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Brca1 Controls Homology-Directed DNA Repair

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Summary

Germline mutations in *BRCA1* confer a high risk of breast and ovarian tumors. The role of BRCA1 in tumor suppression is not yet understood, but both transcription and repair functions have been ascribed. Evidence that BRCA1 is involved in DNA repair stems from its association with RAD51, a homolog of the yeast protein involved in the repair of DNA double-strand breaks (DSBs) by homologous recombination. We report here that Brca1-deficient mouse embryonic stem cells have impaired repair of chromosomal DSBs by homologous recombination. The relative frequencies of homologous and nonhomologous DNA integration and DSB repair were also altered. The results demonstrate a caretaker role for BRCA1 in preserving genomic integrity by promoting homologous recombination and limiting mutagenic nonhomologous repair processes.

Introduction

Chromosomal double-strand breaks (DSBs) can arise from cellular DNA metabolism and from exogenous DNA-damaging agents such as ionizing radiation (IR). Although IR causes an array of genotoxic damage, including rapidly repaired single-strand breaks and oxidative damage, cellular lethality results from unrepaired or misrepaired DSBs (Ward, 1995). In mammalian cells, two major pathways exist to repair chromosomal DSBs, homologous recombination and nonhomologous end joining (NHEJ) (Liang et al., 1998). The first IR-sensitive mammalian cell mutants that were characterized were found to be defective for NHEJ, as demonstrated by impaired V(D)J recombination (Fulop and Phillips, 1990; Taccioli et al., 1993) or chromosomal NHEJ (Liang et al., 1996). Recently, two IR-sensitive cell lines were shown to have homologous repair defects (Johnson et al., 1999; Pierce et al., 1999). These lines are deficient in the RAD51-related proteins XRCC2 and XRCC3 (Cartwright et al., 1998; Liu et al., 1998), confirming a contribution of the RAD51 pathway to homologous repair in mammalian cells.

The products of both hereditary breast cancer genes, BRCA1 and BRCA2, colocalize with RAD51 to nuclear foci following DNA damage in mitotic cells and at forming synaptonemal complexes in meiotic cells (Scully et al., 1997; Chen et al., 1998a). Although clearly a direct interaction for BRCA2 (Mizuta et al., 1997; Sharan et al., 1997; Wong et al., 1997; Chen et al., 1998b), the association of BRCA1 with RAD51 is likely to be indirect, perhaps mediated by BRCA2 (Chen et al., 1998a). BRCA1 has also been found associated with another repair protein RAD50, colocalizing with it upon IR damage in nuclear foci that are distinct from RAD51 foci (Zhong et al., 1999). Brca1-deficient (Gowen et al., 1998; Shen et al., 1998; Abbott et al., 1999) and Brca2-deficient (Connor et al., 1997; Sharan et al., 1997; Chen et al., 1998b; Patel et al., 1998) murine and human cells are sensitive to DNA-damaging agents, including IR, further implicating these proteins in DSB repair.

In mammals, as in other organisms, homologous recombination can maintain genomic integrity through the precise repair of a chromosomal DSB using the sister chromatid as the repair template (Johnson et al., 1999). However, homologous repair of chromosomal DSBs using other homologous templates such as alleles or repeated sequences is potentially deleterious since it can result in loss of heterozygosity (LOH) or chromosomal translocations, respectively (Moynahan and Jasin, 1997; Richardson et al., 1998). Thus, loss of genes that regulate or participate in DSB repair processes can disturb the balance of various repair pathways that have evolved in mammalian cells to guard the genome from perturbation. In this study, we sought to determine if Brca1 mutation affects homologous recombination or nonhomologous repair events.

Results

Brca1$^{-/-}$ Cells Exhibit Gene Targeting Defects and Increased Nonhomologous Integration

The Brca1-deficient murine embryonic stem (ES) cell line 236.44 is hypersensitive to IR and hydrogen peroxide (but not UV) and has normal growth characteristics and normal levels of p53 (Gowen et al., 1998; L. C. Gowen and B. H. K., unpublished data). We have taken two approaches to determine if homologous recombination is perturbed in this cell line. The first approach was to evaluate pathways of integration of transfected DNA; the second was to directly examine chromosomal DSB repair at the locus of integration. The *Brca1*$^{-/-}$ and control *Brca1*$^{+/-}$ 310.7 cell lines were evaluated for gene targeting proficiency at two loci, *Rb* on chromosome 14 and *pim1* on chromosome 17. The *pim1-Q* targeting vector (Figure 1A) contains a promoterless hygromycin resistance (*hyg*^{R}) gene cloned in frame with *pim1* coding sequences (te Riele et al., 1990). The *hyg* gene is expressed when the vector correctly gene targets at the *pim1* locus or when a fortuitous nonhomologous integration occurs adjacent to promoter sequences. The *pim1-Q* targeting vector was electroporated into both cell lines, and *hyg*^{R} colonies were selected. Southern

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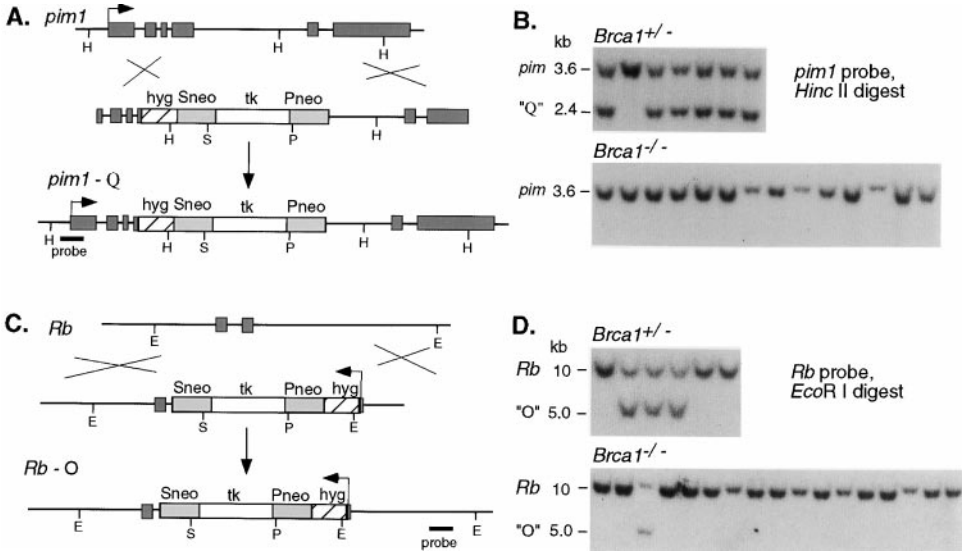

Figure 1. The Efficiency of Gene Targeting Is Reduced in *Brca1*$^{-/-}$ Cells

(A) Gene targeting strategy at the *pim1* locus. In the *pim1-Q* targeting vector, the promoterless *hyg*^{R} gene is cloned in frame with *pim1* exons (black boxes) and has 0.5 kb of *pim1* homology on the left arm and 3.7 kb of homology on the right arm (te Riele et al., 1990). Although promoterless in the targeting vector, the *hyg*^{R} gene is expressed from the endogenous *pim1* promoter when it has homologously integrated. The targeting vector also contains a substrate for DSB repair that consists of two defective *neo* genes, *Sneo* and *Pneo*, separated by the 2.2 kb *pgk-tk*$^{+}$ gene. Mutations in the *Sneo* and *Pneo* genes are linker insertions of I-SceI (S) and PacI (P) endonuclease recognition sites, respectively. The *Sneo* gene has a polyoma/*tk* promoter; the *Pneo* gene is promoterless. H, HincII site.

(B) Southern blot analysis of *hyg*^{R} clones derived from transfection of *Brca1*$^{-/-}$ and *Brca1*$^{+/-}$ cell lines with the *pim1-Q* targeting vector. DNA was digested with HincII and hybridized with the *pim1* probe shown in (A). The wild-type and targeted *pim1-Q* alleles are 3.6 and 2.4 kb, respectively.

(C) Gene targeting strategy at the *Rb* locus in the region of *Rb* exons 20 and 19 (black boxes). In the *Rb-O* targeting vector, the *pgk-hyg*^{R} gene is inserted into exon 19 and has 7 kb of *Rb* homology on the left arm and 2.4 kb of homology on the right arm (te Riele et al., 1992). The targeting vector also contains the same DSB repair substrate described in (A). E, EcoRI site.

(D) Southern blot analysis of *hyg*^{R} clones derived from transfection of the *Brca1*$^{-/-}$ and *Brca1*$^{+/-}$ cell lines with the *Rb-O* targeting vector. DNA was digested with EcoRI and hybridized with the *Rb* probe shown in (C). The wild-type and targeted *Rb-O* alleles are 10 and 5.0 kb, respectively.

analysis demonstrated that most of the *hyg*^{R} *Brca1*$^{-/-}$ clones were derived from random integration of the *pim1-Q* targeting vector (Figure 1B), with only 3% of clones derived from gene targeting (Table 1). By contrast, efficient gene targeting was observed in the *Brca1*$^{+/-}$ cell line (Figure 1B) with 70% of the clones correctly targeted (Table 1), similar to what has previously been observed with this targeting design (te Riele et al., 1990).

To confirm that this 23-fold decrease in gene targeting efficiency in the *Brca1*$^{-/-}$ cell line was not related to the promoterless targeting vector design or locus of integration, gene targeting was also examined at *Rb*, a standard test locus for recombination (te Riele et al., 1992; de Wind et al., 1995). The *Rb-O* targeting vector (Figure 1C) contains an intact *hyg*^{R} gene expressed from a *pgk* promoter. As at the *pim1* locus, most of the *hyg*^{R} *Brca1*$^{-/-}$ clones were derived from random integration of the targeting vector (Figure 1D), with less than 1% of clones derived from gene targeting at the *Rb* locus (Table 1). Conversely, the *Brca1*$^{+/-}$ cell line efficiently integrated the *Rb-O* targeting vector at the *Rb* locus (Figure 1D), with 48% of the clones correctly targeted (Table 1). This is within the range of what has previously been observed for this locus (te Riele et al., 1992; de Wind et al., 1995). Thus, the *Brca1*$^{-/-}$ cells had a 53-fold decrease in gene targeting efficiency as compared with the *Brca1*$^{+/-}$ cell line (Table 1).

Since the efficiency of gene targeting is expressed as the ratio of the number of gene targeted to total *hyg*^{R} clones analyzed, two parameters may be affected, the frequency of gene targeting and/or the frequency of random integration. We observed that the total number of *hyg*^{R} clones in the *Brca1*$^{-/-}$ cells was increased as compared to the *Brca1*$^{+/-}$ control cells following transfection with the linear *Rb-O* targeting construct (2-fold) or with a circular *hyg*^{R} control plasmid (5-fold). With the *Rb-O* targeting construct, therefore, the 53-fold difference in targeting efficiency can be attributed to a 13-fold decrease in gene targeting and a 4-fold increase in random integration. Thus, both parameters were affected, although a greater effect was seen on the frequency of gene targeting.

Table 1. Gene Targeting Efficiency of *Brca1*$^{+/-}$ and *Brca1*$^{-/-}$ Cells at Two Genomic Loci

	Targeting Construct	
Cell Line	*pim1-Q*	*Rb-O*
Brca1$^{+/-}$	70% (50/71)	48% (90/192)
Brca1$^{-/-}$	3% (2/68)	0.9% (4/460)

The efficiency is expressed as the percentage of clones with a homologous integration relative to the total number of clones analyzed. Absolute numbers are given in parentheses.

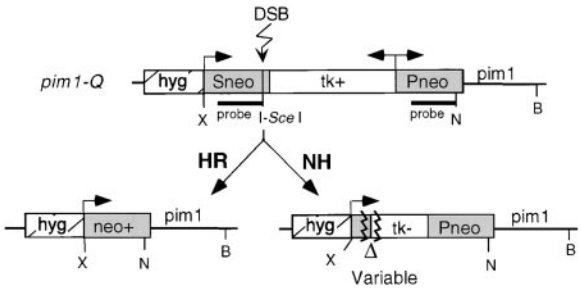

Figure 2. Induction of FIAU^R by DSB Repair

Depiction of a portion of the *pim1-Q* allele highlighting DSB induction at the I-SceI site in *Sneo*. FIAU^R can be acquired through DSB repair by homologous recombination (HR) between the direct *Sneo* and *Pneo* repeats or nonhomologous (NH) events such as deletions of variable size (Δ). To distinguish these two broad classes of events, Southern blots were done on genomic DNA from individual FIAU^R clones restricted with XhoI (X) and BglII (B) and probed with a 526 bp EagI/NcoI *neo* fragment. With this digest, HR events have a 1.8 kb band that contains an NcoI (N) site, whereas a product of variable size or complete loss of *neo* hybridization is seen in NH events (data not shown). Complete loss of *neo* hybridization requires a deletion of at least 4 kb.

To further investigate potential factors that may contribute to either a decrease in gene targeting or an increase in nonhomologous integration, DNA uptake and cell cycle distribution of transfected cells were examined. A vector expressing a nuclear localized green fluorescent protein (GFP) from a chicken β-actin promoter was transfected into the *Brca1*$^{-/-}$ and *Brca1*$^{+/-}$ cell lines. Cells were analyzed for GFP expression and cell cycle distribution by flow cytometry 24 hr following electroporation. Green fluorescence and cell cycle distribution, as measured in both unfixed and ethanol-fixed cells, were equivalent in the *Brca1*$^{-/-}$ and *Brca1*$^{+/-}$ cell lines (data not shown), indicating similar DNA uptake and cell cycle kinetics.

Brca1 Controls Homology-Directed Repair of Chromosomal DSBs

We next investigated the Brca1-deficient cells for the repair of chromosomal DSBs. In the gene targeting experiments, a direct repeat recombination substrate containing the cleavage site for a rare-cutting endonuclease was integrated at the *pim1* (Figure 1A) and *Rb* loci (Figure 1C). The direct repeat consists of two differentially mutated neomycin phosphotransferase (*neo*) genes, *Sneo* and *Pneo*, separated by a thymidine kinase (*tk*) gene. Inserted into the *Sneo* gene was the 18 bp recognition site for the I-SceI endonuclease (Figures 1A and 1C), which disrupts the gene. When the I-SceI site is cleaved in vivo, homologous recombination with the *Pneo* gene will repair the DSB. Alternatively, nonhomologous processes can be used to repair the DSB. Since each process can give rise to multiple products, two different assays were used to score DSB repair events.

The first assay was based on loss of the *tk* gene that causes cells to become resistant to the nucleotide analog FIAU (Figure 2). A deletional homologous recombination event would precisely remove one of the *neo* repeats along with the *tk* gene. A *neo*+ gene would also be restored, but since a *neo* gene was used to knockout one of the *Brca* alleles in the *Brca1*$^{-/-}$ cells (Gowen et al., 1996), G418 selection was not informative for these cells. Deletional homologous recombination can occur by two mechanisms, single-strand annealing, in which the intervening *tk* gene is physically deleted, or reciprocal exchange between repeats on either the same chromatid or sister chromatid, in which the *tk* gene is present on the reciprocal product. Nonhomologous events would also give rise to FIAU^R by *tk* gene deletion, but these deletions would be variable, removing part or all of the gene.

Table 2. Frequency of Thymidine Kinase Loss in *pim1-Q* Cell Lines as Measured by Resistance to the Nucleotide Analog FIAU

Cell Line	Frequency of FIAU^R Colonies	
	Spontaneous	DSB-Induced
Brca1$^{+/-}$		
310.7.Q4	2.4×10^{-4}	6.9×10^{-3}
310.7.QA4	2.7×10^{-4}	7.2×10^{-3}
Brca1$^{-/-}$		
236.44.QC12	5.8×10^{-5}	1.4×10^{-3}

Brca1$^{-/-}$ *and Brca1*$^{+/-}$ cell lines containing the *pim1-Q* or *Rb-O* allele were electroporated with the pCβASce plasmid in which I-SceI endonuclease is expressed from a chicken β-actin promoter. Cell viability following I-SceI expression did not differ between the *Brca1*$^{-/-}$ and *Brca1*$^{+/-}$ cell lines. Cells were grown in nonselective media for 6 days to ensure loss of residual tk protein, followed by replating and selection in FIAU. DSBs substantially increased the frequency of FIAU^R clones in both the *Brca1*$^{-/-}$ and *Brca1*$^{+/-}$ cell lines containing the *pim1-Q* allele. However, the *Brca1*$^{-/-}$ *pim1-Q* cell line had an approximately 5-fold lower frequency of both spontaneous and DSB-induced FIAU^R colonies as compared to either of the *Brca1*$^{+/-}$ *pim1-Q* cell lines (Table 2). In the *Rb-O* cell lines, whether *Brca1*$^{-/-}$ or *Brca1*$^{+/-}$, spontaneous loss of *tk* expression was extremely high, possibly due to occlusion or epigenetic modification of the *pgk* promoter, with only a minimal induction of tk loss by I-SceI expression. Therefore, the *Rb-O* clones were not examined further using the tk assay.

To identify the molecular events leading to FIAU^R, Southern blot analysis was performed on genomic DNA from the individually expanded *pim1-Q* clones using the *neo* gene as probe (data not shown). For both the *Brca1*$^{-/-}$ and *Brca1*$^{+/-}$ *pim1-Q* cell lines, the major pathway leading to FIAU^R was a deletional recombination event. This event was found in 93.2% of the *Brca1*$^{+/-}$ FIAU^R clones (123/132) and 93.8% of the *Brca1*$^{-/-}$ FIAU^R clones (106/113). Consistent with this, G418 selection (*neo*+) of the *Brca1*$^{+/-}$ cell lines demonstrated a concordance in the frequency of deletional recombination events as measured by combined G418 and FIAU selection or FIAU selection only. The remaining clones had a *neo* band of different size from the parental clone or complete loss of the *neo* hybridization signal, each indicative of NHEJ involving a large deletion. Since the major event leading to *tk* loss was homologous recombination, the 5-fold lower frequency of DSB-induced FIAU^R clones indicates that there is a homologous repair defect in the Brca1-deficient cells.

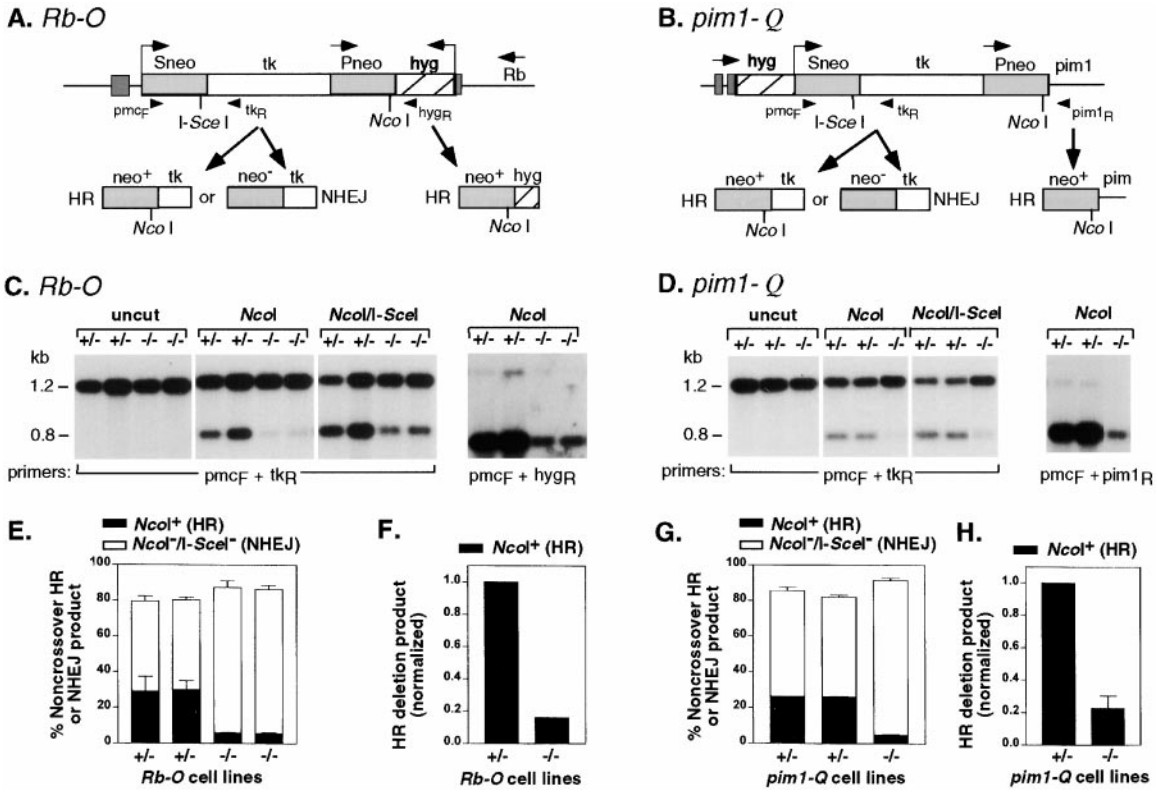

Figure 3. *Brca1*$^{-/-}$ Cells Have Impaired Homologous Repair of Chromosomal DSBs

(A and B) To analyze DSB repair, *Brca1*$^{-/-}$ and *Brca1*$^{+/-}$ cell lines containing the *Rb-O* (A) and *pim1-Q* (B) alleles were electroporated with an I-SceI expression vector. Primers pmc_F and tk_R were used to amplify repair products resulting from HR or NHEJ, as shown. The HR event detected by these primers is a noncrossover gene conversion of the cleaved *Sneo* gene by the downstream *Pneo* gene. Noncrossover HR results in an NcoI$^+$ *neo*+ gene followed by *tk* sequences. By contrast, NHEJ of the DSB results in an NcoI$^-$/I-SceI$^-$ *neo*− gene. The other primer set, pmc_F and hyg_R (*Rb-O*) or pmc_F and $pim1_R$ (*pim1-Q*), was used to amplify the HR deletion product that is followed by *hyg* (A) or *pim* (B) sequences, as shown.

(C and D) A consistent reduction in the HR product is seen in the *Brca1*$^{-/-}$ cell lines, as demonstrated by Southern blot analysis of the *Rb-O* (C) and *pim1-Q* (D) DSB repair products. Two independently derived cell lines were analyzed for each genotype, except for the *Brca1*$^{-/-}$ *pim1-Q* genotype in which only one cell line was analyzed. DSB repair products were amplified by PCR using the indicated primers, digested with NcoI, I-SceI (data not shown), or both enzymes, and analyzed by Southern blotting using the *neo* probe shown in Figure 2. Primers pmc_F and tk_R amplify a 1.2 kb *neo/tk* gene fragment that is cleaved with NcoI in the HR events to produce an 0.8 kb fragment. NHEJ products are uncleaved by both NcoI and I-SceI, maintaining the 1.2 kb fragment. The increase in hybridization signal of the 0.8 kb band in the NcoI/I-SceI lanes as compared with the NcoI lanes is the result of amplification of a residual amount of I-SceI$^+$ material. Amplification with primers pmc_F and hyg_R (C) or pmc_F and $pim1_R$ (D) results in a product that is almost exclusively NcoI$^+$, as expected.

(E–H) Quantitation of the HR and NHEJ products after DSB repair demonstrating impaired HR in the *Brca1*$^{-/-}$ cell lines. Both noncrossover HR (primer pair pmc_F and tk_R, [E and G]) and HR resulting in a deletion (primer pairs pmc_F and hyg_R, [F]; pmc_F and $pim1_R$, [H]) are reduced in the *Brca1*$^{-/-}$ *Rb-O* (E and F) and *pim1-Q* (G and H) cell lines. The relative percentage of the noncrossover HR product (black boxes) and the NHEJ (stacked white boxes) product obtained in each cell line indicates a 5-fold decrease in HR and 1.6-fold increase in NHEJ in the *Brca1*$^{-/-}$ cell lines as compared with the *Brca1*$^{+/-}$ control cell lines (E and G). HR resulting in a deletion product is reduced in the *Brca1*$^{-/-}$ cell lines approximately 6-fold, as determined by quantitating the amount of the NcoI$^+$ product and normalizing it to the amount of product obtained in the *Brca1*$^{+/-}$ cell lines (black boxes, [F and H]). Error bars are present in all samples except the normalized *Brca1*$^{+/-}$ cell lines in (F) and (H) and the *Brca1*$^{-/-}$ cell lines in (F), for which only one experiment was performed. In (E) and (G), the bar graphs do not end at 100%, since a portion of the amplified product is I-SceI$^+$.

Brca1-Deficient Cells Have Impaired Homologous Repair of DSBs but Are Proficient at Nonhomologous Repair

Our previous work demonstrated that the major portion of DSB repair events are from homologous or NHEJ repair that lead to minimal changes to the locus, with only a few percent of events resulting in gross chromosomal rearrangements (Liang et al., 1998). Therefore, a second assay was employed to detect more subtle changes to the locus and to assess the relative contributions of NHEJ and homologous recombination to DSB repair. PCR with primers that flank the DSB site (pmc_F and tk_R; Figures 3A and 3B) detects minimal changes to the locus after repair, such as those resulting from homologous repair by noncrossover gene conversion and from NHEJ, which usually involves small deletions or insertions. PCR with a second set of primers was used to measure the appearance of the deletional homologous recombination product. This would confirm the *tk* gene loss results obtained in *pim1-Q* cell lines and allow detection of events in the *Rb-O* cell lines. With this second set of primers, the downstream primer was specific to each locus (hyg_R for *Rb-O*, $pim1_R$ for *pim1-Q*; Figures 3A and 3B).

The *pim1-Q* and *Rb-O* cell lines were transfected with or without the I-SceI expression vector and grown in

nonselective media. Genomic DNA was isolated either immediately after transfection or 48 hr later and then cleaved with commercially available I-SceI endonuclease to reduce amplification of genomic DNA that maintained an intact I-SceI site. PCR was performed with each set of primers, and the products were digested with NcoI and/or I-SceI and analyzed by Southern blotting to identify homologous ($NcoI^+$/$I\text{-}SceI^-$) and NHEJ ($NcoI^-$/$I\text{-}SceI^-$) repair events. In control transfections without the I-SceI expression vector, only a small amount of product was amplified with either primer set, and it was $I\text{-}SceI^+$, demonstrating a requirement for in vivo cleavage and repair to generate $I\text{-}SceI^-$ products (data not shown).

Southern blots from one transfection of the I-SceI expression vector are shown in Figures 3C and 3D. With the pmc_F and tk_R primer set, approximately 90% of the amplified product was $I\text{-}SceI^-$ (data not shown). The $NcoI^-$/$I\text{-}SceI^-$ NHEJ product was readily detected in all of the cell lines, with an equal or slightly higher amount in the *Brca1*$^{-/-}$, as compared with the *Brca1*$^{+/-}$, cell lines. By contrast, a consistently lower level of the $NcoI^+$ gene conversion product was detected in the *Brca1*$^{-/-}$ cell lines (*Rb-O*, Figure 3C; *pim1-Q*, Figure 3D). The second set of primers, pmc_F and either hyg_R or $pim1_R$, gave a predominantly $NcoI^+$ product (≥97%), as expected. As with the gene conversion product, this deletional recombination product was consistently lower in the *Brca1*$^{-/-}$ cell lines at both the *Rb-O* and *pim1-Q* loci as compared with the *Brca1*$^{+/-}$ cell lines.

Since G418 selection was informative in the *Brca1*$^{+/-}$ cells, the relative contribution of the two recombination products could be assessed. Southern blot analysis of *neo*+ clones demonstrated that gene conversions and deletional homologous recombination events occurred at similar frequencies (approximately 40% and 60%, respectively; data not shown). The similar frequency of the two products, rather than a predominance of the deletion product (Liang et al., 1998), suggests that a major pathway leading to the deletional homologous product is a conservative recombination pathway (Szostak et al., 1983), rather than single-strand annealing. Control experiments using cell lines containing either *Sneo* or *Pneo* alone (Moynahan and Jasin, 1997) demonstrated the dependence on having two homologous *neo* genes for the high frequency of restoration of an intact *neo* gene. Upon I-SceI expression, *neo*+ colonies were not obtained in cell lines containing *Pneo* alone and were obtained at a frequency more than two orders of magnitude lower in cell lines containing *Sneo* alone (4×10^{-5}) as compared with the *Rb-O* cell lines (8×10^{-3}). These *neo*+ clones derived from the *Sneo* cells arose from NHEJ (Moynahan and Jasin, 1997).

To quantitate the defect in homologous recombination due to *Brca1* mutation, several experiments were performed in the *pim1-Q* and *Rb-O* cell lines. As summarized in Figures 3E–3H, the *Brca1*$^{-/-}$ cells have a consistently 5- to 6-fold lower level of homologous repair of the chromosomal DSB, by both noncrossover gene conversion and deletion. No gross defect in NHEJ was observed in the *Brca1*$^{-/-}$ cells, as NHEJ was increased by 1.5- to 1.6-fold in the *Brca1*$^{-/-}$ cells as compared to the *Brca1*$^{+/-}$ cells.

Discussion

These results are the first to directly demonstrate that the hereditary breast cancer gene *Brca1* controls homologous recombination. Cells deficient in Brca1 have both decreased gene targeting and, significantly, impaired homologous repair of a damaged chromosome. Nonhomologous recombination is not reduced in these cells, but rather is somewhat elevated, especially as measured by increased random integration of transfected DNA. Although the mechanism of nonhomologous integration is not known, it is possible that as a result of the homologous repair defect the *Brca1*$^{-/-}$ cells have an elevated level of spontaneous DSBs genome-wide into which transfected DNA can insert.

Brca1 disruption by targeted mutagenesis has been reported by several groups to result in embryonic death in mice, with embryos exhibiting severe growth retardation (Gowen et al., 1996; Hakem et al., 1996; Liu et al., 1996; Ludwig et al., 1997; Shen et al., 1998). Heterogeneity is observed in the timing of embryonic death both within a mouse line containing a particular disrupting mutation and between mouse lines containing different mutations. Most *Brca1* mutations result in lethality prior to embryonic day 9 (Hakem et al., 1996; Liu et al., 1996; Ludwig et al., 1997; Shen et al., 1998), whereas the *Brca1* mutation present in our ES cell line allows mice to survive to embryonic day 9 or 10 (Gowen et al., 1996). (In rare instances, when combined with p53 mutation, live births are obtained [Cressman et al., 1999b].) The mutant *Brca1* gene is disrupted by replacement of the terminal portion on intron 10 and the beginning of exon 11 with a selectable marker gene, resulting in loss of the full-length *Brca1* transcript (Gowen et al., 1996). The mutation does not result in a complete null allele, since an exon 10–12 splice variant is expressed that results in an in-frame fusion between the two exons (Cressman et al., 1999a; L. C. Gowen and B. H. K., unpublished data). Prolonged survival of mouse embryos containing an exon 10–12 splice variant generated by lox–cre recombination has also been reported (Xu et al., 1999).

Exon 11 of *Brca1* encodes approximately 60% of the protein (Bennett et al., 1995; Lane et al., 1995), including the RAD51 interacting domain as mapped in the human protein (Scully et al., 1997). Consistent with this, our *Brca1*$^{-/-}$ ES cell line is partially defective in Rad51 focus formation following DNA damage with the cross-linking agent cisplatin (D. K. Bishop, personal communication). We expect that the homologous repair defect we observed in this mutant would be further magnified by complete loss of Brca1 protein and that a more severe repair defect could contribute to the cellular lethality observed in other *Brca1* mutants, due to an inability to repair spontaneous lesions. Alternatively, growth control and homologous repair may be separable, such that cells may be viable but exhibit genomic instability and a propensity to tumorigenesis. Along with a role for *Brca1* in homologous repair in mitotically growing cells, a role in homologous recombination during meiosis is supported by the block in spermatogenesis observed in the rare animals that have survived to adulthood with this mutation (Cressman et al., 1999b).

In addition to the homologous repair defect reported here, *Brca1* has been implicated in transcription-coupled repair (TCR) of oxidative DNA damage (Gowen et

al., 1998; Abbott et al., 1999; Cressman et al., 1999b). Other mammalian repair genes have been implicated in TCR of oxidative damage, including *CSA* and *CSB* (Leadon and Cooper, 1993), which are also involved in TCR of UV damage (Bootsma et al., 1998). Mutations in these genes lead to Cockayne syndrome, which is not characterized by cancer predisposition, suggesting that a defect in TCR of oxidative damage is not sufficient to promote tumorigenesis. Mutation of another gene, *MSH2*, which is involved in mismatch repair, also leads to impaired TCR of oxidative damage (Leadon and Avrutskaya, 1997). In this case, the mutation predisposes individuals to colorectal cancer due to mismatch repair deficiency (Boland, 1998). One possibility is that poor repair of oxidative DNA damage results in a general inhibition of transcription, which thereby abrogates strand-specific repair.

The importance of homologous recombination as a DNA repair pathway is well established in yeast, whereas its role in mammalian cells is just emerging. A significant fraction of DSB-promoted recombination events between direct repeats in mammalian chromosomes have recently been demonstrated to involve sister chromatids (Johnson et al., 1999). Thus, we expect that the homologous repair defect observed in the *Brca1*$^{-/-}$ cells may reflect, at least in part, decreased repair from the sister chromatid. High levels of BRCA1 are found in proliferating cells in S phase (Chen et al., 1996; Vaughn et al., 1996), as would be predicted for a protein involved in sister chromatid repair. Although repair substrates necessarily measure homologous recombination between unequally positioned sequences on sister chromatids, repair of DSBs or other types of DNA damage from an equivalently positioned sequence on the sister chromatid would be predicted to be as or more frequent and would precisely restore the sequence that was present before the chromosome was damaged.

An impaired ability to precisely repair DSBs from the sister chromatid would be predicted to result in genetic instability in mammalian cells. Consistent with a role for Brca1 in maintaining genetic stability are the recent observations of chromosomal aberrations in Brca1-deficient mouse (Xu et al., 1999) and BRCA1-deficient human cells (Tirkkonen et al., 1997; Tomlinson et al., 1998). A dramatic increase in gross chromosomal aberrations is a hallmark of the chromosome instability disorders including Bloom syndrome, Werner syndrome, Nijmegen breakage syndrome, and ataxia-telangiectasia (Meyn, 1997). Although the function of the genes defective in each of these syndromes has not been conclusively determined, they each appear to be involved in DNA damage recognition and/or repair. Two mammalian cell mutants have been demonstrated directly (irs1; Johnson et al., 1999) and indirectly (irs1SF; Pierce et al., 1999) to have impaired sister chromatid homologous repair, and they are genetically unstable, exhibiting a high frequency of spontaneous chromosomal aberrations, including chromatid-type breaks (Jones et al., 1987; Tebbs et al., 1995; Cartwright et al., 1998; Liu et al., 1998). Notably, a nonhomologous DSB repair mutant, *xrs-6*, does not have elevated levels of spontaneous chromosomal aberrations (Kemp and Jeggo, 1986). This suggests that homologous recombination may play a particularly important role in maintaining chromosomal stability in the absence of exogenous damage. Thus, when homologous repair is defective, nonhomologous processes may promote aberrant types of repair.

In summary, the homologous repair defect demonstrated here supports the hypothesis of a caretaker role for the *BRCA1* hereditary breast cancer gene (Kinzler and Vogelstein, 1997), whereby mutation of the gene leads to a highly penetrant predisposition to early breast and ovarian cancer.

Experimental Procedures

DNA Manipulations

The *pim1-Q* targeting vector was constructed by modifying the previously described gene targeting vector, p59 (te Riele et al., 1990), to contain the *Sneo* and *Pneo* genes (Moynahan and Jasin, 1997). An EcoRI/BamHI fragment containing a promoterless *Pneo* gene was cloned adjacent, but in opposite orientation, to the *HSV-tk* gene that is driven by a *pgk* promoter. A XhoI/HindIII fragment containing the entire *Sneo* gene was inserted downstream of *pgk-tk*, creating the DSB repair substrate *Sneo-tk+-Pneo*. This repair substrate was inserted at a unique NotI site downstream of the p59 *hyg* coding sequence to create *pim1-Q*. The *Rb-O* targeting vector was constructed by modifying the previously described "M" gene targeting vector (Moynahan and Jasin, 1997). *Sneo-tk+-Pneo* and a BglII *pgk-hyg* fragment (te Riele et al., 1992) were inserted into the BglII/EcoRV sites of the *Rb* M allele, deleting exon 19 and a portion of intron 19.

The forward primer, pmc_F (5′-GAGCAGTGTGGTTTTGCAAG-3′) was 865 bp upstream of the I-SceI site. The reverse primers tk_R (5′-GGAATGGTTTATGGTTCGTGG-3′), hyg_R (5′-TACTCGCCGATAGTGGAAACC-3′), and $pim1_R$ (5′-TTCCAGGCCGGCACCTTG-3′) were 352 bp, 3.4 kb, and 3.4 kb downstream from the I-SceI site, respectively. After deletional homologous recombination, the hyg_R and $pim1_R$ primers hybridized 342 and 322 bp, respectively, downstream of the NcoI site in the *neo+* gene. Quantitation of PCR products was performed with Phosphoimager analysis and ImageQuant software.

Cell Transfections

Brca1$^{-/-}$ and *Brca1*$^{+/-}$ ES cell lines have previously been described (Gowen et al., 1996). The two *Brca1* alleles used to construct the *Brca1*$^{-/-}$ cell line were disrupted by *hprt* and *neo* selectable markers. The *Brca1*$^{-}$ allele in the *Brca1*$^{+/-}$ cell line is disrupted by *hprt*. ES cells were electroporated with 75 μg of the linear *pim1-Q* or *Rb-O* targeting fragments and then selected in hygromycin (110 μg/ml for *pim1-Q*; 200 μg/ml for *Rb-O*). For $FIAU^R$ experiments, ES cells were electroporated at 250 V/960 μF with 100 μg of pCβASce (Richardson et al., 1998) or mock DNA and plated in nonselective media at 1×10^4 cells/plate. After 6 days, cells were replated in media containing 0.6 μM FIAU at a density of 5×10^4 cells/plate (experiments 1 and 2) or 1.0 μM FIAU at a density of 1×10^5 cells/plate (experiment 3). $FIAU^R$ clones were picked and expanded in 96-well plates for Southern analysis or stained and counted for derivation of spontaneous and DSB-induced repair frequencies. Conditions were altered in experiment 3 to allow for molecular analysis of a larger number of $FIAU^R$ clones.

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References

Abbott, D.W., Thompson, M.E., Robinson-Benion, C., Tomlinson, G., Jensen, R.A., and Holt, J.T. (1999). BRCA1 expression restores

radiation resistance in BRCA1-defective cancer cells through enhancement of transcription-coupled DNA repair. J. Biol. Chem. *274*, 18808–18812.

Bennett, L.M., Haugen-Strano, A., Cochran, C., Brownlee, H.A., Fiedorek, F.T., Jr., and Wiseman, R.W. (1995). Isolation of the mouse homologue of BRCA1 and genetic mapping to mouse chromosome 11. Genomics *29*, 576–581.

Boland, C.R. (1998). Hereditary nonpolyposis colorectal cancer. In The Genetic Basis of Human Cancer, B. Vogelstein and K.W. Kinzler, eds. (New York: McGraw-Hill), pp. 333–346.

Bootsma, D., Kraemer, K.H., Cleaver, J.E., and Hoeijmakers, J.H.J. (1998). Nucleotide excision repair systems: Xeroderma Pigmentosa, Cockayne syndrome, and Tichothiodystrophy. In The Genetic Basis of Human Cancer, B. Vogelstein and K.W. Kinzler, eds. (New York: McGraw-Hill), pp. 245–274.

Cartwright, R., Tambini, C.E., Simpson, P.J., and Thacker, J. (1998). The XRCC2 DNA repair gene from human and mouse encodes a novel member of the recA/RAD51 family. Nucleic Acids Res. *26*, 3084–3089.

Chen, Y., Farmer, A.A., Chen, C.F., Jones, D.C., Chen, P.L., and Lee, W.H. (1996). BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle–dependent manner. Cancer Res. *56*, 3168–3172.

Chen, J., Silver, D.P., Walpita, D., Cantor, S.B., Gazdar, A.F., Tomlinson, G., Couch, F.J., Weber, B.L., Ashley, T., Livingston, D.M., and Scully, R. (1998a). Stable interaction between the products of the *BRCA1* and *BRCA2* tumor suppressor genes in mitotic and meiotic cells. Mol. Cell *2*, 317–328.

Chen, P.L., Chen, C.F., Chen, Y., Xiao, J., Sharp, Z.D., and Lee, W.H. (1998b). The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment. Proc. Natl. Acad. Sci. USA *95*, 5287–5292.

Connor, F., Bertwistle, D., Mee, P.J., Ross, G.M., Swift, S., Grigorieva, E., Tybulewicz, V.L., and Ashworth, A. (1997). Tumorigenesis and a DNA repair defect in mice with a truncating Brca2 mutation. Nat. Genet. *17*, 423–430.

Cressman, V.L., Backlund, D.C., Hicks, E.M., Gowen, L.C., Godfrey, V., and Koller, B.H. (1999a). Mammary tumor formation in p53- and BRCA1-deficient mice. Cell Growth Differ. *10*, 1–10.

Cressman, V.L., Backlund, D.C., Avrutskaya, A.V., Leadon, S.A., Godfrey, V., and Koller, B.H. (1999b). Growth retardation, DNA repair defects, and lack of spermatogenesis in BRCA1-deficient mice. Mol. Cell. Biol. *19*, 7061–7075.

de Wind, N., Dekker, M., Berns, A., Radman, M., and te Riele, H. (1995). Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. Cell *82*, 321–330.

Fulop, G.M., and Phillips, R.A. (1990). The *scid* mutation in mice causes a general defect in DNA repair. Nature *347*, 479–482.

Gowen, L.C., Johnson, B.L., Latour, A.M., Sulik, K.K., and Koller, B.H. (1996). *Brca1* deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities. Nat. Genet. *12*, 191–194.

Gowen, L.C., Avrutskaya, A.V., Latour, A.M., Koller, B.H., and Leadon, S.A. (1998). BRCA1 required for transcription-coupled repair of oxidative DNA damage. Science *281*, 1009–1012.

Hakem, R., de la Pompa, J.L., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmair, A., Billia, F., et al. (1996). The tumor suppressor gene *Brca1* is required for embryonic cellular proliferation in the mouse. Cell *85*, 1009–1023.

Johnson, R.D., Liu, N., and Jasin, M. (1999). Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. Nature *401*, 397–399.

Jones, N.J., Cox, R., and Thacker, J. (1987). Isolation and cross-sensitivity of X-ray-sensitive mutants of V79–4 hamster cells. Mut. Res. *183*, 279–286.

Kemp, L.M., and Jeggo, P.A. (1986). Radiation-induced chromosome damage in X-ray-sensitive mutants (xrs) of the Chinese hamster ovary cell line. Mutat. Res. *166*, 255–263.

Kinzler, K.W., and Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. Nature *386*, 761, 763.

Lane, T.F., Deng, C., Elson, A., Lyu, M.S., Kozak, C.A., and Leder, P. (1995). Expression of Brca1 is associated with terminal differentiation of ectodermally and mesodermally derived tissues in mice. Genes Dev. *9*, 2712–2722.

Leadon, S.A., and Avrutskaya, A.V. (1997). Differential involvement of the human mismatch repair proteins, hMLH1 and hMSH2, in transcription-coupled repair. Cancer Res. *57*, 3784–3791.

Leadon, S.A., and Cooper, P.K. (1993). Preferential repair of ionizing radiation-induced damage in the transcribed strand of an active human gene is defective in Cockayne syndrome. Proc. Natl. Acad. Sci. USA *90*, 10499–10503.

Liang, F., Romanienko, P.J., Weaver, D.T., Jeggo, P.A., and Jasin, M. (1996). Chromosomal double-strand break repair in Ku80 deficient cells. Proc. Natl. Acad. Sci. USA *93*, 8929–8933.

Liang, F., Han, M., Romanienko, P.J., and Jasin, M. (1998). Homology-directed repair is a major double-strand break repair pathway in mammalian cells. Proc. Natl. Acad. Sci. USA *95*, 5172–5177.

Liu, C.Y., Fleskin-Nikitin, A., Li, S., Zeng, Y., and Lee, W.H. (1996). Inactivation of the mouse BRCA1 gene leads to failure in the morphogenesis of the egg cylinder in early postimplantation development. Genes Dev. *10*, 1835–1843.

Liu, N., Lamerdin, J.E., Tebbs, R.S., Schild, D., Tucker, J.D., Shen, M.R., Brookman, K.W., Siciliano, M.J., Walter, C.A., Fan, W., et al. (1998). XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages. Mol. Cell *1*, 783–793.

Ludwig, T., Chapman, D.L., Papaioannou, V.E., and Efstratiadis, A. (1997). Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. Genes Dev. *11*, 1226–1241.

Meyn, M.S. (1997). Chromosome instability syndromes: lessons for carcinogenesis. Curr. Top. Microbiol. Immunol. *221*, 71–148.

Mizuta, R., LaSalle, J.M., Cheng, H.L., Shinohara, A., Ogawa, H., Copeland, N., Jenkins, N.A., Lalande, M., and Alt, F.W. (1997). RAB22 and RAB163/mouse BRCA2: proteins that specifically interact with the RAD51 protein. Proc. Natl. Acad. Sci. USA *94*, 6927–6932.

Moynahan, M.E., and Jasin, M. (1997). Loss of heterozygosity induced by a chromosomal double-strand break. Proc. Natl. Acad. Sci. USA *94*, 8988–8993.

Patel, K.J., Vu, V.P.C.C., Lee, H., Corcoran, A., Thistlethwaite, F.C., Evans, M.J., Colledge, W.H., Friedman, L.S., Ponder, B.A., and Venkitaraman, A.R. (1998). Involvement of Brca2 in DNA repair. Mol. Cell *1*, 347–357.

Pierce, A.J., Johnson, R.D., Thompson, L.H., and Jasin, M. (1999). XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev., in press.

Richardson, C., Moynahan, M.E., and Jasin, M. (1998). Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations. Genes Dev. *12*, 3831–3842.

Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T., and Livingston, D.M. (1997). Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell *88*, 265–275.

Sharan, S.K., Morimatsu, M., Albrecht, U., Lim, D.-S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P., and Bradley, A. (1997). Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking *Brca2*. Nature *386*, 804–810.

Shen, S.X., Weaver, Z., Xu, X., Li, C., Weinstein, M., Chen, L., Guan, X.Y., Ried, T., and Deng, C.X. (1998). A targeted disruption of the murine Brca1 gene causes gamma-irradiation hypersensitivity and genetic instability. Oncogene *17*, 3115–3124.

Szostak, J.W., Orr-Weaver, T.L., Rothstein, R.J., and Stahl, F.W. (1983). The double-strand-break repair model for recombination. Cell *33*, 25–35.

Taccioli, G.E., Rathbun, G., Oltz, E., Stamato, T., Jeggo, P.A., and Alt, F.W. (1993). Impairment of V(D)J recombination in double-strand break repair mutants. Science *260*, 207–210.

Tebbs, R.S., Zhao, Y., Tucker, J.D., Scheerer, J.B., Siciliano, M.J.,

Hwang, M., Liu, N., Legerski, R.J., and Thompson, L.H. (1995). Correction of chromosomal instability and sensitivity to diverse mutagens by a cloned cDNA of the XRCC3 DNA repair gene. Proc. Natl. Acad. Sci. USA *92*, 6354–6358.

te Riele, H., Maandag, E.R., Clarke, A., Hooper, M., and Berns, A. (1990). Consecutive inactivation of both alleles of the *pim-1* proto-oncogene by homologous recombination in embryonic stem cells. Nature *348*, 649–651.

te Riele, H., Maandag, E.R., and Berns, A. (1992). Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. Proc. Natl. Acad. Sci. USA *89*, 5128–5132.

Tirkkonen, M., Johannsson, O., Agnarsson, B.A., Olsson, H., Ingvarsson, S., Karhu, R., Tanner, M., Isola, J., Barkardottir, R.B., Borg, A., and Kallioniemi, O.P. (1997). Distinct somatic genetic changes associated with tumor progression in carriers of BRCA1 and BRCA2 germ-line mutations. Cancer Res. *57*, 1222–1227.

Tomlinson, G.E., Chen, T.T., Stastny, V.A., Virmani, A.K., Spillman, M.A., Tonk, V., Blum, J.L., Schneider, N.R., Wistuba, II, Shay, J.W., et al. (1998). Characterization of a breast cancer cell line derived from a germ-line BRCA1 mutation carrier. Cancer Res. *58*, 3237–3242.

Vaughn, J.P., Davis, P.L., Jarboe, M.D., Huper, G., Evans, A.C., Wiseman, R. W., Berchuck, A., Iglehart, J.D., Futreal, P.A., and Marks, J.R. (1996). BRCA1 expression is induced before DNA synthesis in both normal and tumor-derived breast cells. Cell Growth Differ. *7*, 711–715.

Ward, J.F. (1995). Radiation mutagenesis: the initial DNA lesions responsible. Radiat. Res. *142*, 362–368.

Wong, A.K.C., Pero, R., Ormonde, P.A., Tavtigian, S.V., and Bartel, P.L. (1997). RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene Brca2. J. Biol. Chem. *272*, 31941–31944.

Xu, X., Weaver, Z., Linke, S.P., Li, C., Gotay, J., Wang, X.-W., Harris, C.C., Ried, T., and Deng, C.-X. (1999). Centrosome amplification and a defective G_2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. Mol. Cell *3*, 389–395.

Zhong, Q., Chen, C.F., Li, S., Chen, Y., Wang, C.C., Xiao, J., Chen, P.L., Sharp, Z.D., and Lee, W.H. (1999). Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. Science *285*, 747–750.