Checkpoint Kinase 2–Mediated Phosphorylation of BRCA1 Regulates the Fidelity of Nonhomologous End-Joining

Jing Zhuang,[1] Junran Zhang,[3] Henning Willers,[4] Hong Wang,[1] Jay H. Chung,[5] Dik C. van Gent,[6] Dennis E. Hallahan,[1,2] Simon N. Powell,[3] and Fen Xia[1,2]

[1]Department of Radiation Oncology, Vanderbilt University Medical Centre; [2]Vanderbilt-Ingram Cancer Center, School of Medicine, Vanderbilt University, Nashville, Tennessee; [3]Washington University School of Medicine, St. Louis, Missouri; [4]Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts; [5]National Heart Lung and Blood Institute, Bethesda, Maryland; and [6]Department of Cell Biology and Genetics, Erasmus Medical Center, Rotterdam, the Netherlands

Abstract

The tumor suppressor gene *BRCA1* maintains genomic integrity by protecting cells from the deleterious effects of DNA double-strand breaks (DSBs). Through its interactions with the checkpoint kinase 2 (Chk2) kinase and Rad51, BRCA1 promotes homologous recombination, which is typically an error-free repair process. In addition, accumulating evidence implicates BRCA1 in the regulation of nonhomologous end-joining (NHEJ), which may involve precise religation of the DSB ends if they are compatible (i.e., error-free repair) or sequence alteration upon rejoining (i.e., error-prone or mutagenic repair). However, the precise role of BRCA1 in regulating these different subtypes of NHEJ is not clear. We provide here the genetic and biochemical evidence to show that BRCA1 promotes error-free rejoining of DSBs in human breast carcinoma cells while suppressing microhomology-mediated error-prone end-joining and restricting sequence deletion at the break junction during repair. The repair spectrum in BRCA1-deficient cells was characterized by an increase in the formation of >2 kb deletions and in the usage of long microhomologies distal to the break site, compared with wild-type (WT) cells. This error-prone repair phenotype could also be revealed by disruption of the Chk2 phosphorylation site of BRCA1, or by expression of a dominant-negative kinase-dead Chk2 mutant in cells with WT BRCA1. We suggest that the differential control of NHEJ subprocesses by BRCA1, in concert with Chk2, reduces the mutagenic potential of NHEJ, thereby contributing to the prevention of familial breast cancers. (Cancer Res 2006; 66(3): 1401-8)

Introduction

Chromosomal double-strand breaks (DSBs), which may arise during physiologic processes, such as replication or V(D)J recombination, or after cellular exposure to ionizing radiation and chemotherapy, are the most dangerous form of DNA lesions (1, 2). DSBs, which can be lethal if unrepaired or mutagenic if misrepaired, can be removed from the genome by two general types of genetically largely independent repair mechanisms—homologous recombination (HR) or nonhomologous end-joining (NHEJ). HR is typically error-free and requires extensive homology on a sister chromatid or a homologous chromosome, which may serve as repair templates to faithfully restore the original DNA sequence (3). HR is most active in the late S and G_2 phases of cell cycle. In contrast, NHEJ, which is traditionally considered error-prone, reseals DSBs efficiently throughout all cell cycle phases with very limited or no homology requirements (4). It has been shown recently that NHEJ can precisely religate DNA ends without sequence change if the ends are cohesive and complementary or blunt [e.g., the signal ends in V(D)J and restriction endonuclease generated compatible ends; refs. 5, 6]. Alternatively, if error-free end-joining is not an option (e.g., ionizing radiation–induced DSBs or restriction endonuclease–induced noncompatible ends), end-joining is error-prone. This error-prone type of rejoining typically involves end modification (which can be limited with loss or insertion of a few nucleotides) or extensive (with deletions up to hundreds of nucleotides). Given that any deletion arising from the break site may destroy coding or regulatory sequences, or may lead to chromosomal aberrations and thereby increase genomic instability, NHEJ processes must be closely regulated to limit the resulting mutagenic potential. However, little is known about the genetic factors that control the extent of sequence alteration during error-prone repair or that prevent error-prone repair mechanisms in scenarios where precise end-joining is a potentially available repair pathway.

Germ line mutations in BRCA1 predispose to breast cancers and other tumors. BRCA1 has been implicated in a variety of cellular processes, including DNA repair, replication, cell-cycle control, apoptosis, transcription, and chromatin remodeling (7, 8). Dysfunction of many or all of these BRCA1 properties may be invoked in cancer development. Through its interaction with BRCA2/Rad51, BRCA1 promotes HR, thereby ensuring error-free repair of DSBs (9–11). It was recently reported that increased breast cancer risk was highly associated with single nucleotide polymorphisms in NHEJ genes (12). Interestingly, significantly increased numbers of high-risk NHEJ genotypes were seen only in women with at least one variant *BRCA1* allele (13). We and others have shown evidence that BRCA1 plays an active role in regulation of NHEJ. However, these studies have generated conflicting observations on the role of BRCA1 in NHEJ, including a promotion of NHEJ, suppressive effects, or no effect (9, 11, 13–19). These results may at least in part be a reflection of different roles of BRCA1 in various subtypes of NHEJ existing in eukaryotic cells (9, 20–22). An increasing body of evidence suggests that in mammalian cells, DNA-PKcs/Ku–dependent NHEJ is the main mechanism by which ends are rejoined precisely or with minimum sequence modification at the junction. Recently, however, several lines of genetic and biochemical evidence have suggested an alternative NHEJ subpathway that is independent of Ku80 but strongly dependent on Mre11 and Rad50 (5, 20, 23–26), which play a central role in sensing and signal

Note: J. Zhuang and J. Zhang contributed equally to this work.

Requests for reprints: Fen Xia, Department of Radiation Oncology, Vanderbilt University Medical Center, 1301 22nd Avenue South, Nashville, TN 37232. Phone: 615-322-2555; Fax: 615-343-6589; E-mail: fen.xia@vanderbilt.edu.

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transduction of DSBs (5, 20, 23–26). This Mre11-dependent alternative process rejoins chromosomal DNA ends by annealing the resected 3′ overhanging strands with imperfect microhomology. In contrast to DNA-PKcs/Ku–dependent NHEJ, the Mre11-mediated repair pathway is always highly mutagenic with reported nonrandom deletions of up to 300 bp (5, 20), which results from searching for microhomology sequences flanking the DSB ends. Although BRCA1 has not been reported to directly interact with proteins in the DNA-PKcs/Ku pathway, it physically interacts with the Mre11/NBS1/Rad50 complex (27). Several recent studies have linked BRCA1 to Mre11 function. For example, BRCA1 suppresses the DSB-induced Mre11 response and decreases the efficiency of random chromosomal integration of exogenous DNA, which is at least in part mediated by the Mre11 complex (9). Therefore, we hypothesize that, besides controlling error-free HR, BRCA1 also maintains genome integrity by promoting precise NHEJ and by preventing highly mutagenic error-prone microhomology-mediated end-joining (MMEJ).

Phosphorylation of BRCA1 is an important means by which its cellular functions are regulated. During the DNA damage response, BRCA1 is phosphorylated by several protein kinases, such as ATM, ATR, Chk2, and MDC1 (28–31). The checkpoint kinase 2 (Chk2) is emerging as a key mediator in the network of genome-surveillance pathways that coordinate cell cycle progression with DNA repair and cell survival. In response to DNA damage, Chk2 is activated and propagates the checkpoint signal along several pathways to cause cell cycle arrest in the intra-S, and G_2-M phases (32). It has been suggested that Chk2 functions upstream of BRCA1 in the same pathway involved in maintaining genome integrity and preventing cancer development (9, 33). Data from our laboratories have shown that Chk2 phosphorylation at the S988 site of BRCA1, but not ATM-mediated phosphorylation, is important for the function of BRCA1 in promoting error-free HR and inhibiting Mre11-mediated random chromosomal integration (9). However, Chk2 function has not yet been directly linked to NHEJ-mediated DSB repair. We have further proposed that phosphorylation of BRCA1 at S988 by Chk2 controls the function of BRCA1 in orchestrating multiple submechanisms of NHEJ (9).

In the current study, we seek to define the role of BRCA1 and its Chk2 phosphorylation site in various NHEJ subpathways at the DNA sequence level using both naked and chromatinized NHEJ substrates. We find that BRCA1 promotes error-free NHEJ while preventing error-prone MMEJ and restricting sequence deletion upon error-prone repair. Both the Chk2 kinase activity and its phosphorylation site are critical for BRCA1 to differentially regulate various sub-NHEJ mechanisms and to prevent large deletion formation by rejoining along long distal microhomologies. Thus, we suggest that BRCA1, in concert with Chk2, actively regulates NHEJ processes to reduce their mutagenic potential.

Materials and Methods

Cell lines, transfections, infections, and Western blots. HCC1937 human breast carcinoma cells were established and maintained as described (9). Briefly, parental HCC1937 cells were stably transfected with an expression plasmid for wild-type (WT) BRCA1, the Chk2 phosphorylation mutant, or an empty vector control (pcDNA3-BRCA1, pcDNA3-BRCA1-S988A, or pcDNA3, respectively). All transfections were done using LipofectAMINE (Invitrogen, Carlsbad, CA) and BRCA1-expressing clones were selected with G418 (300 μg/mL, Mediatech, Inc., Herndon, VA). Clones were screened for comparable expression level of WT or mutant BRCA1 protein by immunoprecipitation and immunoblotting (Ab-3 and Ab-1, respectively; Oncogene, San Diego, CA). For transient expression of WT Chk2 or kinase-dead (KD) Chk2 mutant, HCC1937 cells were infected with recombinant adenovirus with cytomegalovirus promoter–driven Chk2-WT or Chk2-KD at 10 plaque-forming units per cell (31, 34). Total cell lysates were prepared 24 hours after infection to confirm comparable protein expression levels of Chk2-WT and Chk2-KD with Western blot probed with mouse anti-Chk2 antibody (Ab-12 Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Plasmid construction. The plasmid pGL3-MCS as a substrate for end-joining–mediated recircularization was derived from pGL3-Control (Promega, Madison, WI). To introduce additional unique restriction cleavage sites between the promoter and the luciferase gene, part of the multiple cloning site of pcDNA3.1/Zeo (Invitrogen) was amplified by PCR. This PCR fragment (106 bp) was inserted into the unique *Hin*dIII site of pGL3-Control. The integrity of the fragment was verified by DNA sequencing. Blunt-ended DSBs were created by *Eco*RV cleavage and cohesive ends were created by digest with *Apa*I or *Sal*I (pGL3-MCS) or *Hin*dIII (pGL3-Control) enzymes.

The plasmid pEPI-HDV6 as the substrate for the transposon excision assay was derived from pDVH6. pDVH6 contains a Tn5 minitransposon that can be excised by Tn5 transposase, thereby creating a blunt-ended DSB. The polyoma sequence in the plasmid enables stable episomal replication in murine cells (6). The plasmid pEPI-GFP, which carries a human scaffold/matrix-attached region and allows sustained episomal replication without chromosomal integration in human cells, was generously provided by Dr. H.J. Lipps (35). The 2.6 kb *Pvu*II fragment of pDVH6 containing all the elements required for the Tn5 transposase excision assay was subcloned into the *Eco*RV site of plasmid pEPI-GFP (35). This generated pEPI-HDV6, which allows for assessment of Tn5 transposon excision and end-rejoining in a chromatinized context in human cells. To confirm the episomal replication of pEPI-HDV6, extrachromosomal plasmids were harvested at days 15, 22, and 35 after transfection of HCC1937 cells using the EZNA plasmid miniprep kit (Omega Bio-tek, Doraville, GA) and subjected to restriction digestion with either *Dpn*I or *Mbo*I. After replication in human cells, the plasmids are sensitive to *Mbo*I but resistant to *Dpn*I digestion (NEB, Ipswich, MA). The mitotic stability of the episomal plasmid was determined by quantifying the copy number of the episomal plasmid using Southern blot analysis of extrachromosomal plasmid extracted up to 35 days after transfection.

Assay of NHEJ by circularization of linear plasmid substrate. pGL3-MCS was cleaved between the promoter and luciferase reporter gene. Linearized DNA was gel purified for human cell transfection. Cells were cotransfected with a pRL-SV40 internal control (renilla luciferase, Promega) and either cleaved substrate or a circular positive control using Lipofect-AMINE. All transfections were done in parallel. Cell extracts were prepared 24 hours later according to the instructions of the manufacturer and assayed in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) using the Dual-Luciferase Reporter assay System (Promega). The relative DSB rejoining activity was obtained by comparison of firefly luciferase activity detected in cells transfected with linearized substrate relative to cells transfected with circular plasmid corrected for transfection efficiency and expression levels.

For physical analysis of repair products, plasmid DNA was extracted 24 hours after transfection as described (36). The DNA was transformed into electrocompetent bacteria (DH10B, Invitrogen), followed by plating onto fresh Luria-Bertani agar plates containing 50 μg/mL ampicillin. Small-scale DNA preparations were made from bacterial colonies (Qiagen, Hilden, Germany) and subjected to restriction fragment analysis, PCR amplification, and sequencing (see text and figure legends for details).

Assay of NHEJ by Tn5 transposon excision. Seven days after transfection of pEPI-HDV6 into HCC1937 cells, cells were infected with recombinant adenovirus expressing either Chk2-WT or Chk2-KD. On the following day, cells were transfected with the Tn5 transposase expression vector pDVG-148. Extrachromosomal plasmids were harvested at day 10 for analysis of NHEJ. To assess the relative level of overall end-joining, recovered plasmids were subjected to *Dpn*I digestion and PCR amplification using primers flanking the transposon region (5′-GGCTAACTAGAGAACCCACTGCTTA-3′ and 5′-GACCGGTGTCAGATTATGCAGCAA-3′). PCR products

were separated on 8% polyacrylamide gel in Tris-borate EDTA buffer, silver stained using Silver Stain Plus from Bio-Rad (Hercules, CA), and analyzed using the Quantity One Analysis Software (Bio-Rad). To determine the nature of NHEJ events, the recovered extrachromosomal DNA were digested with *Bam*HI and *Nhe*I before PCR amplification to remove plasmids that did not undergo transposon excision. PCR products digested with *Eco*RV endonuclease, which cleaves precise rejoining products, or *Bst*XI, which cleaves the imprecise NHEJ products using a designed 6 bp microhomology sequence flanking the DSB (Fig. 3), were subjected to gel separation and quantification.

Results

BRCA1 promotes overall DSB end rejoining. To study the role of BRCA1 on NHEJ at the DNA sequence level in living human cells, we started out by using a commonly used reporter reactivation assay (15, 17, 37). The plasmid substrate pGL3-MCS was cleaved by the *Eco*RV restriction endonuclease *in vitro* between the promoter and the luciferase reporter gene, thereby preventing expression of the reporter *in vivo* (Fig. 1*A*). The linearized plasmid was introduced together with an internal control plasmid into a previously well-characterized isogenic pair of HCC1937 breast carcinoma cells with or without stable expression of WT BRCA1 (for the BRCA1 S988 mutant, see below; Fig. 1*B*; ref. 9). Intracellular recircularization of linearized DNA was detected by measuring luciferase activities following 24 hours of incubation. The rejoining levels in BRCA1-deficient cells were significantly reduced compared with cells with restored WT BRCA1 (i.e., 13.4% versus 24.1% rejoining of linear substrate relative to circular control transfected in parallel, respectively; Fig. 1*C*). The effect of BRCA1 on NHEJ was not dependent on the type of double-stranded ends present: The difference in the rejoining of blunt ends (*Eco*RV) between cells with and without WT BRCA1 (Fig. 1*C*) was comparable with the difference in the repair of cohesive ends, which was 12.8% versus 27.6% ($P < 0.01$; data not shown). Thus, WT BRCA1 is required for proficient rejoining of linearized extrachromosomal plasmid substrates.

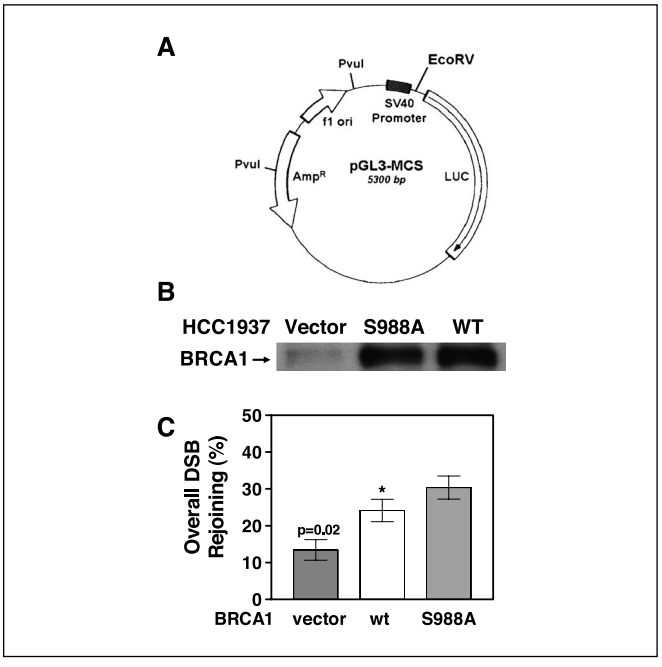

Figure 1. Intramolecular NHEJ measured by an episomal plasmid assay. *A,* the luciferase (*LUC*)-based reporter plasmid, pGL3-MCS, offers a set of unique restriction enzyme cleavage sites, including *Eco*RV, between the promoter and the *LUC* gene. *Pvu*I sites are used for deletion size mapping (see Fig. 6*A* for further details). *B,* the protein expression levels of WT BRCA1 and the S988A mutant in HCC1937 cells. *C,* the rejoining of *Eco*RV-cleaved plasmid ends was measured by the LUC activity detected in cells transfected with linear plasmid substrate relative to circular plasmid control.

It has been speculated that the mechanisms of recircularization of "naked" extrachromosomal linearized DNA may be different from the rejoining of chromosomal DSBs, which are packed in highly organized chromatin. For example, the status of the *DNA-PKcs* or *XRCC4* genes, which are essential for V(D)J recombination and cellular resistance to X-rays, only have a mild effect on recircularization of linearized substrate DNA (22, 37–39).

Therefore, to verify the results obtained with naked, non-replicating DNA substrates, we used an assay based on the rejoining of blunt-ended DSBs generated by expression of Tn5 transposase in a chromatinized episomal replicating plasmid substrate pEPI-DVH6 in human cells (Fig. 2*A*). This transposon excision assay has been developed previously and has been successfully used to show the roles of DNA-PK, XRCC4, and MDC1 in the control of NHEJ in murine cells (6, 40). Briefly, transient expression of Tn5 transposase in HCC1937 cells carrying the episomally replicating pEPI-DVH6 substrate generates blunt-ended DSBs at the defined Tn5 transposon recognition sites within the substrate (Fig. 2*A*). Transposon excision and end-joining can be determined by PCR amplification of the region containing the transposon. Without transposon excision, PCR amplification of pEPI-DVH6 gives rise to a product of 487 bp, whereas after transposon excision and end-rejoining the PCR fragments are shorter (146 bp; Fig. 2*B*). Consistent with the previous recircularization assay (Fig. 1*C*), the loss of BRCA1 resulted in a 3.2-fold reduction in the relative efficiency of DSB rejoining, e.g., 2.4 $\pm$ 0.16% in vector-transfected BRCA1-null HCC1937 cells compared with 7.7% $\pm$ 0.01% in cells with WT BRCA1 (Fig. 2*C*). We also determined that BRCA1 status had no significant effect on either the copy number of the episomally replicating pEPI-HDV6 plasmid, measured using both Southern blot analysis and *Escherichia coli* transformation efficiency, or the transfection efficiency, determined by an YFP expression vector (data not shown).

To determine if the phosphorylation of BRCA1 regulates its function in NHEJ, we used a phosphorylation mutant of BRCA1, which contained a serine 988 to alanine substitution (S988A), thereby abrogating phosphorylation of BRCA1 by Chk2 (9, 41). We examined the relative overall rejoining efficiencies in HCC1937 cells stably expressing either WT BRCA1 or the S988A mutant (Fig. 1*B*). As shown in Fig. 1*C* and 2*C*, the levels of overall NHEJ in S988A-expressing cells were increased by almost 2-fold compared with vector-alone transfected BRCA1-deficient cells, although for the transposon assay the NHEJ level in S988A-expressing cells did not reach the same rejoining proficiency produced by the presence of WT protein, as observed with the luciferase assay (Fig. 1*C*). It is possible that this difference is due to a different degree of usage of the various NHEJ subpathways in the repair of DSBs in naked DNA versus chromatinized DNA (6).

Chk2 kinase activity has not been directly linked to NHEJ. We reasoned that if the effect of the S988A mutant on overall NHEJ truly resulted from its resistance to Chk2 kinase–mediated phosphorylation, disruption of the kinase activity of Chk2 would result in decreased NHEJ in BRCA1-proficient cells but would not affect overall NHEJ in cells deficient for BRCA1 or expressing the S988A mutant. Therefore, we infected HCC1937 cells with different BRCA1 status with recombinant adenovirus expressing either Chk2-WT or Chk2-KD, which inhibits the kinase activity of endogenous WT Chk2

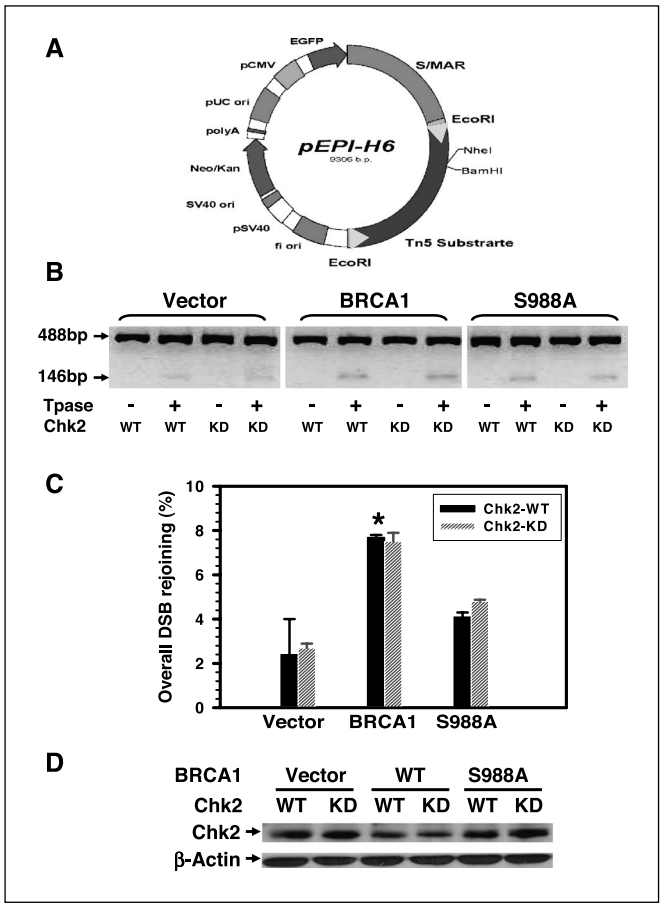

Figure 2. Intramolecular NHEJ measured by a chromatinized episomal plasmid assay (*A*). The transposon excision assay plasmid, pEPI-HDV6, contains two Tn5 ends in inverted orientation (*triangles*) and the scaffold/matrix-attached region (*S/MAR*) sequence, which enables sustained episomal replication of the plasmid in human cells. After transfection of Tn5 transposase expression plasmid, the Tn5 minitransposon is excised. *B,* products obtained by PCR amplification over the Tn5 substrate. In the absence of Tn5 transposase (*Tpase*) expression, the intact transposon region amplified by PCR produced a 488 bp fragment. Upon Tn5 transposase expression, the excision of the transposon and the rejoining of the flaking ends produced a shorter fragment. *C,* the quantity of the PCR fragment was analyzed using the Quantity One Analysis Software from Bio-Rad. The relative efficiencies of DSB rejoining were calculated accordingly. *Columns,* mean of three to seven independent experiments; *bars,* SE. Comparisons were made between isogenic HCC1937 cells stably transfected with WT BRCA1, the Chk2 phosphorylation site mutant (S988A), or empty control vector, and the additional transient expression of WT or KD Chk2. *, *P* values for comparison with WT BRCA1 (Fisher's exact test). *D,* the protein expression levels of Chk2 WT and Chk2 KD in HCC1937 cells with different BRCA1 status as indicated.

in a dominant-negative fashion (31, 34). Comparable expressing levels of Chk2-WT or Chk2-KD were achieved (Fig. 2*D*). Consistent with the observed rejoining proficiency of S988A-expressing cells, we found that expression of Chk2-KD had no additional effect on the overall NHEJ efficiency regardless of the BRCA1 status (Fig. 1*C* and 2*C*). We conclude that despite the dependence of BRCA1 promotion of HR on Chk2-mediated phosphorylation, Chk2 plays a less important role in the promotion of BRCA1-dependent overall NHEJ proficiency, which represents the combined DSB rejoining efficiencies of various NHEJ subpathways.

Promotion of error-free NHEJ by BRCA1 depends on Chk2 phosphorylation at the S988 site. Different subpathways of NHEJ may dictate the repair of the plasmid substrate by either error-free direct ligation or by error-prone MMEJ. To determine whether the fidelity of end-joining is controlled by Chk2 phosphorylation of BRCA1, we used the pEPI-HDV6 substrate. The sequences flanking the Tn5 transposon have been designed in such a way that precise rejoining of the DSB ends would generate an *Eco*RV restriction site (Fig. 3*A*), whereas imprecise MMEJ using a 6-bp microhomology that directly flanks the transposon would generate a novel *Bst*XI restriction site (6). This sequence arrangement allowed us to detect the relative efficiencies of these different NHEJ repair events by digestion of the PCR products with either *Eco*RV or *Bst*XI (Fig. 3*B*). The relative efficiencies of precise rejoining were assessed in HCC1937 cells with different BRCA1 status (Fig. 3*C*; ref. 9). Loss of

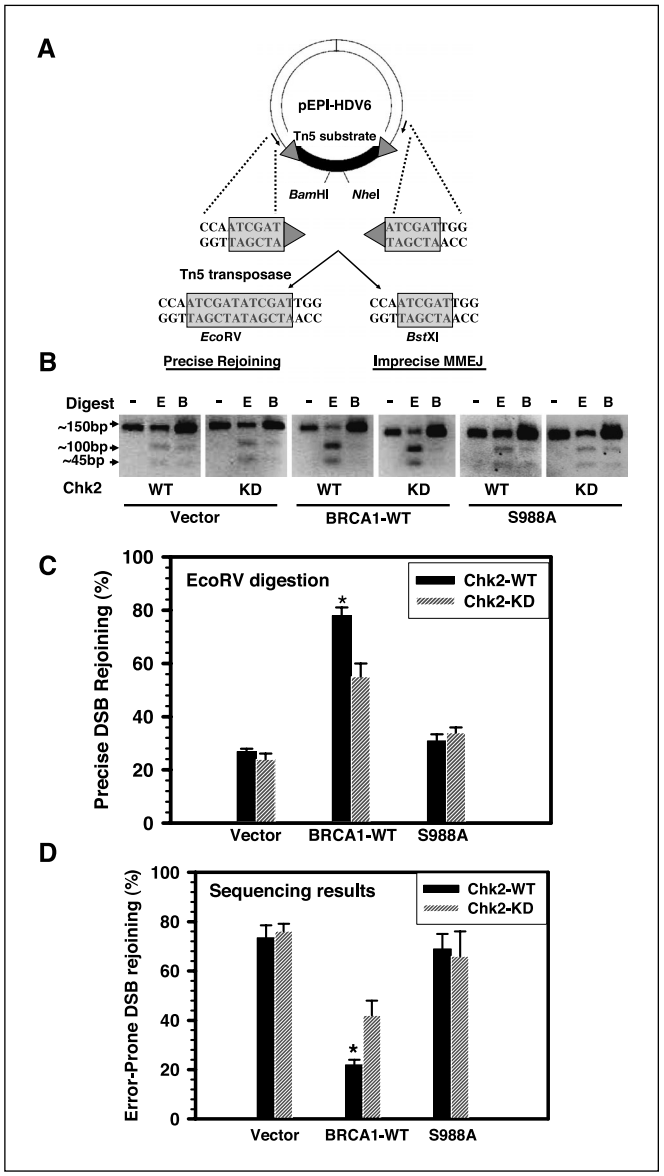

Figure 3. Influence of BRCA1 and its phosphorylation site on fidelity of NHEJ. *A,* schematic representation of the transposon excision reaction to assess error-free versus error-prone NHEJ. *B,* the precise repair of the DSB ends without alteration of sequence creates a novel *Eco*RV restriction site. Imprecise end-joining using a stretch of 6 bp microhomology (MMEJ) flanking the DSB creates a novel *Bst*XI site. Digestion of PCR product with *Eco*RV (*E*) or *Bst*XI (*B*) enzymes generates two shorter fragments, respectively. *C,* the relative levels of precise rejoining per *Eco*RV digestion of recovered pEPI-HDV6 were quantified similarly as described in Fig. 2*C*. *D,* the relative levels of imprecise rejoining determined by sequencing analysis of individual recovered pEPI-HDV6 molecules. *Columns,* mean of three to seven independent experiments; *bars,* SE.

BRCA1 WT function or mutation at the Chk2 phosphorylation site resulted in a 2.5- to 3-fold decrease in the efficiency of precise religation compared with cells with WT BRCA1 (27 ± 0.5% and 31 ± 3% versus 78 ± 3%, respectively). In addition, by using sequencing analysis of the break junction, we confirmed an increased percentage of imprecise rejoining events (i.e., resistant to *Eco*RV digestion) in cells expressing the S988A mutant (Fig. 3*D*), which mirrors the reduced frequency of precise events measured by *Eco*RV digestion (Fig. 3*C*). To confirm that Chk2 phosphorylation at the S988 site is critical for the role of BRCA1 in precise rejoining, we also showed that expression of the dominant-negative Chk2-KD mutant led to a significant 25% reduction of religation in cells with WT BRCA1 ($P = 0.02$), but not in cells deficient for BRCA1 or expressing the S988A mutant, compared with cells expressing exogenous WT Chk2 (Fig. 3*C* and *D*). Thus, we provide genetic evidence for a promotion of precise DSB ligation and inhibition of imprecise end-joining by BRCA1 that is controlled by Chk2-mediated phosphorylation.

In a second approach, we also used the recircularization assay with the linearized pGL3-MCS substrate to examine the role of BRCA1 phosphorylation in the fidelity of rejoining. Twenty-four hours after transfection of the linearized substrate, plasmids were extracted from the cells and amplified in bacteria. Precisely rejoined plasmid retained the *Eco*RV restriction site as determined by PCR with primers flanking the DSB and susceptibility to *Eco*RV digestion (Fig. 4*A*). A total of 689 colonies were analyzed. Interestingly, different from the transposon assay, decreased levels of error-free NHEJ were only seen in BRCA1-deficient cells (34%), but not in cells with S988A compared with cells expressing the WT protein (45-49%, $P = 0.04$; Fig. 4*B*). Again, it is possible that the differential effect of the S988A mutant on end-joining using the pGL3-MCS substrate versus pEPI-HDV6 is linked to the previously reported use of different NHEJ mechanisms in the repair of naked versus chromatinized DSBs (6) but this was not investigated further (see also Discussion).

BRCA1-mediated suppression of error-prone rejoining and deletional MMEJ is controlled by Chk2 phosphorylation. Using the chromatinized pEPI-HDV6 substrate in HCC1937 cells with different BRCA1 status, we next studied the role of BRCA1 in deletional imprecise MMEJ. We used the designed 6 bp microhomology sequence directly flanking the DSB described above (Figs. 3*A* and *B*). Consistent with the increased levels of imprecise rejoining in BRCA1-deficient cells (Figs. 3*C, D* and 4*B*), we found a strong 13-fold and 11-fold induction of the relative levels of deletional MMEJ in cells deficient for WT BRCA1 (5.5 ± 1%, $P = 0.013$) or expressing the S988A mutant (5.1 ± 0.8%, $P = 0.023$) compared with cells with WT protein (0.5 ± 0.2%; Fig. 5*A*). To confirm that Chk2-mediated phosphorylation is required for the apparent inhibition of error-prone MMEJ by WT BRCA1, we expressed Chk2-KD, which resulted in a 7.3-fold increase of MMEJ levels only in BRCA1-proficient cells compared with Chk2-WT expressing control cells (3.5 ± 0.4% versus 0.5 ± 0.2%, $P < 0.01$). By contrast, there was no effect in BRCA1-deficient cells or in cells expressing the S988A mutant (Fig. 5*A*). Thus, our results suggest that Chk2 controls the function of BRCA1 in the prevention of imprecise end-joining and deletional MMEJ through phosphorylation at the S988 site.

Mutation of Mre11 in yeast has been reported to shift the spectrum of nonhomologous repair products toward a decreased proportion of rejoining mediated by 8 to 10 bp of microhomology (20). Given the observation that BRCA1 inhibits the function of

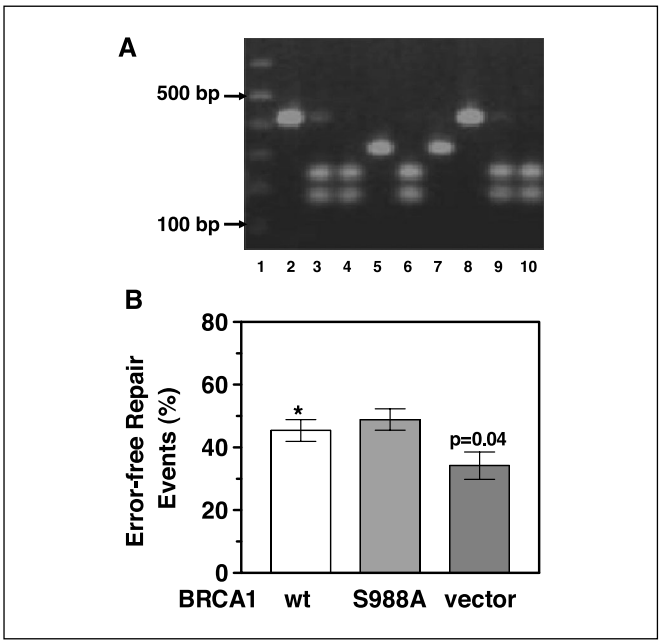

Figure 4. BRCA1 and its phosphorylation site are required for error-free NHEJ. *A,* a representative spectrum of individual repair products recovered from transfected cells for measuring the precise recircularization of pGL3-MCS plasmid. Preservation of the cleavage site upon error-free religation of the break ends generates two bands of 176 and 242 bp from PCR amplification of the region including the junction (*lanes 3, 4, 6, 9*, and *10*). *B,* the proportion of error-free events among all repair events with pGL3-MCS. *Columns,* mean of three to seven independent experiments; *bars,* SE.

Mre11 *in vivo* (9) and *in vitro* (42), we therefore sought to determine whether an opposite shift (a relative increase of longer stretches of microhomology) could be detected upon loss of BRCA1 function. For this approach, we preferred to use pGL3-MCS over pEPI-HDV6 because pGL3-MCS offers various stretches of microhomology around the cleavage site (see Fig. 5*D* and below). The break junction of a sample of 204 error-prone repair products was sequenced (i.e., products that had lost the *Eco*RV site). In the presence of BRCA1, 43% of all repair events were mediated by flanking microhomologies (Fig. 5*B*). In contrast, in the absence of BRCA1 or upon loss of the Chk2 phosphorylation site, the proportion of rejoining events using any microhomology length was significantly elevated to 65% to 72% ($P \leq 0.02$). The break junctions of a representative sample of 204 error-prone repair products were sequenced and analyzed. We showed that the vast majority of microhomologies in cells with WT BRCA1 were of 1 to 5 bp length, as commonly observed in NHEJ (Fig. 5*C*; ref. 4). Some typically used microhomologies are illustrated in Fig. 5*D*. In contrast, in BRCA1-deficient cells or in cells expressing the S988A mutant, there was a significant shift toward use of a stretch of 8 bp microhomology that flanked the break site at a distance of ~50 bp on either side (i.e., an increase to 43% to 75% in the presence of S988A versus 7% in cells with WT BRCA1; $P \leq 0.04$). The majority of 1 to 5 bp microhomologies used for rejoining were located between these 8 bp stretches. Taken together, the data indicate that the Chk2 phosphorylation site controls deletion size and microhomology usage during error-prone NHEJ.

BRCA1-mediated restriction of deletion formation is dependent on the Chk2 phosphorylation site. Error-prone NHEJ often involves limited deletion of no more than a few nucleotides at the site of rejoining (4), but more extensive loss of sequence can

also be observed (20). To study the effect of BRCA1 phosphorylation on deletion formation, we subjected the error-prone repair products to further analysis. Virtually all products were found to have arisen from deletion events (data not shown). Initial experiments categorized the deletions into three arbitrary classes (i.e., ≤175 bp versus 176 to 2,000 bp versus >2,000 bp; Fig. 6*A* and *B*). In the presence of WT BRCA1, almost 50% of deletions were ≤175 bp, whereas only in a few cases (8%) the deletion size exceeded 2,000 bp (Fig. 6*C*). Conversely, BRCA1-deficient cells showed the opposite pattern, with a lower percentage of ≤175 bp deletions and a higher percentage of large deletions (i.e., 30% and 20%, respectively; $P < 0.03$). Interestingly, the distribution of deletion sizes in the presence of the S988A mutant revealed a phenotype that was almost identical to the spectrum of products seen with BRCA1 deficiency. Of note, in the S988A-expressing cells, the 12% relative increase in the proportion of >2,000 bp deletions, which extended into the promoter and luciferase gene regions, could not be detected as a reduced level of luciferase activity (Fig. 1*C*). We estimated that such a 12% increase would have only led to an ~3% drop in luciferase activity compared with WT cells, which would have been within the error-margin of the assay (for WT cells, mean luciferase activity, 24.1%; 95% confidence intervals, ±8.5%).

To determine whether cell cycle kinetics might have contributed to the observed differences, we examined cell cycle distributions by flow cytometric analysis as described previously (9). The proportion of cells in the G_1, S, and G_2 phases was similar in control and BRCA1-expressing cells (data not shown). Thus, the spectrum of deletion sizes was not due to variations in cell cycle distribution of the cell lines under study.

Discussion

A growing body of evidence implicates that BRCA1 actively regulates both principal DSB repair mechanisms, HR and NHEJ. We have previously shown that abrogation of Chk2-mediated phosphorylation of BRCA1 at the S988 site or absence of WT BRCA1 protein affected NHEJ as measured by random chromosomal integration of a plasmid substrate (9). However, the effect of BRCA1 on integration frequencies could have been the result of multiple factors. Whether a particular NHEJ subpathway was controlled by BRCA1 was unknown. In this study, we investigated the effect of BRCA1 on the proficiency and fidelity of DSB repair through various NHEJ subpathways and the role of posttranslational modification of BRCA1 by Chk2 phosphorylation. The evidence presented here shows that BRCA1 controls two aspects of end-joining: It promotes error-free NHEJ while inhibiting error-prone MMEJ and minimizing the size of deletions found in association with rejoining. Furthermore, the Chk2-mediated phosphorylation of BRCA1 was critical for the differential roles of BRCA1 in controlling precise end-joining and mutagenic MMEJ.

A strength of our study was the use of a strictly isogenic cell system with genetic manipulation on two different protein levels, BRCA1 and Chk2, as well as the use of two different repair assays, which yielded largely comparable results. Interestingly, the overall rejoining levels seen with "naked" DNA (pGL3-MCS; 15-20%) seemed higher than with chromatinized DNA (pEPI-H6; <10%), which may represent a differential accessibility of the substrate DNA for repair enzymes (see also below). We acknowledge that that imprecise repair of the plasmid ends in pGL3-MCS could have potentially affected luciferase expression levels. For example, large deletions may extend toward the viral promoter compromising transcription or destroy the downstream translational initiation site. As a result, measuring luciferase expression levels might have underestimated the true overall rejoining proficiency of the cell. However, the use of a PCR-based approach in the chromatinized pEPI-H6 system, which is less influenced although not completely independent of sequence changes originating from the break junction (Fig. 2*C*), corroborated the luciferase expression results.

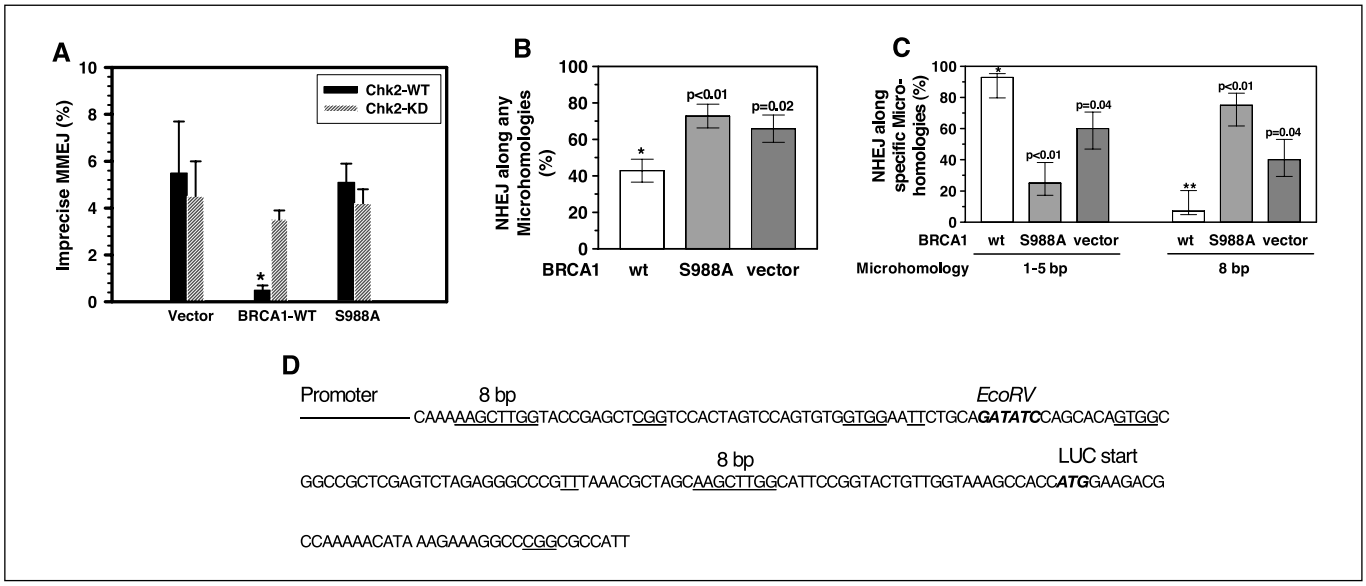

Figure 5. Function of BRCA1 in prevention of error-prone MMEJ depends on Chk2-mediated phosphorylation. *A,* the relative levels of deletional MMEJ using a flanking 6 bp microhomology in the transposon excision substrate pEPI-HDV6 in cells with different BRCA1 and Chk2 status were determined as described in Fig. 3. *B,* proportion of DSB rejoining along microhomologies of any length among all error-prone repair products, as determined by DNA sequencing of the recircularized pGL3-MCS. *C,* proportion of DSB rejoining according to length of microhomologies in the ≤175 bp deletion class. Central confidence intervals (68%) were calculated because some of the proportions approached 0% or 100%. *D,* microhomology usage around the *Eco*RV cleavage site. Typical 2 to 4 bp microhomologies as well as the 8 bp stretches are underlined. Indicated is the start codon for the luciferase open reading frame. *Columns,* mean of three to seven independent experiments; *bars,* SE.

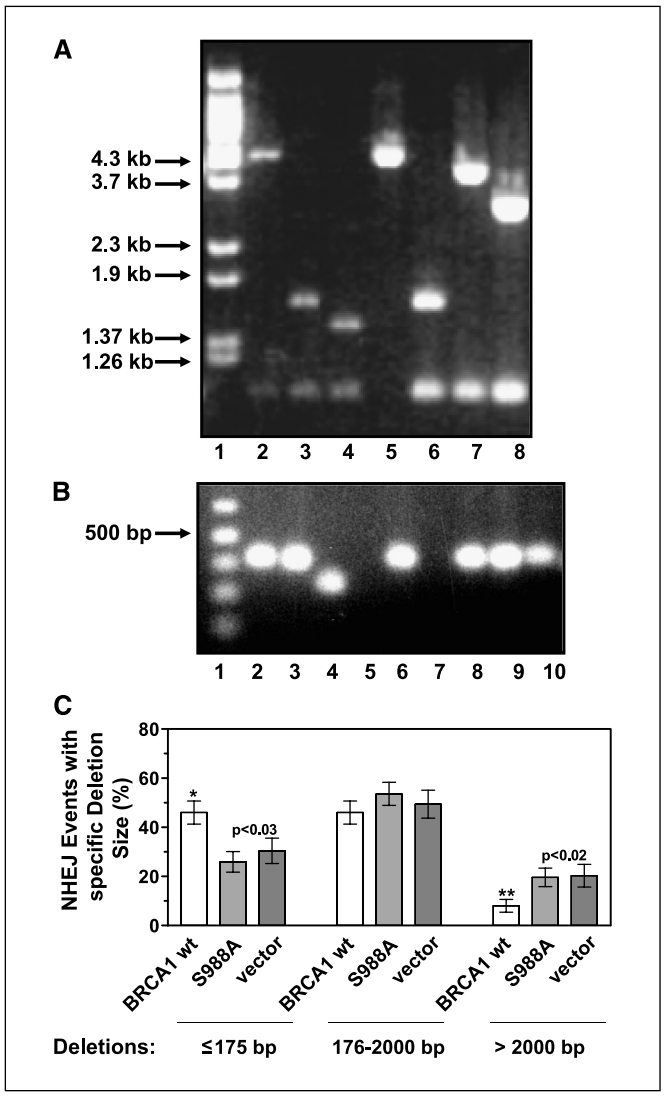

Figure 6. The restriction of deletion size by BRCA1 is controlled by Chk2 phosphorylation. *A,* 0.8% agarose gel with representative repair spectrum depicting deletions of >175 bp. Extracted plasmid was subjected to *Pvu*I cleavage, which generates two bands of ~1 and 4.3 kb size (see Fig. 1*A* for *Pvu*I sites). Deletions arising from the *Eco*RV break site mostly reduced the size of the 4.3 kb fragment. If the deletion extended 600 bp or more upstream, one of the *Pvu*I cleavage sites was lost. The downstream cleavage site was located at a 3.7 kb distance. *Lanes 2, 5, 7, 8,* deletions of 176 to 2,000 bp; *lanes 3, 4, 6,* deletions of >2,000 bp. *Lane 1,* λ *Hin*dIII/*Bst*II marker; additional DNA ladders not shown. *B,* 1.2% agarose gel to detect deletions of ≤175 bp, which are too small to be resolved on gels as in (*A*). An ~400 bp fragment around the *Eco*RV site was amplified by PCR and subjected to direct DNA sequencing to verify deletion size. *Lane 4,* visible deletion of ~100 bp. *Lane 5,* seven asymmetric deletions extending to one of the PCR primer sites 200 bp away from the *Eco*RV site. *Lanes 2, 3, 6, 8, 9, 10,* small deletions detected by subsequent DNA sequencing. *C,* quantitative analysis of deletion products. *Columns,* mean of three to seven independent experiments; *bars,* SE.

In support of our findings, another study using a different NHEJ substrate and cell lines observed that the promotion of BRCA1 on precise DSB end-joining was dependent on Chk2 kinase to phosphorylate BRCA1 at S988 site (44). Our results are also consistent with previous reports demonstrating a promotion of end-joining activity by BRCA1 using retroviral or plasmid substrates (13, 15, 16). In our system as well as in other reports, DNA-PKcs or XRCC4 deficiency did not significantly reduce the efficiency of recircularization of cleaved plasmid (data not shown; refs. 5, 6, 22, 37–39). The recircularization efficiency has been shown to be relatively less dependent on a DNA-PKcs/Ku–mediated mechanism but more readily affected by a MMEJ-based mechanism. Our observation that the effect of BRCA1 on error-free end-joining was evident only in repairing chromatinized DNA but not naked DNA implies that (*a*) the promoting function of BRCA1 on error-free end-joining seems to be at least partially associated with the DNA-PKcs/Ku–dependent pathway; and (*b*) the presence of an alternative NHEJ pathway such as MMEJ. The physical and functional interrelationship between BRCA1 and the components of DNA-PKcs/Ku pathway needs to be defined in future investigations.

Our results also suggest that the Chk2 phosphorylation site of BRCA1 controls some but not all of the functions of BRCA1 in NHEJ. It seems that the complete absence of the BRCA1 protein causes a more severe overall repair phenotype than the presence of a protein with the Chk2 phosphorylation site mutated (Fig. 1). We speculate that this phosphorylation site is required for the modification of NHEJ fidelity by BRCA1. A possible downstream mechanism for this effect involves the activity of the Mre11 protein, which interacts with BRCA1 (27, 42, 43). Interestingly, when we analyzed the repair spectra with regard to microhomology usage, we detected a shift in the utilization of regions of 1 to 5 bp to a more distal region of 8 bp in BRCA1-null and S988A-expressing cells, compared with cells with WT protein. This repair phenotype is reminiscent of a Mre11-mediated repair activity in light of recent observations in yeast, which have revealed that error-prone NHEJ utilizing flanking 8 to 10 bp microhomologies was strongly dependent on Mre11 (20). Deletion formation and usage of distant microhomologies is likely linked to the biochemical properties of Mre11, including its exonuclease and annealing activities (20, 45). BRCA1 strongly binds to double-stranded DNA, thereby inhibiting the nucleolytic activity of the Mre11 complex (42). Impaired phosphorylation at the S988 site, which resides in the DNA-binding region of BRCA1, may disrupt the direct inhibitory effect on Mre11 function, although this remains to be shown directly (9, 42). The mechanisms by which the interaction between Chk2, BRCA1, and Mre11 affects the frequency and fidelity of NHEJ processes await further study.

In conclusion, as a central regulator of the cellular response to DSBs, BRCA1 may influence DSB processing and repair at an early stage. The BRCA1 protein becomes rapidly hyperphosphorylated after induction of DNA damage (45, 46). Chk2-mediated phosphorylation of BRCA1 seems critical for the reported active regulation of both principal repair mechanisms HR and NHEJ (9). This suggests that BRCA1 functions at a point upstream in the response pathway where it helps determining whether a break is channeled toward an error-free homologous or a error-prone nonhomologous repair process. In addition, BRCA1 may modulate NHEJ processes to restrict the extent of sequence deletion at the break site. We suggest that in addition to its promotion of HR, the detailed control of NHEJ processes by BRCA1, in concert with Chk2, reduces the mutagenic potential of NHEJ, thereby contributing to the prevention of familial breast cancers.

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