BRCA1-Ku80 Protein Interaction Enhances End-joining Fidelity of Chromosomal Double-strand Breaks in the G₁ Phase of the Cell Cycle*†‡§¶

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Quality control of DNA double-strand break repair is vital in preventing mutagenesis. Non-homologous end-joining (NHEJ), a repair process predominant in the G₁ phase of the cell cycle, rejoins DSBs either accurately or with errors, but the mechanisms controlling its fidelity are poorly understood. Here we show that BRCA1, a tumor suppressor, enhances the fidelity of NHEJ-mediated DSB repair and prevents mutagenic deletional end-joining through interaction with canonical NHEJ machinery during G₁. BRCA1 binds and stabilizes Ku80 at DSBs through its N-terminal region, promotes precise DSB rejoining, and increases cellular resistance to radiation-induced DNA damage in a G₁ phase-specific manner. These results suggest that BRCA1, as a central player in genome integrity maintenance, ensures high fidelity repair of DSBs by not only promoting homologous recombination repair in G₂/M phase but also facilitates fidelity of NHEJ repair during G₁.

Background: Quality control of DNA double-strand break repair is poorly understood.

Results: BRCA1 enhances the fidelity of NHEJ repair and prevents mutagenic deletional end-joining through interaction with canonical NHEJ machinery during G₁.

Conclusion: BRCA1 ensures high fidelity repair of NHEJ and thus prevents deletional end-joining of chromosomal DSBs during G₁.

Significance: BRCA1 not only promotes homologous recombination in G₂/M phase but also facilitates fidelity of NHEJ repair during G₁.

DNA double-strand breaks (DSBs) are lethal if unrepaired or can lead to mutagenesis and genomic instability if misrepaired (1). There are two major processes for repair of DSBs in mammalian cells: homologous recombination (HR) and non-homologous end-joining (NHEJ) (2, 3). HR repairs DSBs precisely using the intact copy of an extensively homologous sequence from a sister chromatid during the late S and G₂ phases of the cell cycle. In the G₁ phase, NHEJ repairs the majority of DSBs without relying on extensive sequence homology (4, 5).

NHEJ consists of two subpathways: the canonical NHEJ pathway (C-NHEJ) and the alternative NHEJ (Alt-NHEJ) pathway (6–14). The C-NHEJ pathway, which is dependent on the Ku80 protein complex, can precisely repair DSBs when the physical structures at the ends are compatible. Ku80 has been shown to be a critical factor in preventing carcinogenesis in conjunction with the tumor suppressor p53 (4). In contrast, the Alt-NHEJ pathway, which is independent of Ku80 and mediated by Mre11 (16–18), repairs DSBs by searching for and using flanking microhomology. This results in extensive deletions at the junction and is highly mutagenic.

To maintain genomic integrity, cells must choose among multiple competing pathways to repair DSBs. The tumor suppressor BRCA1, whose mutation in individuals is associated with familial breast and ovarian cancers (19), is a factor common to each of these pathways. In HR, BRCA1 promotes high fidelity repair in response to DNA damage during the late S and G₂ phases and may serve as the key regulator of this process. BRCA1 dysfunction leads to deficiencies in HR, causing genomic instability and accelerated carcinogenesis (20–22). Despite the lack of critical role in Rag1/2 induced V(D)J recombination (23), BRCA1 has also been shown to promote the fidelity of NHEJ repair (13, 14, 24–26) and to repress Alt-NHEJ repair in episomal plasmid-based NHEJ assays (27). We sought to investigate whether BRCA1, as a tumor suppressor, promotes fidelity of end-joining of DSBs through interaction with the C-NHEJ machinery, a function different from its known role in HR.
EXPERIMENTAL PROCEDURES

Cell Culture, siRNAs, Plasmids, and Antibodies—HEK293/pPHW1 cells were cultured in complete DMEM medium. pcDNA3β-HA-BRCA1 has been described previously (13). pcDNA3β-HA-BRCA1-ΔF2 was constructed by deletion of a BRCA1 fragment between EcoR1 and Taq1. The in-frame deletion of the HA-BRCA1-ΔF2 was verified by sequencing. Mouse monoclonal anti-BRCA1 (Ab-1) antibody, rabbit anti-BRCA1 (C-20), rabbit anti-Ku80, and polyclonal anti-β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), whereas anti-γ-H2AX (Ser-139) was purchased from Millipore. BRCA1 siRNA or Ku80 siRNA were transfected using Oligofectamine (Invitrogen). pCMV-I-SceI or its control vector were transfected using FuGENE 6 (Roche Applied Science) 24 h after siRNA transfection. Cells were collected 48 h later for protein extraction, genomic DNA isolation, or chromatin preparation. The siRNA sequences for BRCA1 are 5′-CCC UAA GUU UAC UUC CUU AUU-3′ and 5′-GCC CCU CUC ACU CUU CAG UUU-3′, and the siRNA sequence for Ku80 is 5′-GAA GUU CUG UCA CAG CUG AUU-3′.

Strategy for NHEJ Assays—Methods for analysis of precise NHEJ have been reported previously (29). Forty-eight hours after I-SceI expression, genomic DNA was extracted with Qiagen’s DNeasy kit for real-time PCR analysis. Quantitative real-time PCRs were carried out with the TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA). Probe C was designed to amplify only the accurate religation product. The probe C sequence was 5′-TGCCG-CCATTACCGGTATTCCTAGATC-3′. The PCR primer sequences for the religation substrate were as follows: probe C forward (5′-GCGGTCCAGGCTTTGCAA-3′) and probe C reverse (5′-TGATATTTCGCTTCGATCT-3′). An RNase P probe was used as an internal control. For quantification of precise NHEJ repair products, we isolated a stable cell line designated RHW1, in which the I-SceI-induced DSBs were repaired by precise end-joining events (C-NHEJ). The standard curve for the number of C-NHEJ events measured using real-time PCR was generated from serial dilution of the genomic DNA from RHW1. The values for C-NHEJ repair events were obtained from the standard curve generated using genomic DNA from RHW1. Calculation of the relative NHEJ repair events was done as follows: ΔCt = Ct_{sample} - Ct_{input} / ΔCt = ΔCt_{I-SceI} - ΔCt_{control vector}. The relative -fold change for NHEJ repair = 2^{-ΔΔCt}. A second NHEJ substrate containing CD4 and CD8 genes as cell markers has also been described previously (9), and the details of its use are described in the text (Fig. 2A).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP was performed as described elsewhere (71). Briefly, after I-SceI induction, human HT1904 cells with a single copy of the NHEJ substrate containing one I-SceI site were fixed in 1% formaldehyde for 10 min at room temperature, and glycerol was added to a final concentration of 125 mM. The chromatin was pelleted by centrifugation at 1800 rpm for 5 min at 4 °C and stored at −80 °C for further use. The chromatin was sonicated into fragments between 200–1000 bp, and then 500 μg of chromatin was used for immunoprecipitation for each sample with 2 μg of normal IgG or 2 μg of anti-Ku80 antibody and incubated overnight in a cold room. The immunocomplex was retrieved by incubating an additional 30 min with 50 μl of protein A/G-agarose beads saturated with BSA/salmon sperm DNA. After a series of standard washes, the chromatin was eluted with SDS/NaHCO3 and de-cross-linked at 65 °C overnight. After the chromatin was treated with Proteinase K and RNase A, DNA was extracted with phenol/chloroform for PCR analysis. Transient expression of I-SceI was achieved by infection with a I-SceI-expressing adenovirus (both the HT1904 cell line and the I-SceI-expressing virus were kindly provided by Dr. Jac A. Nickoloff). The PCR products spanned the 230-bp region upstream from the I-SceI insertion site; the upstream primer sequence is 5′-TGCGCTCCC TCG CC GG GT TC TGG-3′, and the downstream primer sequence is 5′-TTCGTTCTTGGCTTGTGC-3′. Quantitative real-time PCR was performed using SYBR Green reagent. All experimental values were normalized to the input DNA using amplification of GAPDH. GAPDH forward primer is 5′-TTCGTTCTTGGCTTGTGC-3′, and GAPDH reverse primer is 5′-CTCCATTTGTCCTTCCACTC-3′ as used before (72).

Results

Purification of GST Fusion BRCA1 Protein Fragments and GST Pulldown—The pGEX-GST-BRCA1 constructs GST-BRCA1 (1–6) were kindly provided by Drs. Lih-Ching Hsu and David Livingston. GST-BRCA1 purification was obtained with the GE GST fusion protein purification kit, and protein pull-down was performed as previously described (58).

Cell Sorting and Synchronization—HEK293/pPHW1 cells were sorted into G1 and S/G2/M phase after Dielectric (Invitrogen) staining according to the manufacturer’s instructions. HT1904 or MCF7 cells were synchronized in G0/G1 phase of I-SceI was achieved by infection with a I-SceI-expressing adenovirus (both the HT1904 cell line and the I-SceI-expressing virus were kindly provided by Dr. Jac A. Nickoloff). The PCR products spanned the 230-bp region upstream from the I-SceI insertion site; the upstream primer sequence is 5′-TGCGCTCCC TCG CC GG GT TC TGG-3′, and the downstream primer sequence is 5′-TTCGTTCTTGGCTTGTGC-3′. Quantitative real-time PCR was performed using SYBR Green reagent. All experimental values were normalized to the input DNA using amplification of GAPDH. GAPDH forward primer is 5′-TTCGTTCTTGGCTTGTGC-3′, and GAPDH reverse primer is 5′-CTCCATTTGTCCTTCCACTC-3′ as used before (72).

RESULTS

Silencing BRCA1 Inhibits Overall NHEJ Repair—Data from previous studies suggest that, in addition to its function in HR, BRCA1 may also promote precise end-joining of DSBs in episomal plasmids based NHEJ assays (13, 14). To investigate whether BRCA1 is implicated in NHEJ repair at the chromosomal level in mammalian cells, we stably introduced a NHEJ substrate in which the two I-SceI sites are separated by an additional 30 min with 50 μl of protein A/G-agarose beads saturated with BSA/salmon sperm DNA. After a series of standard washes, the chromatin was eluted with SDS/NaHCO3 and de-cross-linked at 65 °C overnight. After the chromatin was treated with Proteinase K and RNase A, DNA was extracted with phenol/chloroform for PCR analysis. Transient expression of I-SceI was achieved by infection with a I-SceI-expressing adenovirus (both the HT1904 cell line and the I-SceI-expressing virus were kindly provided by Dr. Jac A. Nickoloff). The PCR products spanned the 230-bp region upstream from the I-SceI insertion site; the upstream primer sequence is 5′-TGCGCTCCC TCG CC GG GT TC TGG-3′, and the downstream primer sequence is 5′-TTCGTTCTTGGCTTGTGC-3′. Quantitative real-time PCR was performed using SYBR Green reagent. All experimental values were normalized to the input DNA using amplification of GAPDH. GAPDH forward primer is 5′-TTCGTTCTTGGCTTGTGC-3′, and GAPDH reverse primer is 5′-CTCCATTTGTCCTTCCACTC-3′ as used before (72).
confirm our previous observations and suggest that BRCA1 is required for chromosomal NHEJ repair.

**Knockdown of BRCA1 Suppresses C-NHEJ Repair Specifically in the G1 Phase of the Cell Cycle**—Given that the majority of cultured cells are in G1 phase of the cell cycle and NHEJ is the predominant mechanism of DSB repair in G1, we further investigated whether BRCA1 controls the fidelity of NHEJ specifically in the G1 phase of the cell cycle by utilizing an assay that can directly detect precise end-joining (C-NHEJ) events by quantitative real-time PCR (qPCR), as described previously (28). We stably integrated a single copy of the NHEJ substrate pHW1 in which the two I-SceI sites are separated by 30 base pairs of intervening sequence (29, 30) into the genome of HEK293 cells. A pair of primers flanking the two I-SceI sites and a probe complementary to the sequence resulting from precise end joining was designed for quantitative real-time PCR (Fig. 2A). HEK293/pPHW1 cells in G1 or G2/S phase were collected through fluorescence-activated cell sorting (FACS) sorting (Fig. 2B, right panel), and C-NHEJ mediated repair of I-SceI-induced DSBs was quantified using real-time PCR analysis. As shown in the left panel of Fig. 2B, knocking down BRCA1 resulted in a 60% reduction of C-NHEJ in G1 phase cells, whereas there was no significant change in C-NHEJ in G2/S phase cells. This observation is consistent with previously well documented findings (31, 32) that a majority of radiation-induced DSBs is rapidly repaired predominantly in the G1 phase of the cell cycle by Ku-dependent NHEJ.

It is possible that a small fraction of the sorted G1 phase cells had moved into the next phase of the cell cycle after sorting and pCMV-I-SceI expression. We thus examined the effect of BRCA1 on NHEJ repair in cells at different phases of the cell cycle using S/G2/M phase-specific CNEP-F staining. CNEP-F is highly expressed in S/G2/M phase, moderately expressed in S-phase, and not expressed in G1 phase (33–35). The quantification of persistent γ-H2AX foci was used to assess NHEJ repair efficiency (31, 36–38). To validate this approach, we examined the effect of inhibition of Ku80 on DSB NHEJ repair. Cells were co-stained for γ-H2AX foci and CENP-F at 1 h after 6 Gy IR (Fig. 2C). Silencing Ku80 led to increased persistent γ-H2AX foci in G1 cells, consistent with previous reports. Similarly, we again found that silencing BRCA1 resulted in a significant increase in cells with persistent γ-H2AX foci during G1 (CENP-F negative) (Fig. 2D). Taken together, these data suggest that BRCA1 plays a critical role in controlling overall NHEJ and may specifically promote the accuracy of C-NHEJ during the G1 phase of the cell cycle.

**Inhibition of BRCA1 Expression Decreases the Accuracy of Chromosomal NHEJ**—If BRCA1 is required for fidelity of NHEJ repair, dysfunction of BRCA1 may lead to increased deletions during NHEJ repair. To directly test this hypothesis, we first analyzed the quality of the junction after NHEJ repair of DSBs. The fragment containing the repair junction was amplified from genomic DNA isolated from the CD4- or CD8-expressing cells, and the pattern of the PCR products was analyzed. Full-length PCR products would represent high fidelity end-joining of the junction, whereas a change in the length of the PCR fragments indicates that deletions (decreased length) or insertions (increased length) were generated during NHEJ (10). In GM639 cells transfected with control siRNA, the expected NHEJ repair product of ~820 bp was detected in all 30 clones.
However, when BRCA1 expression was silenced, 5 of 30 clones exhibited shorter PCR products, suggesting a deletion had occurred during NHEJ-mediated repair in BRCA1-depleted cells.

We next analyzed the sequence at the end-joining junction. Twenty-five clones with control siRNA transfection and 23 clones with BRCA1 siRNA transfection were analyzed (Fig. 3B). We found that 52% (13/25) of the clones transfected with control siRNA exhibited precise end-joining compared with 22% (5/23) of the clones when BRCA1 was silenced. Conversely, the number of deletions at the junction increased from 44% (11/25) with control siRNA to 70% (16/23) with BRCA1 knockdown (Fig. 3B). Importantly, suppression of BRCA1 also affected the size of the deletions (Fig. 3C). The mean size of deletion increased 3-fold, from 13 nucleotides with control siRNA to 38 nucleotides with siRNA directed against BRCA1. In addition, although the maximum deletion size was 34 nucleotides long with control siRNA, events with more extensive deletions (from 38 to 134 nucleotides) occurred when BRCA1 was silenced (Fig. 3C). This deletion pattern corresponds to the phenotype of Ku80-deficient cells when using the same NHEJ repair substrate (10). Interestingly, the insertion events were not affected by BRCA1 silencing in this analysis (data not shown). These results indicate a potential role for BRCA1 in promoting the precise end-joining of chromosomal breaks, similar to our and others’ previous reports with episomal plasmid based NHEJ assays (13, 14, 24, 25, 27).

To further characterize the function of BRCA1 in NHEJ and the mechanisms involved, we compared the effects of BRCA1 with Ku80 specifically on C-NHEJ using the established C-NHEJ quantitative real-time PCR assay. As shown in Fig. 4A, BRCA1 knockdown reduced precise C-NHEJ by 50% compared with control siRNA. However, knockdown of Ku80 led to a greater than 25-fold suppression of precise end-joining (Fig. 4A), consistent with the results of others (10, 11). Western blot analysis confirmed that Ku80 and BRCA1 protein expression were suppressed by targeted siRNAs (Fig. 4A, lower panel). As repair processes can be affected by cell cycle phase or cell viability, we also confirmed that the effects of silenced BRCA1 on repair were not due to changes in cell viability or changes in cell cycle distribution with siRNA knockdown (supplemental Fig. 1), consistent with a previous report (25). Interestingly, when cell cycle-dependent NHEJ repair was analyzed using co-staining of persistent γ-H2AX foci with the S/G2/M phase-specific
marker CENP-F, we found that the inhibition of NHEJ-mediated DSB repair is of significantly higher magnitude when silencing Ku80 than silencing BRCA1 in the G1 phase cells (Fig. 2D). Furthermore, simultaneous knockdown of both BRCA1 and Ku80 did not further increase the number of persistent γ-H2AX foci, indicating that BRCA1 may control C-NHEJ in the G1 phase of the cell cycle through interaction with and regulation of Ku80. Taken together, our data support the notion that BRCA1 promotes high fidelity NHEJ-mediated repair and suppresses mutagenic deletional end-joining repair, which probably happens in the G1 phase of the cell cycle.

**BRCA1 Interacts with and Stabilizes Ku80 Binding at DSB Sites during G1 Phase**—BRCA1 is able to interact with many proteins with its N-terminal ring domain, central DNA binding domain, or C-terminal domain of BRCA1 protein. Therefore, it is quite possible that there is interaction between BRCA1 and the Ku-dependent NHEJ repair machinery. To test this hypothesis, we examined whether a functional interplay exists between BRCA1 and the Ku70/Ku80 heterodimer by assessing the binding of Ku80 to chromosomal DSB ends using ChIP-qPCR. Kinetic analysis of Ku80 binding to DSBs induced by infection of I-SceI-expressing adenovirus revealed that silencing BRCA1 compromises the retention of Ku80 on DSB sites during NHEJ repair. A, silencing BRCA1 results in a decreased number of end-joining clones carrying full-length PCR products. Representative PCR patterns after NHEJ-mediated repair in control or BRCA1 knockdown human SV-40 fibroblasts are shown. The bold arrow indicates PCR products after NHEJ repair. B, shown is analysis of the junction sequences of the end-joining products from cells transfected with control or BRCA1 siRNAs. C, silencing BRCA1 results in a decrease in high fidelity end-joining and an increase in the size of the deletions.
DSB ends as early as 2 h after Adeno-I-SceI infection; however, BRCA1 knockdown did not completely abolish Ku80 recruitment to DSB ends (Fig. 4B and supplemental Fig. 2). These data suggest that BRCA1 may be critical for stabilizing Ku80 binding at DSB sites, which may facilitate Ku80-mediated protection of DSB ends from resection (39, 40) and subsequently inhibit deletion-prone Alt-NHEJ.

Given that BRCA1 promotes fidelity of NHEJ mainly in G1 phase and that BRCA1 has functional interactions with the NHEJ protein Ku80 in chromosomal DSB sites after damage, we hypothesized that interaction of BRCA1 and Ku80 occurs mainly in G1 phase. To test this, we examined the BRCA1-Ku80 interaction during the cell cycle. pPHW1-HEK293 cells were lysed immediately for immunoprecipitation after being sorted into G1 or S/G2 phases of the cell cycle. As shown in Fig. 4, C and D, the in vivo interaction of BRCA1 and Ku80 occurs mainly in G1 phase. Reciprocal BRCA1 and Ku80 co-immunoprecipitations (IP) were performed after lysis of DyeCycle-sorted G1 or S/G2 phase HEK293 cells. The amount of Ku80 or BRCA1 pulled down was normalized to its input. Note that the input amount of BRCA1 was lower in G1, than in G2/S phase. E, Ku80 binding to DSB ends in different phases of the cell cycle. Ku80 ChIP-qPCR assays were performed at 3 h after Adeno-I-SceI infection in serum starvation-synchronized G1 cells or G2/S cells with or without BRCA1 knockdown. The levels of Ku80-DNA binding were measured using ChIP-qPCR assays and expressed as levels relative to IgG control after normalization with DNA input before ChIP assays as described under “Experimental Procedures.”

It has been reported that BRCA1 expression level peaks in the late S/G2 phase (41–43). Accumulated evidence also consistently demonstrates that BRCA1 is expressed at decreased levels and is hypophosphorylated in G1 phase (44–47). To further rule out the possibility that the Western blot result was due to contamination of S/G2 cells in G1 cell lysates, we confirmed G1 phase BRCA1 expression by BRCA1 immunostaining. As shown in supplemental Fig. 4, the specificity of BRCA1 immunostaining was shown by the fact that a peptide containing the
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BRCA1 N-terminal sequence (and the specific epitope for the binding of antibody Ab-1) completely blocked positive staining of BRCA1 by antibody Ab-1. Co-immunostaining using both Ab-1 (for BRCA1) and CENP-F (for G2/M/S phase-specific marker) demonstrated clear positive staining of BRCA1 in both G1 cells and G2/S cells (supplemental Fig. 5).

To assess the function of BRCA1-Ku80 interaction during Ku80 binding to DSBs throughout the cell cycle, Ku80 ChIP-qPCR was performed with synchronized cells, as shown in Fig. 4E. When BRCA1 was knocked down, Ku80 binding to DSBs was reduced in G1 phase cells but not in S/G2 phase cells 3 h after Adeno-I-SceI infection. These data suggest that BRCA1 regulates C-NHEJ through multiple ways, including differential interaction with protein partners such as Ku80, Mre11, and CtIP and affects the DNA binding capacity of Ku80 during the cell cycle.

Localization of the Physical Domain in BRCA1 Critical for Binding with Ku80 and Promoting High Fidelity End-joining of DSBs—We next sought to investigate whether the functional interaction of BRCA1 with Ku80 at DSB ends takes place through a physical interaction between these two proteins. As shown in Fig. 5A, a similar level of BRCA1 and Ku80 immunocomplex formed in vivo regardless of the presence or absence of I-SceI-induced DSBs. To assess whether the BRCA1-Ku80 association is indirectly mediated by DNA, we performed the similar experiment in the presence of DNase to deplete genomic DNA and found that BRCA1 coimmunoprecipitation with Ku80 was not dependent on the presence of DNA in the immunocomplex (Fig. 5B). We further demonstrated that Rad51 protein, which does not interact with Ku80, was not detected in the same immune complex with Ku80 regardless of DNase treatment (Fig. 5B) and that EtBr incorporation did not affect the proportions of BRCA1 and Ku80 in the complex (Fig. 5C). These data confirm that BRCA1 specifically associates with Ku80 and that the interaction is not mediated through DNA.

To further characterize BRCA1 interaction with Ku80, we purified six GST-tagged BRCA1 fragments (BF1–6) (Fig. 5, D and E), which span the entire BRCA1 open reading frame (4, 16). Using these purified fragments, in vitro pulldown studies revealed that GST-BF2, which contains BRCA1 amino acids 262–552, pulled down Ku80, whereas GST-BF3-(504–803) partially pulled down Ku80 from nuclear extracts obtained from HCC1937 human breast cancer cells, which is hemizygous for the BRCA1 5382insC mutation (Fig. 5F). This mutation renders the BRCA1 protein unstable, and for this reason these cells are considered BRCA1 null. These results suggest that the Ku80 binding region of BRCA1 resides between residues 262 and 803.

To address the functional significance of the BF2 region of BRCA1 in vivo, we constructed the plasmid pcDNA3β-HA-BRCA1ΔF2 (BRCA1-ΔF2), in which the partial BF2 region (from amino acids 242 to 513) was deleted. We transiently expressed this deletion mutant or wild-type BRCA1 (BRCA1-
WT) in HCC1937 cells. After co-immunoprecipitation with anti-HA antibody, the immunoprecipitates were probed with anti-Ku80 or anti-BRCA1. IP, immunoprecipitation. B, BRCA1-ΔF2 fails to rescue the compromised binding of Ku80 to DSBs in cells with endogenous BRCA1 protein depletion. Quantification of Ku80-DNA binding are expressed relative to the IgG control after normalization with DNA input before ChIP assays as described under “Experimental Procedures.” **, p < 0.01. C, the BF2 region is required for BRCA1 function in promoting fidelity of NHEJ in vivo. Precise end-joining was assessed using real-time PCR as described in Fig. 1. The values were expressed relative to the level of precise C-NHEJ in cells rescued with wild-type BRCA1. **, p < 0.01. D, cells with stable expression of BRCA1-ΔF2 are more sensitive to IR. HEK293/pPHW1 cells with stable expression of vector, BRCA1-WT, and BRCA1-ΔF2 were subjected to 6 Gy IR after endogenous BRCA1 was knocked down. After 24 h, cells were collected, stained, and subjected to flow cytometric analysis for apoptosis. Shown is the average of three independent experiments. **, p < 0.01. E, BRCA1-ΔF2-expressing cells are proficient in IR-induced G2/M checkpoint activation. HCC1937 cells expressing control vector, BRCA1-WT, or BRCA1-ΔF2 were subjected to 6 Gy IR. One hour later the cells were collected, and the mitotic indices were analyzed by Phospho-Ser10 H3 staining. The number of cells positively labeled with phospho-Ser-10 H3 was quantified by flow cytometric analysis (**, p < 0.01).

The BF2 region is also essential for high fidelity C-NHEJ repair in vivo. Similar to vector control cells lacking BRCA1 expression, cells expressing BRCA1-ΔF2 had a ~4-fold reduction of high fidelity end-joining compared with cells expressing exogenous BRCA1-WT (Fig. 6C) when endogenous BRCA1 was silenced with siRNA (supplemental Fig. 6). Accordingly, expression of the BRCA1-ΔF2 mutant increased IR-induced apoptosis compared with BRCA1-WT (Fig. 6D), indicating the functional significance of BRCA1-mediated interaction with and stabilization of Ku80 at the DSB ends for both genome maintenance and cell survival in response to genotoxic stress. It is possible that a destructive conformational change caused by the deletion of the amino acids 242–513 sequence renders the mutant BRCA1 protein completely nonfunctional. Therefore, we examined the function of the deletion mutant in DNA damage-induced checkpoint activation. As shown in Fig. 6E, the BRCA1-ΔF2 mutant was proficient in G2/M check-
point, which affirms that the defective C-NHEJ upon deletion of the BF2 region was not due to complete disruption of BRCA1 function. These results suggest that the BF2 region in BRCA1 is specifically required for BRCA1 binding to Ku80 and precise NHEJ repair.

Taken together, our data suggest that BRCA1 promotes the fidelity of NHEJ repair during the G1 phase of the cell cycle through physical interaction with Ku80. Our data demonstrate that BRCA1, as a main tumor suppressor, maintains genome stability throughout the cell cycle by facilitating not only error-free HR in S/G2/M phase but also fidelity of NHEJ repair during G1 phase.

**DISCUSSION**

In this study we provide evidence that, in addition to controlling HR repair in S/G2 phase, BRCA1 may be essential for the fidelity of NHEJ-mediated repair of DSBs during the G1 phase of the cell cycle. BRCA1 may act as an upstream regulator in NHEJ by interacting with and stabilizing the binding of Ku80, a key component of the C-NHEJ machinery, to DSB ends. Our data strongly support the notion that BRCA1 serves as a central player in maintenance of genomic stability by promoting not only Rad51-dependent HR repair in late S/G2 phase but also Ku80-dependent precise C-NHEJ in G1 phase. However, our data can not exclude the possibility that interaction between Ku80 and BRCA1 may be mediated by an intermediary protein partner, although it seems that the presence of DNA is not required. Furthermore, Ku80 function in precise end-joining may be only partially regulated by BRCA1 as BRCA1 silencing did not completely disrupt Ku80 binding to DSB ends and the effect of loss of Ku80 in precise NHEJ is much greater than that of BRCA1 (Fig. 4B).

The significance of high fidelity repair of DSBs during G1 was not appreciated until recently (48). In contrast to the extensive research efforts and well characterized HR regulation during S/G2, the molecular mechanisms that control DSB repair quality during G1 are yet to be defined. It has been reported that DSBs observed in G1 phase can be inherited from the unrepaired DNA lesions shielded by 53BP1 in G2/S phase (49). In this case, BRCA1-Ku80-dependent precise end-joining may provide a second chance for accurate repair during G1 phase (49). DNA-PKcs has recently been shown to be required for arrest of RNA polymerase II during stalled transcription (50), and given that BRCA1 forms a complex with RNA polymerase II to repress transcription (51–54), it is possible BRCA1 may also be involved in DSBs repair during stalled transcription (52, 53, 55, 56). Strikingly, another recent report showed that BRCA1 maintained heterochromatin-mediated silencing, which involved in silencing satellite RNA transcripts (57). It would be interesting to investigate whether BRCA1 represses transcription during C-NHEJ repair in G1.

BRCA1 protein expression has been reported to peak in the S/G2 phase of the cell cycle (41–43), with a comparatively low level of expression in G1 phase (44–47). Our results are consistent with others’ reports and clearly demonstrated the expression of BRCA1 in G1 phase by using both Western blot analysis of synchronized G1 or S/G2 cells and co-immunostaining of BRCA1 with an S/G2 phase-specific marker.

Recent studies have shown evidence that HR and NHEJ is mutually controlled by the regulators in a cell cycle-dependent fashion (59, 60). We observed that the inhibitory effect on C-NHEJ by BRCA1 silencing is similar to, but at a lesser magnitude, than that in Ku-deficient cells (8–10, 30), indicating that BRCA1 may control C-NHEJ via regulation of Ku80 in the G1 phase. Although more biochemical evidence is needed to determine the mechanism of BRCA1 in DNA repair during the cell cycle, data from this study and previous studies support the notion that the role of BRCA1 in differential DSB repair pathways may depend on its association with different binding partners during the cell cycle. For example, during the G1 phase of the cell cycle, BRCA1 protein may form a complex specifically with Ku80 protein, stabilizing it at the DSB ends, and consequently prevent end resection and deletion events during end joining. In contrast, during S/G2/M phase, BRCA1 may preferentially form a complex with CtIP/Mre11, accelerating end resection, and thereby promote HR repair. Intriguing models have been proposed in which phosphorylated CtIP disrupts Ku80-BRCA1 interaction or cyclin-dependent kinase-mediated phosphorylation of BRCA1 during S/G2 phase causes its dissociation from Ku80 and increases the affinity of BRCA1 for CtIP (41, 61).

The critical region for interaction between BRCA1 and Ku80 resides in the N-terminal half of BRCA1, specifically residues 262–803. Partial deletion of this interacting domain (from amino acids 242–513) compromised recruitment of Ku80 to DSBs as well as diminished fidelity of NHEJ capacity compared with wild-type BRCA1. Because this region encompasses the DNA and Rad51 binding regions of BRCA1 (62), one might expect HR to be slightly compromised. We observed a modest inhibitory effect on HR (data not shown). Similarly, a recent study reported a slightly reduced HR capacity upon mutation in this region (63). Deletion mutations within the BF2 region have been reported to decrease the fidelity of NHEJ in a plasmid-based assay in a lymphoblastoid cell line established from a breast cancer patient (25). Furthermore, several missense mutations in the BF2 region of BRCA1 were reported in some populations of familial breast cancer patients, including BRCA1 I379M and Q356R. The data from our study may provide the molecular basis for the tumorigenesis observed in these breast cancer patients (64, 65).

Several proteins have been shown to contribute to cell commitment to a specific NHEJ subpathway, including Ku80, XRCC4, and Mre11 (10, 16–18, 28, 66). In particular, Ku80 and XRCC4 have been shown to be essential for fidelity of NHEJ repair (10, 67), whereas we and others have discovered that the mutagenic Alt-NHEJ is dependent on Mre11 (16–18, 28). A recent report showed that Ku80 is required to recruit BRCA1 to DSB sites after DNA damage (68). It is possible that Ku80 binds to broken ends immediately after DNA damage and recruits BRCA1, which in turn stabilizes the association of Ku80 with exposed DNA ends. Although BRCA1 has been shown to be important only for HR (69), our data and others’ support a model that BRCA1 may play a central role in repressing mutagenic Alt-NHEJ and promoting high fidelity C-NHEJ repair (13, 14, 27, 70). With its capacity to interact with different functional partners at different phases of the cell cycle, BRCA1 may...
maintain genome stability throughout the cell cycle by facilitating not only error-free HR in S/G2/M phase but also fidelity of NHEJ repair during G1 phase.

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