spun down and resuspended in ~70 µl reisdual volume, then dropped onto microscope slides at 22.9º C/52% humidity using CDS-5 model Thermotron controlled environment chamber. The metaphase slides were kept in a 37ºC incubator overnight, or until FISH could be peformed.

2.9 Fluorescence In Situ Hybridization (FISH):

To prepare probes for PNA FISH, 2µl of a Cy3-labeled peptide nucleic acid telomere probe (Cy3-CCCTAACCCTAACCCTAA) was mixed with 7µl deionized formamide (Ambion 9342) at 37ºC in a Thermomixer with shaking for 1 hr. 7µl prewarmed FISH Master Mix is then added to the mixture and incubated at 37ºC for a further 1 hr period in the Thermomixer. (FISH Master Mix is prepared by mixing 40 ml of 50% dextran sulfate (Intergen S4030), 20 ml of 20x SSC (Sigma S6639) and 40 ml dH_2O). Probes are then denatured at 80ºC without shaking, and then incubated at 37ºC for pre-annealing for a further 60 mins.

Meanwhile metaphase chromosomes on microscope slides were pretreated for hybridization. The slides are first placed in a Coplin Jar with 2xSSC for 5 min at RT. 2 µL 100mg/ml pepsin (Sigma P6887-5g) is added to a second prewarmed

Coplin Jar at 37ºC. 60 ml of 0.01M HCL (Sigma H9892), prewarmed to 37 ºC, was added to make a solution of pepsin. Slides were incubated in this mixture for 90 sec, washed with 1xPBS two times for 5 mins, then for a further 5 mins in 0.05M $MgCl_2$ (J.T.Baker 2444-01) in 1xPBS. Slides were then fixed in 1% formaldehyde in 1xPBS/0.05M $MgCl_2$ at RT for 10 min. After washing with PBS, slides were dehydrated using a series of 70%, 90%, and 100% ethanol solutions in which slides were incubated for 3 min each. Dehydrated slides were air-dried.

To denature chromosomes on microscope slides, 120µl 70% deionized formamide/2xSSC was applied to a 24mm x 60 mm coverslip and placed on top of each slide. Denaturation was at 80 ºC on a hot plate for 1.5 min. After denaturation, slides were dehydated in an ethanol series as before and allowed to dry. Using a humid chamber, pre-annealed probes were added to the slides and incubated at 37 ºC for 1 hr. Slides were then washed, first with 45ºC prewarmed 50% formamide / 2x SSC (three times for 5 min with shaking), then with 45ºC prewarmed 1xSSC (three times for 5 min with shaking) and finally with 45ºC prewarmed 4x SSC/0.1%Tween 20 (three times for 5 min with shaking). Slides were counter-stained in a Coplin jar containing 80ng/ml DAPI in 2xSSC for 3 min, then washed with 2xSSC for 5 min. 40 µL Mowiol solution was added to mount, using 24 mmx 60 mm coverslips. Images of metaphase chromosomes were captured automoatically using a Zeiss fluorescent microscope powered by Metasystems Autocapt software. Image analysis was performed with Adobe PhotoShop.

3. Results

3.1 The *BRCA1*^{neo} allele is transcribed to produce a variant *BRCA1* splice form lacking Exon 2 sequence.

In 1997, Dr Thomas Ludwig described a disruption of the mouse BRCA1 gene, which caused embryonic lethality in homozygous mice (Ludwig T, Genes Dev, 1997). This gene targeting involved replacement of BRCA1 exon 2 with a *neo* cassette (Fig. 7a). As the initiator codon for BRCA1 protein is found in exon 2, this allele has been considered to be a null allele of BRCA1.

More recently, research in our lab demonstrated that deletion of the *53BP1* gene was sufficient to rescue embryonic lethality in these *BRCA1*$^{neo/neo}$ mice (Bunting et al., 2012). While characterizing *BRCA1*$^{neo/neo}$,*53BP1*$^{-/-}$ mouse embryonic fibroblasts (MEFs), we observed that it was possible to amplify an RT-PCR product corresponding to *BRCA1* exon 11 (Fig 7b). This PCR product is not a genomic DNA PCR product, because it was not amplified from a control sample prepared without reverse transcriptase (RT). Using primer sets specific for other parts of the *BRCA1* gene, we found that *BRCA1*$^{neo/neo}$,*53BP1*$^{-/-}$ cells express a *BRCA1* mRNA in which exon 1 is spliced directly to exon 3, forming a splice form which does not include exon 2. In particular, with forward and reverse primers specific for exons 1 and 3, we amplified a 200bp product from *BRCA1*$^{+/+}$,*53BP1*$^{-/-}$ cDNA (Fig 7c, lane 3) and a 100bp product from

BRCA1$^{neo/neo}$,*53BP1*$^{-/-}$ cDNA (Fig7c, lane 1). The difference in size corresponds to the missing exon 2 in the *BRCA1*$^{neo/neo}$,*53BP1*$^{-/-}$ cells.

We were not able to amplify a product using RT-PCR with a forward primer specific for BRCA1 exon 1 and a reverse primer in the *neo* cassette, although we were able to detect *neo* transcription using a primer pair annealing within the *neo* gene (data not shown). Our results indicate that *BRCA1* and *neo* are transcribed independently, and RNA transcribed from *BRCA1* exon 1 is not spliced to the *neo* transcript.

To test whether the mRNA transcript of *BRCA1*^{neo} allele is translated, we made protein lysates from *BRCA1*$^{neo/neo}$,*53BP1*$^{-/-}$ MEFSs and performed Western blotting using antibodies against BRCA1 (Fig. 7d). As expected, a ~220kDa band was identified from WT cells, which was absent in *BRCA1*$^{\Delta 11/\Delta 11}$ mutant control cells (X. Xu et al., 1999). A band running at a slightly smaller size was detected in *BRCA1*$^{neo/neo}$,*53BP1*$^{-/-}$ MEFs, whereas *BRCA1*$^{neo/+}$,*53BP1*$^{-/-}$ cells showed two bands, which appear very close together at around 220kDa. We interpret the lower MW band observed with *BRCA1*$^{neo/neo}$,*53BP1*$^{-/-}$ and *BRCA1*$^{neo/+}$,*53BP1*$^{-/-}$ cells to be a product of the *BRCA1*^{neo} allele, presumably arising from translation initiating at a downstream, in-frame ATG site (see Discussion).

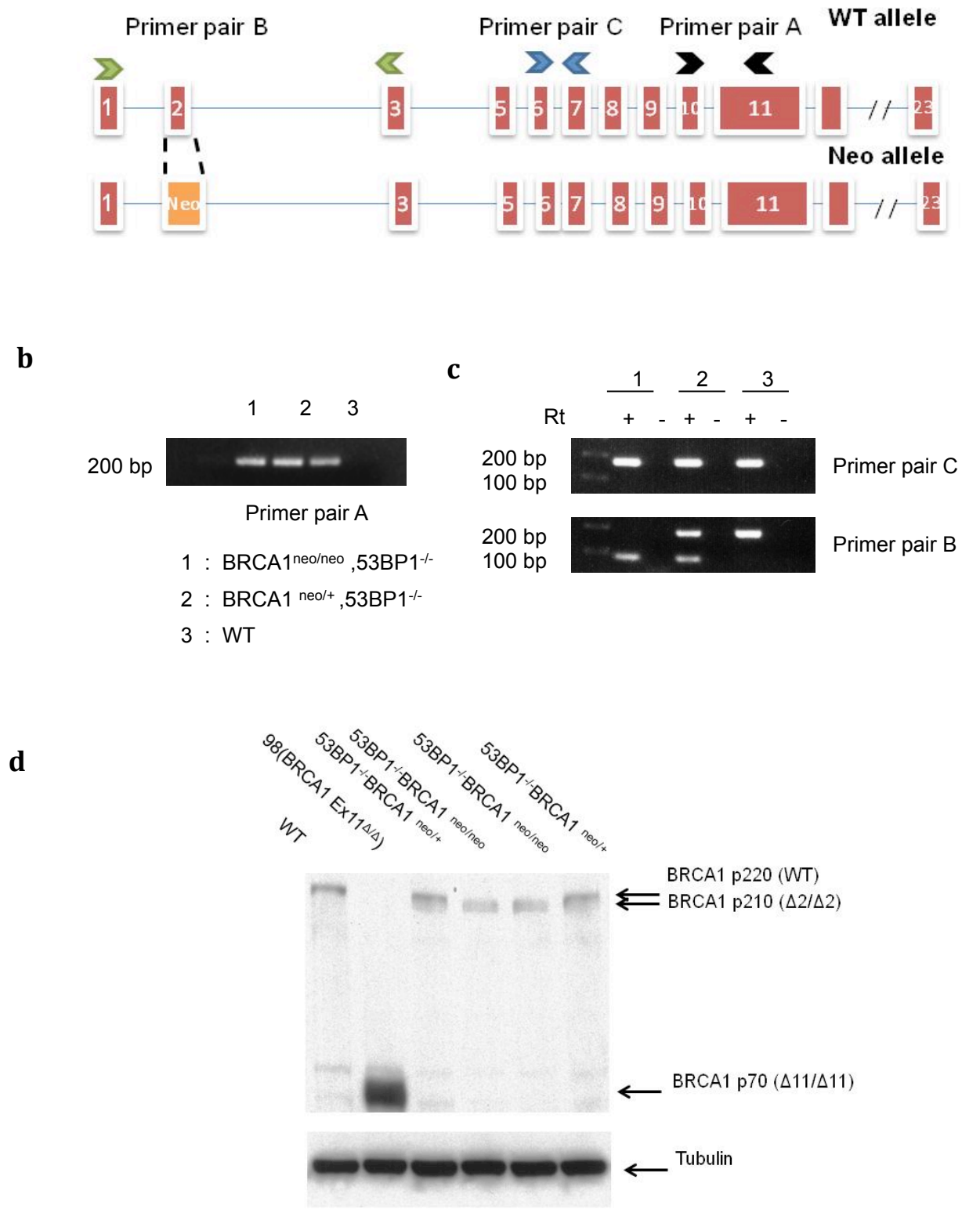


Figure 7. BRCA1^{neo} allele and BRCA1 RING mutant protein product. a) A gene map represents *BRCA1* wild type and *BRCA1*^{neo} allele along with three different pairs of primers pair (pair A 5' and 3'aligned to exons 10 and 11 respectively. Pair B 5' and 3' aligned to exons 6 and 7 respectively. Pair C 5' and 3' aligned to exons 1 and 3 respectively. b and c) RT-PCR gel images show BRCA1 allele amplification band using pair b and c primes +/- reverse transcriptase .d) A western blot showing protein bands of wild type and Neo BRCA1 protein product . Note: No exon 4 is recognized in current annotations of *BRCA1*.

3.2 Conditional deletion of *BRCA1* exon 2 produces a RING domain mutant allele

The BRCA1 RING domain is encoded in part by exon 2, hence a protein arising from an exon 2-deleted *BRCA1* transcript will not contain a functional RING domain. The importance of the BRCA1 RING domain is a major question in the field of DNA repair, as various reports indicate that E3 ligase activity associated with the RING domain is either absolutely essential (Zhu et al., 2011)or dispensable (Reid et al., 2008) for the role of BRCA1 in tumor suppression. The $BRCA1^{neo}$ allele is normally homozygous embryonic lethal, and homozygous animals can only be obtained by breeding to a $53BP1^{-/-}$ background. To study the importance of the RING domain on a $53BP1^{+/+}$ background, we therefore used a BRCA1 conditional allele, in which exon 2 is flanked by LoxP sites ($BRCA1^{flox2/flox2}$) (Fig. 8a). We hypothesized that conditional deletion of exon 2 would lead to production of a *BRCA1* transcript lacking exon 2, similar to the transcript that is produced from the $BRCA1^{neo}$ allele.

To characterize the BRCA1 transcripts produced from the exon2-conditional allele, we crossed conditional mice to a $CD19^{Cre}$ line in which Cre recombinase is expressed specifically in B cells. B cells were isolated from the spleens of conditional mice with $CD19^{Cre}$ and the mRNA product was tested by RT-PCR. We observed that these cells produce the same splice variant of BRCA1 as was observed with the $BRCA1^{neo}$ allele in which exon1 is spliced directly to exon 3. We call this allele $BRCA^{\Delta 2}$.

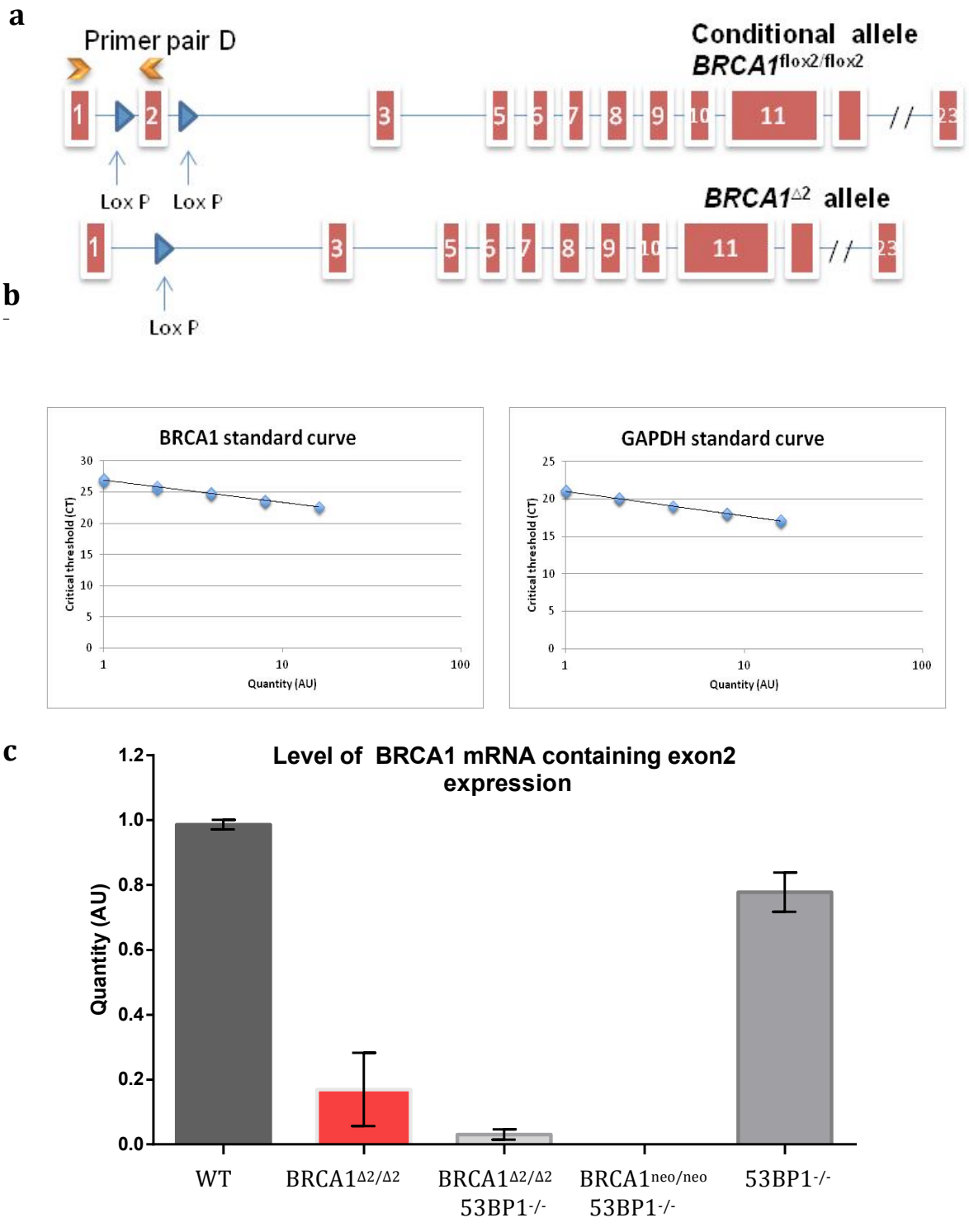


Figure 8. *BRCA1*$^{\Delta 2}$ allele and *BRCA1* mRNA containing exon 2 expression. a) A gene map represents *BRCA1* wild type and *BRCA1*$^{\Delta 2}$ allele. b, c and d) qPCR approach to amplify cDNA containing exon 2 from BRCA$^{\Delta 2/\Delta 2}$ and BRCA$^{neo/neo}$ cell type relative to GAPDH mRNA expression. Note: exon 4 of BRCA1 was mis-annotated formerly and does not exist.

To confirm that Cre-recombination leading to deletion of exon 2 was efficient in *BRCA1*$^{\Delta 2/\Delta 2}$ homozygous mice, we designed PCR primers to detect exon 2 sequence (Figure. 8a, Primer pair D). Quantitative PCR was performed using a SYBR Green reporter to measure the abundance of exon 2 sequence in cDNA from *BRCA1*$^{\Delta 2/\Delta 2}$ B cells. The relative quantification method was used to compare the abundance of exon 2 amplification between samples, with standard curves constructed to calibrate product amplification (Figure 8b). We detected *BRCA1* transcript containing exon 2 at around 10% and 2% expression of the WT level in *BRCA1*$^{\Delta 2/\Delta 2}$ and *BRCA1*$^{\Delta 2/\Delta 2}$,*53BP1*$^{-/-}$ B cells respectively (figure. 8c). We conclude that exon 2 is deleted in at least 90% of *BRCA1* alleles in *BRCA1*$^{\Delta 2/\Delta 2}$cells.

For further identification of the protein product in conditional deleted BRCA1 cells, we used B cell protein lysate for immunoblotting. *BRCA1*$^{\Delta 2/\Delta 2}$ cells gave a BRCA1 band that ran slightly lower than the BRCA1 band from WT cells (Figure 9). A fainter band corresponding to BRCA1 protein of the WT size was also detected in *BRCA1*$^{\Delta 2/\Delta 2}$ cells. This upper band could be explained because, as seen by quantitative PCR, deletion of exon 2 is not 100% efficient. The *BRCA1*$^{\Delta 2/\Delta 2}$ cells therefore mainly express a mutant form of BRCA1, but express a small amount of BRCA1 p220.

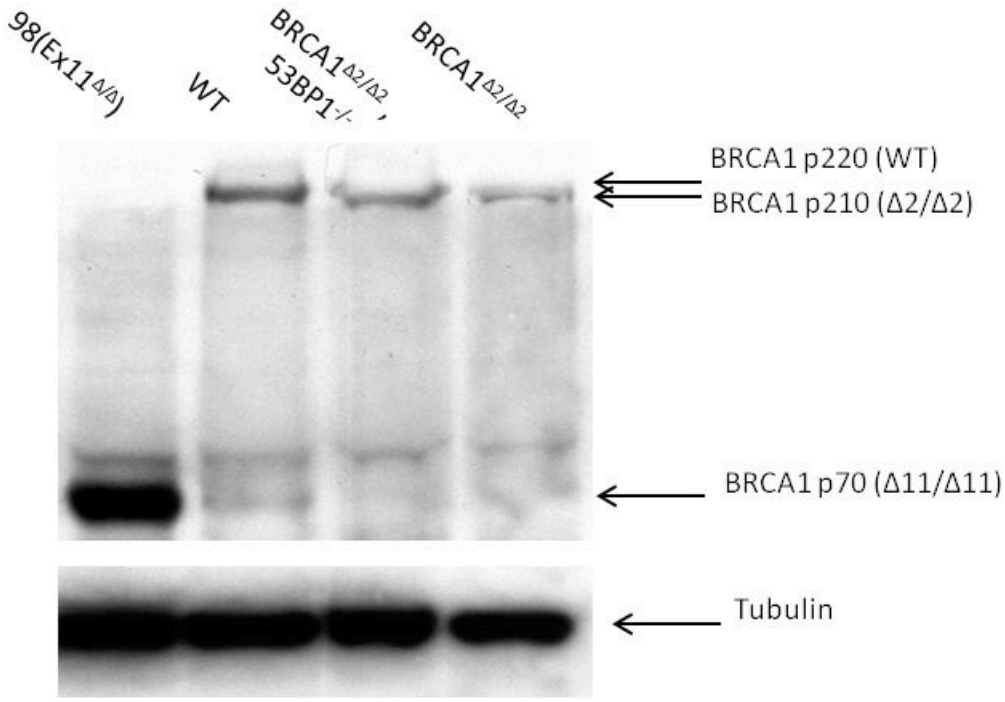


Figure 9. Western blot showing BRCA1 RING mutant protein band in B lymphocytes from either WT or BRCA1 exon 2-conditional ($BRCA1^{\Delta 2/\Delta 2}$) B cells. The size of the WT (p220) protein band and mutant protein product (p210) are shown. Samples from a cell line (98) that expresses a different BRCA1 allele, in which the very large exon 11 is deleted, are shown at left. (This cell line expresses a much smaller protein product, which runs around 70kDa.)

3.3 The BRCA1 RING domain is essential for genomic integrity.

BRCA1 has a significant role in tumor supression and homologous recombination-mediated DNA repair(Moynahan, Chiu, Koller, & Jasin, 1999) .BRCA1$^{\Delta 11/\Delta 11}$ cells show high sensitivity to DNA inter-strand cross-linking (ICL) agents and poly(ADP-Ribose) inhibitors (PARPi) (Bunting et al., 2012). PARP is important for detection and repair of DNA single-strand breaks, which arise spontaneously in all cells. PARPi treatment increases the frequency of DNA double-strand breaks in growing cells, because DNA replication forks collapse when they encounter single-strand breaks. Cells lacking functional DNA double-strand break repair activities are therefore hypsersensitive to PARPi treatment, because they cannot repair the increased load of double-strand breaks. For our studies, we have made use of olaparib, a potent inhibitor of multiple cellular PARP enzymes, which is currently a candidate for phase III clinical trials targeting cancers with suspected defects in homologous recombination.

To test whether the RING domain of BRCA1 has a role in maintaing genoming stability and DNA repair, we exposed B cells from *BRCA1*$^{\Delta 2/\Delta 2}$ mice, which express BRCA1 lacking the RING domain, to DNA damaging agents. After fixing chromosomes from *BRCA1*$^{\Delta 2/\Delta 2}$ B cells arrested at metaphase, we applied a telomere PNA-FISH staining technique to more easily identify chromosome breaks and aberrations (Figure 10). We observed that *BRCA1*$^{\Delta 2/\Delta 2}$ B cells that are exposed to Olaparib or Mitomycin C (MMC- which produces DNA

interstrand crosslinks) develop a significantly elevated rate of radial chromsome fusions, chromosome breaks (CSB), chromatid breaks (CTB) and DNA fragments (Figure 11) relative to WT controls. Chromosome aberrations are also detectable at a lower frequency in untreated *BRCA1*$^{\Delta 2/\Delta 2}$ cells. Our results indicate a requirement for an intact BRCA1 RING domain for the maintainance of genomic integrity.

We further observed a rescue of genomic instability after exposure to PARPi or MMC in *BRCA1*$^{\Delta 2/\Delta 2}$ cells in which *53BP1* was co-deleted. *53BP1* deletion was previously shown to be sufficent to rescue the hypersensitivity of hypomorphic BRCA1 mutant cells **(***BRCA1*$^{\Delta 11/\Delta 11}$) to PARP inhibition (Bunting et al., 2010). However, *BRCA1*$^{\Delta 11/\Delta 11}$ cells are still hypersensitive to MMC, even after deletion of *53BP1*, whereas *BRCA1*$^{\Delta 2/\Delta 2}$ cells show only mild sensitivity.

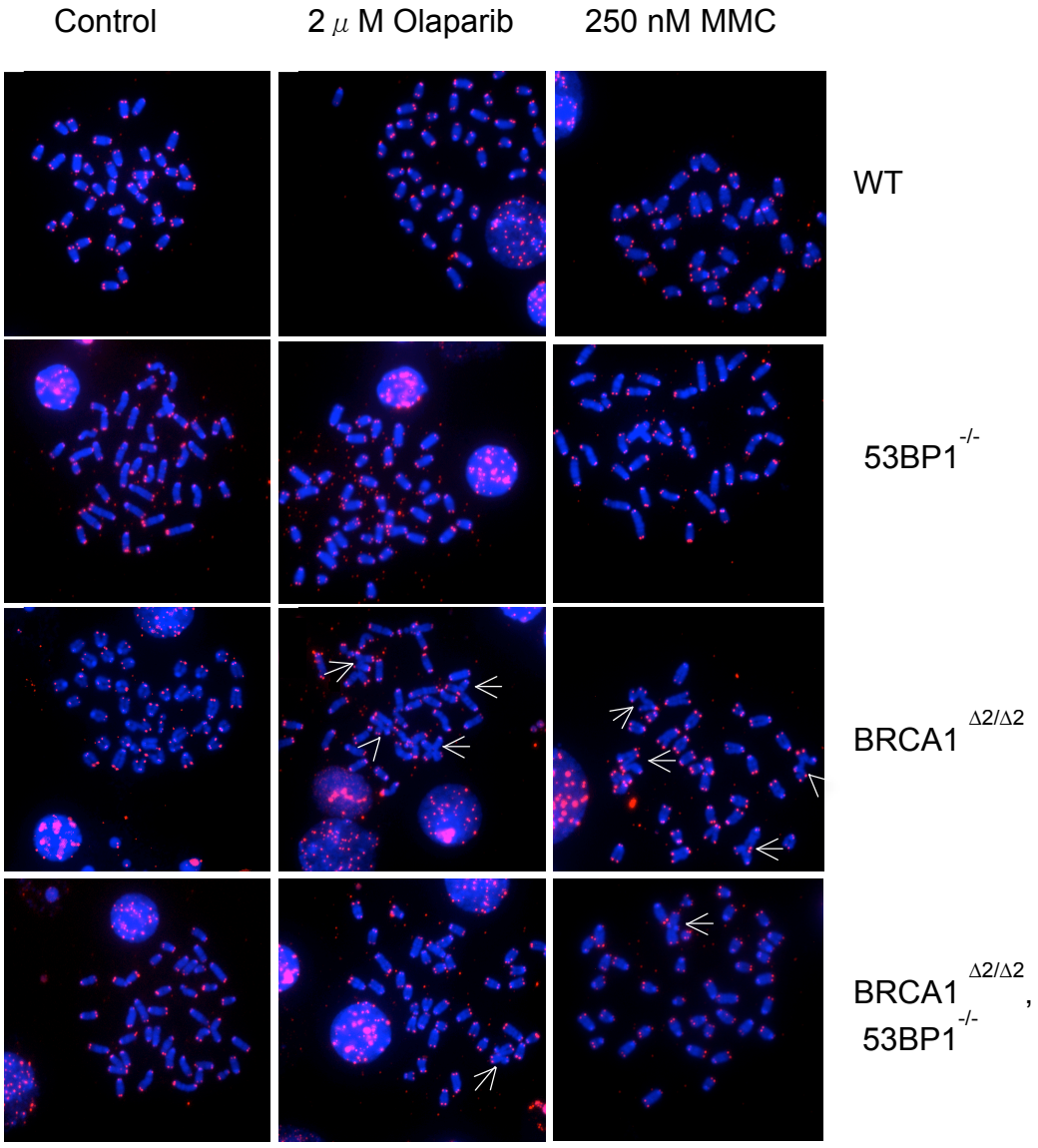


Figure 10. Telomere PNA-FISH images showing radial chromosome structures in B cell metaphase caused by applying PARPi (Olaparib) and DNA interstrand crosslinker (Mitomycin C).

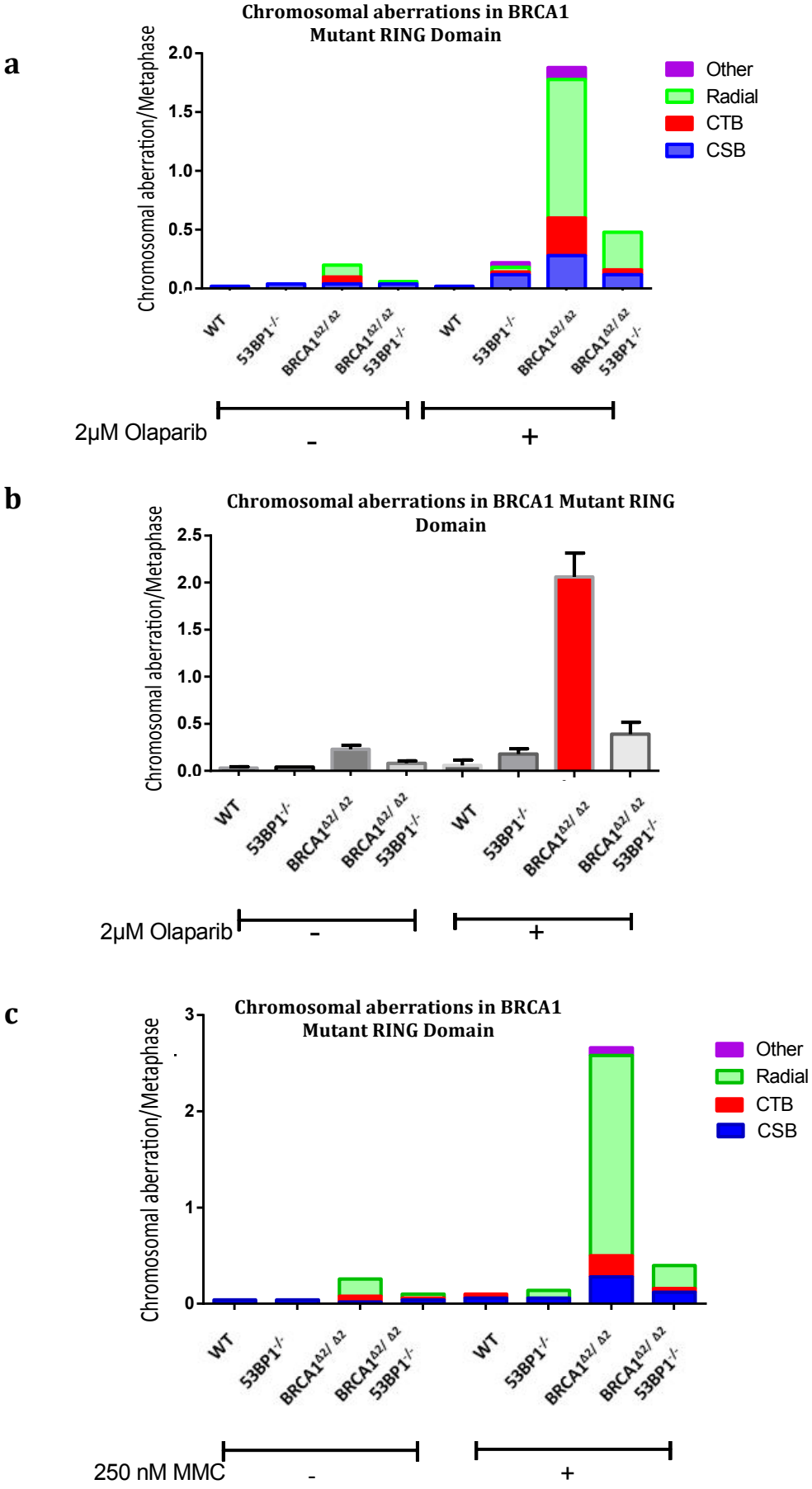
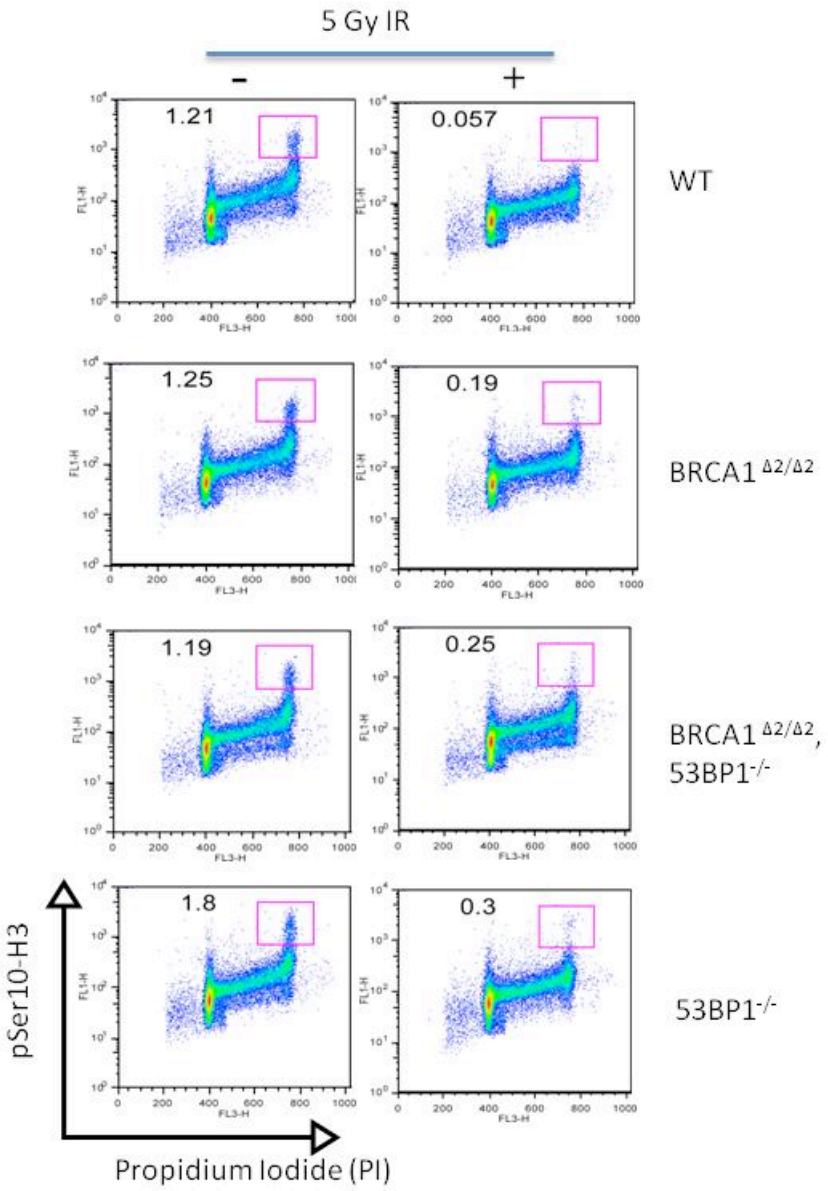


Figure 11. Frequencies of different chromosomal aberrations in B cells metaphase chromosomes caused by adding (a, b) 2μM Olaparib (two separatedexperiments, n=50 cells per experiment) and (c) 250 nM Mitomycin C.

3.4 The BRCA1 RING domain is important for initiation of the G2/M cell cycle checkpoint in response to DNA damage.

DNA damage triggers cellular signaling pathways to induce G2/M cell cycle checkpoints including activation of a G_2M checkpoint that prevents G2 cells with chromosome abberations from entering mitosis. This G_2M checkpoint has been shown to be defective in BRCA1-deficient cells (X. Xu et al., 1999). We tested the activity of the G_2M checkpoint in *BRCA1*$^{\Delta 2/\Delta 2}$ B cells by measuring the percentage of pSer10-H3^{+} (mitotic) cells 60 mins after 5 Gy ionizing radiation (IR) treatment (figure.12a). *BRCA1*$^{\Delta 2/\Delta 2}$ cells showed a G_2M checkpoint defect relative to WT controls. Other genotypes tested did not show a statistically significant difference based on data from two experiments (figure. 12b). Our data suggest that in addition to mediating genomic integrity, the RING domain of BRCA1 is essential for mediating the G_2M checkpoint in response to IR.

Notably, this G_2M checkpoint defect in *BRCA1*$^{\Delta 2/\Delta 2}$ B cells was not rescued in the absence of *53BP1. 53BP1* deletion therefore appears to have a different effect on the phenotype of *BRCA1*$^{\Delta 2/\Delta 2}$ cells with respect to the G_2M checkpoint compared to the rescue achieved by *53BP1* deletion in assays of chromosome aberrations.



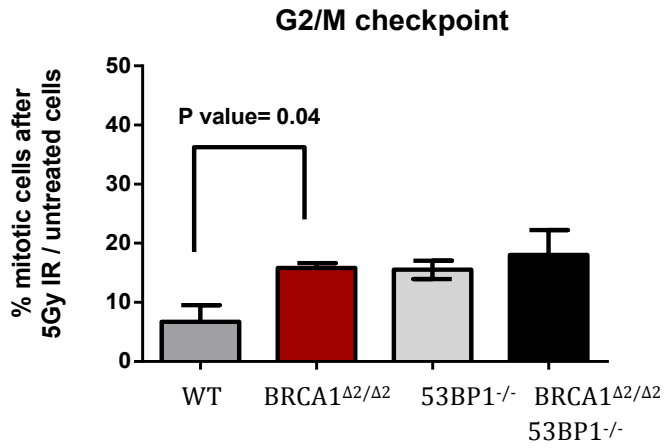


Figure 12. G2M checkpoint activity in B cells after 5 Gy ionizing radiation exposure. a) Percentage of mitotic cells after 60 mins recovery from IR. b) Average of Mitotic cells from two experiments compared to WT.

3.5 RING domain mutant BRCA1 affects class switch recombination.

Class switch recombination (CSR) in B lymphocytes mediates switching of IgM immunoglobulin to IgG, IgA, and IgE antibody isotypes by replacing the Cμ region of the immunoglobulin heavy chain gene (*IgH*) with Cɣ, Cα and Cε respectively. This process occurs through DSB induction at two sites in the *IgH* gene, mediated by the AID enzyme, followed by intrachromosomal repair by nonhomologous end joining (NHEJ) (Figure.13a) (Li, Woo, Iglesias-Ussel, Ronai, & Scharff, 2004). CSR has an essential role in immunity by diversifying the effector functions of antibodies, but mis-repair of induced double-strand breaks can lead to chromosome translocations which cause different types of lymphoma (Guikema et al., 2006).

Successful CSR generates $IgG1^+$ B cells, which can be detected by flow cytometry. We tested CSR in $BRCA1^{\Delta 2/\Delta 2}$ B cells, and found that mutant BRCA1 B cells have a significant decrease in $IgG1^+$ B cells, to around 50% of the WT level (Figure.13 b,c). This was unexpected, because BRCA1 has not previously been implicated in repair processes linked to CSR. 53BP1-deficient B cells showed low levels of Ig isotype switching in our assay, as has been reported previously (Ward et al., 2004). $BRCA1^{\Delta 2/\Delta 2}$ $53BP1^{-/-}$ cells showed a further reduction in CSR, suggesting that loss of BRCA1 RING function is necessary for the low level of switching that occurs in $53BP1^{-/-}$ cells. These findings indicate, for the first time, an important role for the BRCA1 RING domain in the CSR process.

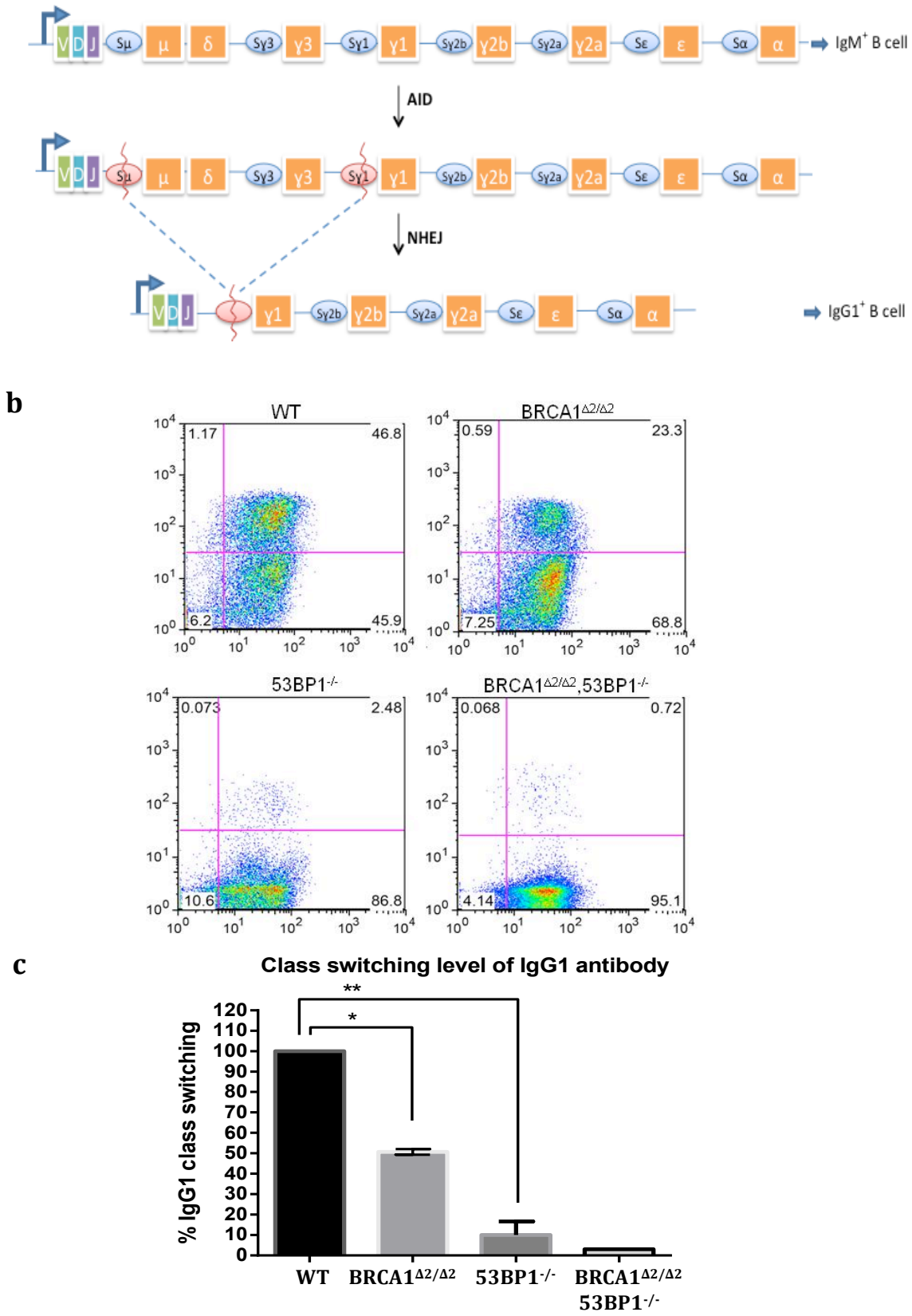
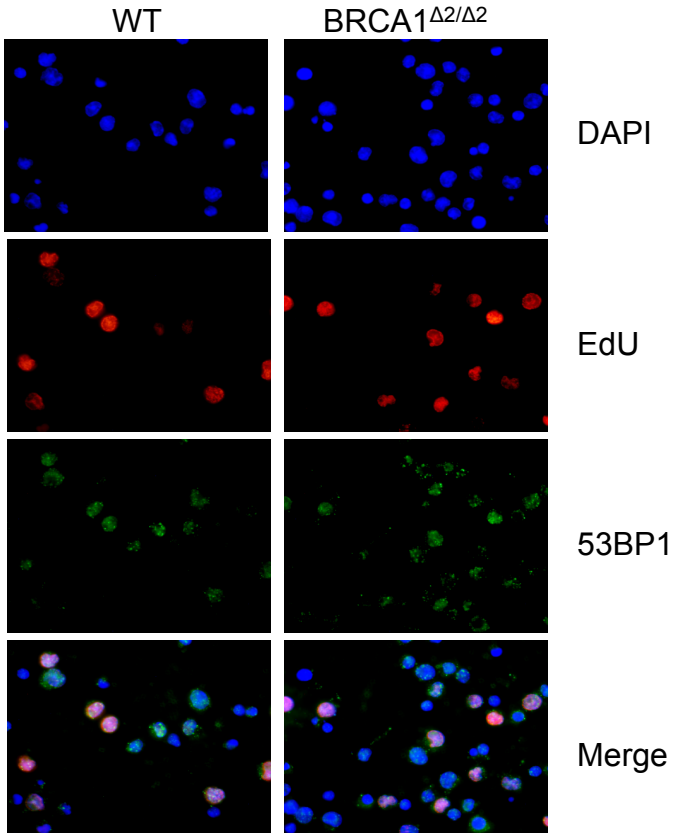


Figure 13. IgG1 class swich recombination .a) a map showing the CSR process to generate IgG1 antibody isotype. b and c) levels of IgG1 antibody in activated B cells as measured by flow cytometry. Statistical results were obtained using Student t–test.
* represents a P value =0.0004, **represents a P value =0.0027.

3.6 The BRCA1 RING domain deletion has no significant impact on 53BP1 accumulation at DNA double-strand break sites.

The genomic instability phenotype observed in *BRCA1*$^{\Delta 2/\Delta 2}$ cells was very dependent on the presence of *53BP1*. This observation suggests that 53BP1 may prevent normal DNA repair in cells lacking functional BRCA1. Previous work has suggested that BRCA1 may act to displace 53BP1 from break sites, eliminating a repressive effect of 53BP1 on DNA repair by the homologous recombination pathway. This model is supported by results using structured-luminescence microscopy, which demonstrated that although BRCA1 and 53BP1 are both recruited to break sites, they do not occupy overlapping domains (Chapman, Sossick, Boulton, & Jackson, 2012).

We measured the appearance of 53BP1 at DNA break sites, where it forms discrete 'foci' after IR treatment (Figure 14). 53BP1 foci were detected in both WT and *BRCA1*$^{\Delta 2/\Delta 2}$ B cells after IR. Using fluorescent microscopy, we quantified the percentage of G1 and G2 cells that show 53BP1 foci in each genotype by pulsing cells with EdU for 15 mins. (G2 cells were detected using Click-Chemistry to stain cells that incorporated EdU.) Our results show no significant differences in 53BP1 foci formation between *BRCA1*$^{\Delta 2/\Delta 2}$ and wild type controls, in either the EdU-positive or EdU-negative cell populations. This study suggests that the effect of the BRCA1 RING domain in modulating genomic instability may be mediated a mechanism independent of 53BP1 recruitment to break sites.



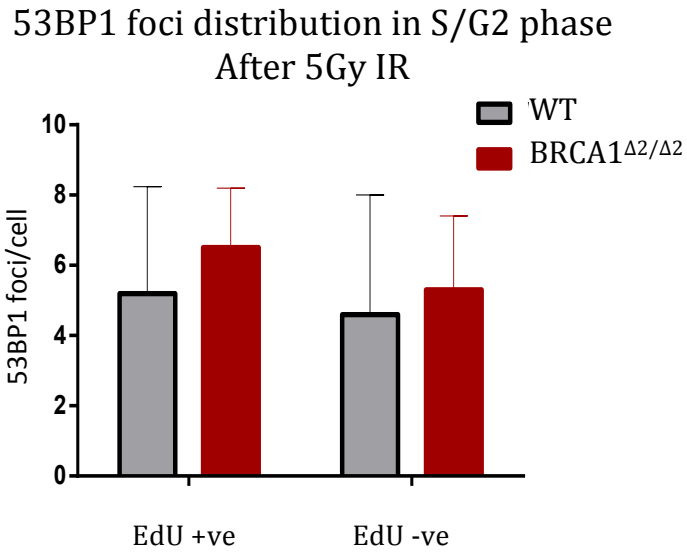


Figure 14. 53BP1 foci formation at double strand break sites in S/G2 phase in BRCA1$^{\Delta 2/\Delta 2}$ and WT cells.

3.7 Genomic Instability in *BRCA1*$^{\Delta 2/\Delta 2}$ cells does not correlate with satellite repeat transcription.

A recent report indicated that the primary role of BRCA1 in tumor suppression is mediated by the E3 ubiquitin ligase activity of its RING domain (Zhu et al., 2011). According to this study, genomic instability, centrosome amplification and aberrant growth of BRCA1-deficient cells arises as a direct consequence of increased expression of non-coding RNAs from genomic satellite repeats. BRCA1-mediated ubiquitylation of histone H2A at satellite repeats normally represses transcription of these regions, thereby mediating genomic integrity. *BRCA1*$^{\Delta 2/\Delta 2}$ cells express an N terminal-deleted form of BRCA1 that is missing the RING domain, therefore we hypothesized that the genomic instability seen in these cells arises because of aberrantly high expression of satellite repeat transcripts.

As *BRCA1*$^{\Delta 2/\Delta 2}$ cells express BRCA1 protein that lacks the E3 ubiquitin ligase catalytic pocket as well as the BARD1 interaction motif, they offer an opportunity to validate the importance of E3 ligase activity for repression of satellite repeat transcription. We tested the degree of satellite repeat expression in *BRCA1*$^{\Delta 2/\Delta 2}$ cells using qPCR with primers as previously described (Zhu et al., 2011). Primers for the constitutively expressed cyclophilin gene were used to normalize for cDNA abundance (Figure. 15). We found that satellite repeat expression is not significantly altered in *BRCA1*$^{\Delta 2/\Delta 2}$ relative to WT cells. In general, there was

substantial variability in satellite repeat expression, even between mice of the same genotype, but in no case did we observe the high levels of repeat expression described previously. We conclude that altered transcription of satellite repeats does not appear to account for the high sensitivity of *BRCA1*$^{\Delta 2/\Delta 2}$ cells to DNA damaging agents.

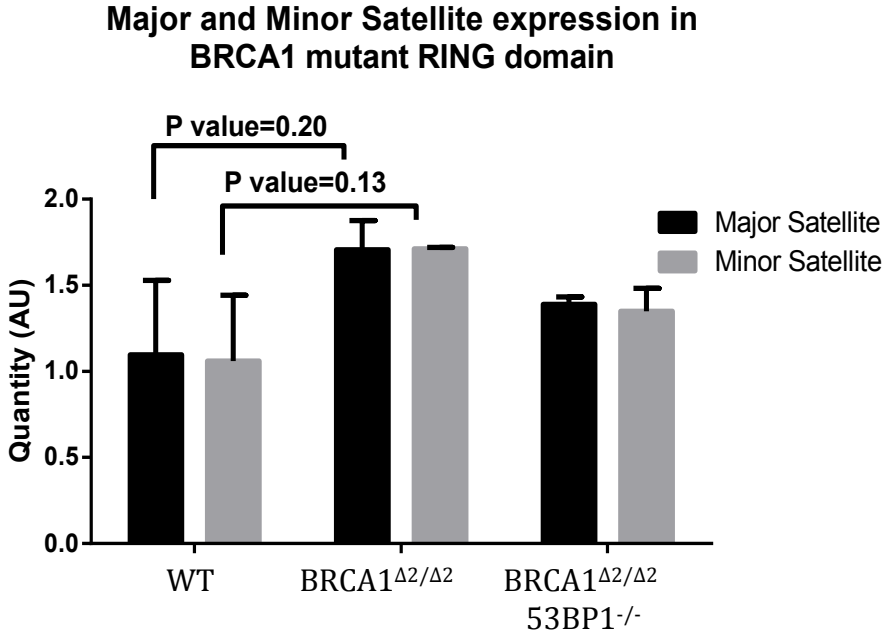


Figure 15. Major Satellite and Minor Satellite repeat expression in BRCA1$^{\Delta 2/\Delta 2}$ compared to WT.

4.Discussion

4.1 RING-less BRCA1 Cannot Maintain Genomic Integrity or Normal G_2M Checkpoint Activation.

In this study we aimed to test whether the BRCA1 RING domain is essential for maintenance of genomic integrity. In the late 1990s, several 'BRCA1 knockout' mice were developed. The *BRCA1*$^{neo/neo}$ mouse was one model for *BRCA1* loss of function. The *BRCA1*^{neo} allele was generated by replacing exon 2, which contains the normal intiator codon for translation of BRCA1 mRNA, with a *neo* cassette. This allele causes homozygous embryonic lethality, as was oberved with other knockout alleles around the same time, and as such has been described as a null (Ludwig, Chapman, Papaioannou, & Efstratiadis, 1997). While characterizing *BRCA1*$^{neo/neo}$53BP1$^{-/-}$ mouse embryonic fibroblasts, however, we discovered that *BRCA1*^{neo} is transcribed as an almost full-length BRCA1 mRNA. We showed that this mRNA contains exon 1 spliced directly to exon 3, omitting sequence from the exogenous *neo* cassette.

In the absence of the regular BRCA1 initiator codon in exon 2, it is not clear how *BRCA1*^{neo} mRNA is translated. Two protein bands were detected in *BRCA*$^{neo/+}$,*53BP1*$^{-/-}$ cells, which run very close to each other at around 220 kDa. The upper band matches the size of WT BRCA1 protein. The lower band only appears in mice with the *BRCA1*^{neo} allele, therefore it seems likely that this band represents mutant BRCA1 protein encoded by the *BRCA*^{neo} allele. Such a protein

might be produced if the *BRCA*^{neo} mRNA was translated from an alternative, downstream, in-frame initator codon. One candidate for this alternative initiator codon exists in exon 5 (Figure 16). If this ATG acts as the start site for translation of the *BRCA*^{neo} mRNA, it would produce a protein of approximately the size that we see in our Western blot (a protein of 1816 residues, with an estimated MW of 209 kDA, as opposed to 1863 residues, 220 kDA for WT BRCA1). The small differerences in the predicted size of these proteins account for the challenge in separating bands from the WT and *BRCA1*^{neo} alleles in the Wetern blot.

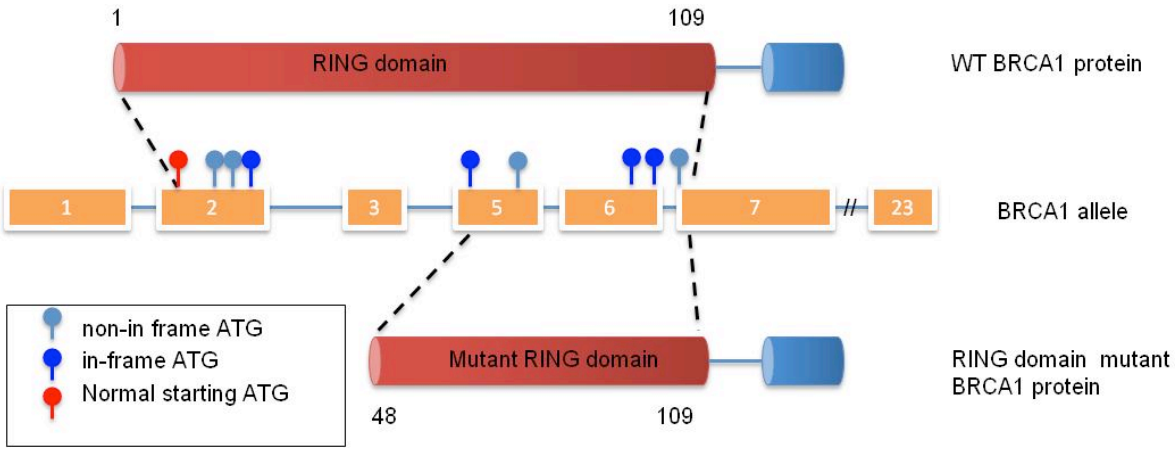


Figure 16. Showing RING domain in wild type and mutant BRCA1protein and the location of all ATG codons. *Note:* BRCA1 does not have an 'exon 4' because of an error in the original annotation of the gene

Importantly, use of the alternative ATG site for translation of BRCA1 would produce a protein that lacks 43 % of the RING domain. This deletion includes the catalytic pocket of the BRCA1 RING domain which is responsible for its E3 ligase activity (Brzovic, Meza, King, & Klevit, 2001). As such, we propose that the

BRCA1^{neo} allele is not a null allele, but instead encodes a RING-less BRCA1 mutant protein. Results in our lab have shown that the mutant protein is not just expressed, but efficiently localizes to DNA double-strand break sites after ionizing radiation (Minxing Li, personal communication).

The embryonic lethality of *BRCA1*$^{neo/neo}$ homozygosity indicates that the RING domain of BRCA1 is of great importance, at least for the role of BRCA1 in embryonic development. Currently, there is some debate about whether the key activity of BRCA1 in tumor suppression and maintenance of genomic integrity lies in the RING domain,in the BRCT domains, or potentially in some other part of the protein. One recent report linked all of the phenotypes associated with BRCA1 loss-of-function to loss of E3 ubiquitin ligase activity associated with the BRCA1 RING domain. (Zhu et al., 2011). Conversely, a second study indicated that a mutation of the RING domain of BRCA1, which inactivated E3 ubiquitin ligase activity, had only a minor impact on genomic integrity (Reid et al., 2008).

As *BRCA1*$^{neo/neo}$ mice are embryonic lethal, we are not able to use these mice to study the function of the RING domain. We were able to get around this problem based on our observation that conditional deletion of BRCA1 exon 2 by a Cre-Lox approach also generates an mRNA lacking exon 2. As was the case with the *BRCA1*^{neo} allele, this mRNA is translated, presumably using the alternative ATG site in exon 4, and producing protein with an equivalent N-terminal truncation. We used this conditional allele to study the effect of deletion

of the BRCA1 RING domain on a *53BP1*$^{+/+}$ background, and discovered that several of the features of BRCA1 loss of function, including genomic instability and a defect in induction of the ionizing-radiation induced G_2M checkpoint, occur in the absence of the RING domain. We conclude that the BRCA1 RING domain is essential for normal BRCA1 activity. At this point, we do not know if the *BRCA1*$^{\Delta 2/\Delta 2}$ mice are susceptible to tumors. To test the tumor predisposition of *BRCA1*$^{\Delta 2/\Delta 2}$ mice, we would need to set up a longitudinal study to test whether they develop cancer more quickly than *BRCA1*$^{+/+}$ control mice.

4.2 Patient mutations in the BRCA1 RING domain

Human patients with BRCA1-associated breast and ovarian cancer have mutations affecting many different parts of the gene. Considering that the sequence encoding the RING domain only makes up a small part of the BRCA1 gene, a disproportionate number of cancer cases are associated with RING mutations (Clark, Rodriguez, Snyder, Hankins, & Boehning, 2012). One mutation that affects the RING domain is the 185delAG mutation, a comon cancer-causing mutation found at high frequency in Ashkenazi Jewish populations, which has been the subject of much study.

The 185delAg mutation is a deletion of two nucleotides in the sequence encoding the RING domain, which causes a frameshift, so that the reading frame of *BRCA1* becomes interrupted by a premature termination codon in exon 3. This

mutant transcript has been considered to be a potential target for nonsense-mediated-decay, however the transcript is stabilized by restart of translation from two downstream ATG start codons, located at Met128 and Met297(Buisson, Anczukow, Zetoune, Ware, & Mazoyer, 2006). Translation from these alternative initator codons leads to production of a BRCA1 protein lacking the RING sequence, as occurs with our RING-less *BRCA1*$^{\Delta 2/\Delta 2}$ mice. Based on this evidence, we hypothesize that *BRCA1*$^{\Delta 2/\Delta 2}$ mice will be susceptible to tumor formation. This prediction fits our observation of signficant genomic instability and checkpoint defects in *BRCA1*$^{\Delta 2/\Delta 2}$ cells.

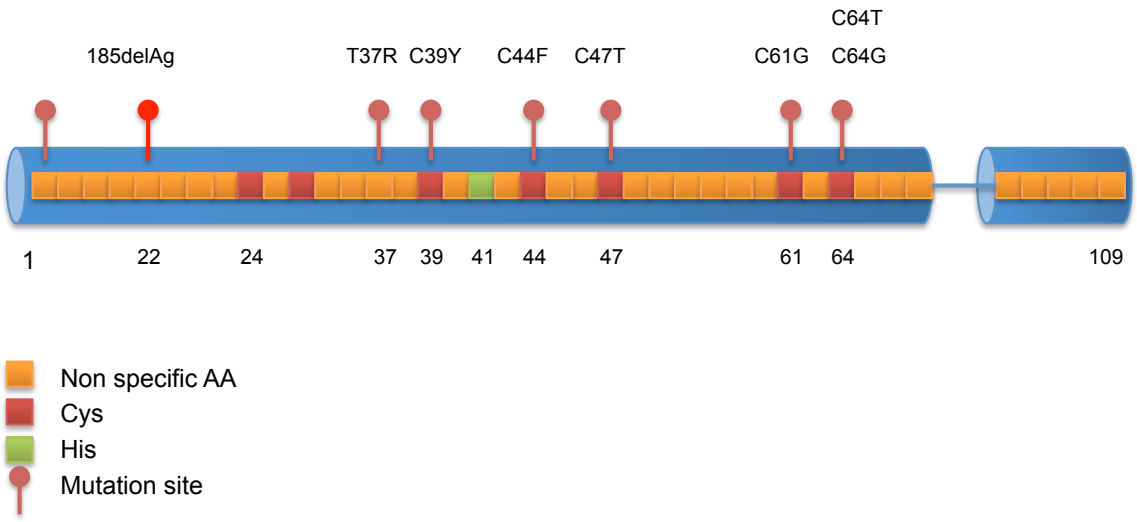


Figure 17. Human BRCA1 RING domain showing mutations most associated with human cancer incidence.

Other patient mutations localizing to the RING domain include mutations of the conserved cysteine residues that coordinate the zinc metal center in the RING domain. These residues include Cys39, Cys44, Cys47 and Cys64 (Figure. 17). These mutant alleles still express BRCA1 protein, but the protein is likely to

be non-functional for E3 ubiquitin ligase activity. These mutant forms of BRCA1 are therefore similar to the mutant protein expressed in our *BRCA1*$^{\Delta 2/\Delta 2}$ mice, because most of the BRCA1 protein is intact, but the RING domain is disrupted.

4.3 Previous Experimental Studies of the BRCA1 RING Domain

Several other groups have previously used different experimental approaches to study the function of the BRCA1 RING domain. Ruffner et al transfected mutant BRCA1 RING constructs into the human ductal primary breast cancer cell line, HCC1937, which is a BRCA1-deficient line. Although a WT BRCA1 construct complemented the BRCA1-deficient cells, RING mutants that caused a loss of E3 ubiquitin ligase activity were associated with hypersensitivity to ionizing radiation as well as a G2M checkpoint defect (Ruffner, Joazeiro, Hemmati, Hunter, & Verma, 2001). Nelson and Holt used the same BRCA1-deficient HCC1937 human cell line, transfected with various constructs containing mutant BRCA1, and reported that BRCA1 recruitment to DNA damage required intact RING and BRCT domains (Nelson & Holt, 2010).

These studies with human cell lines and the large number of patient mutations affecting the RING domain strongly suggest that integrity of the BRCA1 RING domain is essential for BRCA1 function. However, to date nobody has reported a mouse model of a RING-less BRCA1. Our results demonstrate that deletion of the RING domain is sufficient by itself to cause genomic instability and checkpoint defects in primary mouse cells.

In addition to having intrinsic low-level E3 ubiquitin ligase activity, the BRCA1 RING domain also forms the site of association of BRCA1 with its heterodimeric binding partner, BARD1. Heterodimeric BRCA1-BARD1 has significantly greater E3 ubiquitin ligase activity than either protein by itself (Hashizume et al., 2001). The *BRCA1*^{I26A} mouse was designed to specifically test the importance of BRCA1 E3 ubiquitin ligase activity as opposed to its ability to bind BARD1 (Reid et al., 2008; Shakya et al., 2011). Based on structural biological information about the association of BRCA1 with the E2 enzyme, UbcH5c, the I26A point mutant was introduced into the RING domain of BRCA1, abolishing E3 ubiquitin ligase activity, but maintaining the interaction with BARD1. *BRCA1*$^{I26A/I26A}$ mice show normal levels of homology directed DNA repair and are resistant to the DNA crosslinking agent, mitomycin C. This stands in contrast to our results, because we saw high rates of chromosomal aberrations in *BRCA1*$^{\Delta 2/\Delta 2}$ cells in response to MMC.

4.4 Importance of BARD1 association for normal BRCA1 activity

Whereas BRCA1 and BARD1 can form a heterodimer in cells from *BRCA1*$^{I26A/I26A}$ mice, which show low tumor predisposition, human cancer mutations affecting the BRCA1 RING domain often disrupt its interaction with BARD1. It is therefore important to consider whether *BRCA1*$^{\Delta 2/\Delta 2}$ protein can bind to BARD1. Work in our lab has revealed that BARD1 protein is absent in cells

from mice expressing our BRCA1 RING mutants (Minxing Li, personal communication). BARD1 is destabilized when the protein cannot interact with BRCA1, as occurs in cells lacking BRCA1 protein (McCarthy, Celebi, Baer, & Ludwig, 2003). Deletion of the RING domain in *BRCA1*$^{\Delta 2/\Delta 2}$ cells is therefore likely to cause genomic insability at least in part because of a defect in interaction of BRCA1 with BARD1.

Loss of interaction with BARD1 does not affect stability or nuclear sublocalization of BRCA1 in *BRCA1*$^{\Delta 2/\Delta 2}$ cells. This observation matches what is seen in experiments in which the C61G RING domain was transfected into BRCA1-deficient cells. Although the C61G mutation disrupts interaction of BRCA1 with BARD1, BRCA1 is nonetheless expressed at normal rates and can localize to the nucleus (Nelson & Holt, 2010).

4.5 Mechanism of BRCA1 RING domain in Mediating Genomic Integrity

To attempt to find a mechanistic basis for the phontypes of *BRCA1*$^{\Delta 2/\Delta 2}$ mutant mice, we tested whether deletion of the RING domain in *BRCA1*$^{\Delta 2/\Delta 2}$ cells causes an increase in transcription of major and minor satellite repeats in the mouse genome. Ectopic transcription of satellite repeats, caused by loss of a silencing effect mediated by BRCA1-dependent H2A ubiquitylation, was recently proposed to be a key mechanism leading to genomic instability in cases of

BRCA1 loss-of-function (Zhu et al., 2011). Using primers described in that study, we repeated the experiment with cDNA prepared from *BRCA1*$^{\Delta 2/\Delta 2}$ B cells. Despite the high levels of genomic instability in the *BRCA1*$^{\Delta 2/\Delta 2}$ cells, we found no statistically significant increase in transcription of either major or minor satellite repeats, as compared to the previous study, which reported a 10-fold increase in major stallite repeat transcription and a 27-fold increase in minor satellite repeat transcription. We hypothesize that the increase in satellite repeat transcription observed in the previous study was a consequence of the specific *BRCA1* allele used, and does not represent a general mechanism underpinning the genomic instability of all BRCA1-mutant cells. Notably, this study was based on analysis of a conditional mouse in which exons 5-13 are deleted, as opposed to our mice, in which exon 2 is deleted.

The DNA damage response factor, 53BP1, has emerged as a major modifier of genomic instability in BRCA1-deficient cells (Bunting et al., 2010). 53BP1 has been proposed to block DNA repair by the HR pathway during the G1 phase of the cell cycle by blocking access of repair factors to the DNA break site, but is displaced by BRCA1 during S and G2 phases of the cell cycle (Bunting & Nussenzweig, 2013). We therefore tested whether deletion of the RING domain of BRCA1 affected 53BP1 recruitment to DNA damage sites. We measured 53BP1 foci formation by immunofluorescence after ionizing radiation in G1 cells and cells in S/G2 phase. Our results to date have not shown a statistically significant effect on 53BP1 foci formaton in *BRCA1*$^{\Delta 2/\Delta 2}$ cells, but these results

are based on relatively small sample sizes. Further experiments will be required to more carefully test whether BRCA1 mediates homologous recombination by altering the ability of 53BP1 to inhibit repair activities at sites of DNA damage.

4.6 Effect of BRCA1 on class switch recombination

Class switch recombination (CSR) involves induced DNA double-strand breakage and repair at the immunoglobulin heavy chain locus, and serves to change the effector properties of antibodies secreted as part of an immune response. As repair of DNA breaks during CSR is dependent on efficient nonhomologous end-joining (NHEJ), measuring the ability of B cells to complete CSR is a useful assay of NHEJ activity. For example, *Ku80*$^{-/-}$ mice, which lack the essential NHEJ factor, Ku80, show a substantial block in CSR (Casellas R, EMBO J, 1998).

As BRCA1 is normally considered to be active in the HR pathway for double-strand break repair, as opposed to in NHEJ, we hypothesized that there would be no CSR phenotype in *BRCA1*$^{\Delta 2/\Delta 2}$ B cells. When we tested CSR in *BRCA1*$^{\Delta 2/\Delta 2}$ mice, however, we observed a defect in CSR whereby only about 50% of the WT level of IgG1^{+} cells were produced at the end of the culture period. This is a surprising result, as defects in CSR in *BRCA1*-deficient mice have not been shown before. On the other hand, few *BRCA1* mice with specific deletion of the RING domain have been studied before. Further characterization of the role of

the BRCA1 RING domain In CSR is required to confirm this result, including a parellel cell proliferation meaurement using the CFSE dilution assay to ensure that the phenotype is not a consequence of an undetected proliferation defect caused by mutant BRCA1 (X. Xu et al., 1999).

4.7 Effect of 53BP1 deletion on *BRCA1*$^{\Delta 2/\Delta 2}$ phenotypes.

Several recent papers indicate that deletion of the *53BP1* gene rescues many phenotypes of BRCA1 mutation (Bunting et al., 2010)(Bouwman et al., 2010). We also observe that *53BP1* deletion rescued hypersensitivity of *BRCA1*$^{\Delta 2/\Delta 2}$ cells to the PARP inhibitor, olaparib. In other BRCA1-deficient mice, *53BP1* deletion was shown to be insufficient to rescue the hypersensitivity of BRCA1-deficient cells to DNA inter-strand crosslinking agents. Specifically, *53BP1* deletion did not rescue the mitomycin C hypersensitivity of BRCA1$^{\Delta 11/\Delta 11}$ cells (Bunting et al., 2012). Based on this observation, BRCA1 has been proposed to have 53BP1-dependent and 53BP1-independent functions in DNA repair, depending on the nature of the DNA damage. Surprisingly, *53BP1* deletion rescues the MMC-hypersensitivity of *BRCA1*$^{\Delta 2/\Delta 2}$ cells. This finding suggests that that the ability of cells to repair MMC-induced DNA damage is to some extent affected by an activity present in the RING domain of BRCA1.

Our finding that the RING domain of BRCA1 in required for the G_2M checkpoint shows another important role of the BRCA1 RING domain besides DNA repair. *BRCA1*$^{\Delta 2/\Delta 2}$ cells show a defect in activation of the G_2M checkpoint,

even in the presence of many chromosome aberrations. The same finding has been shown by other groups who observerd a defective G_2M checkpoint in other mutated BRCA1 isoforms (Ruffner et al., 2001; X. Xu et al., 1999). No checkpoint activation rescue was observed by co-deletion of *53BP1*, which suggests that BRCA1 plays different roles in regulating G2M checkpoint and homologous recombination.

4.8 A model for BRCA1 phosphorylation as an essential step in DNA inter-strand crosslink repair.

53BP1 and BRCA1 appear to act in a common pathway to regulate DNA repair in mammalian cells, although the exact activity of either protein is not fully understood. Previous results indicated that deletion of *53BP1* could rescue the hypersensitivity of *BRCA1*$^{\Delta 11/\Delta 11}$ cells to PARP inhibitors, but not to DNA crosslinking agents like MMC (Bunting et al., 2012). Based on this observation, it was proposed that BRCA1 could affect the efficiency of two separate DNA repair pathways: the HR pathway, which repairs DNA double-strand breaks, and the Fanconi Anemia pathway, which repairs DNA crosslinks. Our latest results show that *BRCA1*$^{\Delta 2/\Delta 2}$ *53BP1*$^{-/-}$ cells show little sensitivity to MMC treatment (Figure 11c). This represents a clear difference to what was previously reported for *BRCA1*$^{\Delta 11/\Delta 11}$*53BP1*$^{-/-}$ cells. Accounting for this difference may provide insight into the contribution of BRCA1 to repair of DNA interstrand crosslinks.

Both BRCA1$^{\Delta 11/\Delta 11}$ and *BRCA1*$^{\Delta 2/\Delta 2}$ cells express hypomorphic BRCA1 proteins. *BRCA1*$^{\Delta 2/\Delta 2}$ cells produce an N-terminal-truncated protein without a functional RING domain (Figure 2). BRCA1$^{\Delta 11/\Delta 11}$ cells produce a protein lacking the peptide sequence encoded by exon 11 (X. Xu et al., 1999). Exon 11 of *BRCA1* is very large, and encodes approximately 50% of the BRCA1 protein, hence the protein expressed by BRCA1$^{\Delta 11/\Delta 11}$ is much smaller than that seen in WT cells (Figure 7d). Residues 224-1365 of BRCA1 make up a serine-rich domain, in which multiple serine residues become phosphorylated after DNA damage. For example, in cells treated with DNA crosslinking agents such as MMC, the kinase ATR becomes activated, leading to phosphorylation of several components of the Fanconi Anemia pathway, and residue Ser988 of BRCA1 (Zou & Elledge, 2003).

Importantly, the BRCA1 phosphorylation sites, including Ser988, are present in the BRCA1 protein expressed in *BRCA1*$^{\Delta 2/\Delta 2}$ cells, but are not present in the BRCA1 protein expressed in *BRCA1*$^{\Delta 11/\Delta 11}$ cells. Our results support a model in which the presence of phosphorylated BRCA1 at the sites of DNA crosslinks is essential for the appropriate repair of these lesions. Even in the absence of the RING domain, the mutant protein expressed in *BRCA1*$^{\Delta 2/\Delta 2}$ *53BP1*$^{-/-}$ cells can be phosphorylated, and contributes to repair of DNA interstrand crosslinks. Conversely, mutant protein expressed in *BRCA1*$^{\Delta 11/\Delta 11}$*53BP1*$^{-/-}$ cells cannot contribute to repair of DNA damage induced by MMC, because it cannot be phosphorylated.

This model does not explain how 53BP1 regulates DNA repair. It is also possible that other motifs within exon 11 other than the phosphorylation sites contribute to interstrand crosslink repair. Nonetheless, our genetic experiments provide additional detail about the domains of BRCA1 that may interact with factors of the Fanconi Anemia pathway, as part of our ongoing efforts to reach a full understanding of the contribution of *BRCA1* to DNA repair.

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