DOI:10.1158/0008-5472.CAN-05-3296

Cancer Research

BRCA1 DNA-Binding Activity Is Stimulated by BARD1

Amanda M. Simons, Andrew A. Horwitz, Lea M. Starita, et al.

Cancer Res 2006;66:2012-2018. Published online February 17, 2006.

DOI:10.1158/0008-5472.CAN-05-3296

Research Article

BRCA1 DNA-Binding Activity Is Stimulated by BARD1

Amanda M. Simons,[1] Andrew A. Horwitz,[1] Lea M. Starita,[1] Karen Griffin,[2] R. Scott Williams,[3] J.N. Mark Glover,[3] and Jeffrey D. Parvin[1,2]

[1]Graduate Program in Biological and Biomedical Sciences, Harvard Medical School; [2]Brigham and Women's Hospital, Boston, Massachusetts; and [3]University of Alberta, Edmonton, Alberta, Canada

Abstract

The breast- and ovarian-specific tumor suppressor BRCA1 has been implicated in numerous cellular processes, including transcription, ubiquitination, and DNA repair. Its tumor suppression activity is tightly linked to that of BARD1, a protein that heterodimerizes with BRCA1. It has been previously shown that BRCA1 binds to DNA, an interesting functional observation in light of the genetic data linking BRCA1 to DNA repair pathways. In this work, we reexamine the DNA-binding properties of BRCA1, comparing them with the DNA-binding properties of the BRCA1/BARD1 heterodimer. Because nuclear BRCA1 exists as a heterodimer with BARD1, it is likely that *in vitro* studies of the heterodimer will provide a more accurate model of physiologic conditions. Our results indicate that whereas BARD1 cannot directly bind DNA, it does enhance DNA binding by BRCA1. This is a surprising observation as both DNA-binding domains are distal to the BARD1-interacting RING domain of BRCA1. Further analysis of the dimerization reveals that the BRCA1/BARD1 interaction is not limited to the amino-terminal RING domains of each protein. The carboxyl terminus of BRCA1 contributes significantly to the stability of the heterodimer. We also show that the presence of BARD1 has a secondary effect, as autoubiquitination of BRCA1/BARD1 heterodimers additionally enhances the affinity of BRCA1 for DNA. Together, these data suggest that BRCA1 and BARD1 heterodimerization is stabilized via domains not previously thought to interact and that BARD1 acts in both ubiquitination-dependent and ubiquitination-independent ways to influence the role of BRCA1 in DNA repair. (Cancer Res 2006; 66(4): 2012-8)

Introduction

Loss of BRCA1 function causes cancer in breast and ovarian cells, but the BRCA1 protein executes a variety of functions required for all cell types. These functions include the regulation of transcription (1–4), homology-directed DNA repair (5–9), control of cell cycle checkpoints (10–12), and the regulation of centrosomes (13–17). The roles for BRCA1 in these processes have been deduced using cell lines that express hypomorphic mutants of BRCA1 but a mechanistic understanding of how BRCA1 regulates these diverse functions remains unknown.

The 1,863 amino acid sequence of BRCA1 bears little homology to other known proteins. Two domains of BRCA1, found at the amino and carboxyl termini of the protein, are exceptions. The carboxyl terminus of BRCA1 contains two tandem BRCT repeats (18). These domains, first identified in BRCA1, are found in many DNA repair proteins. Recently, they have been shown to bind polypeptides in a phosphorylation-dependent manner (19–22). Interestingly, many clinically relevant mutations of BRCA1 disrupt or delete the BRCT domains (23, 24). At the amino terminus of BRCA1 is a RING domain. This is a class of zinc finger domains that has an ubiquitin ligase activity in many different proteins (25). BRCA1 heterodimerizes with another RING-containing protein, BARD1, forming an E3 ubiquitin ligase (26–28).

BRCA1 has also been shown to have transcriptional coactivation function (29–31). Although it would be tempting to attribute its tumor suppression phenotype to indirect downstream effects of transcription and/or ubiquitination, that may be only an incomplete picture. BRCA1 has also been shown to interact with many DNA repair proteins (32) as well as DNA lesions themselves. Two domains of BRCA1 exhibit direct DNA-binding activity. A central region of BRCA1, encompassing approximately amino acids 452 to 1,079, binds with structure specificity to branched, or cruciform, DNA (33), whereas the carboxyl-terminal BRCT repeats bind with specificity to DNA ends (34). This suggests that BRCA1 may play a direct as well as indirect role in DNA repair and in maintaining genomic stability.

One defining characteristic of BRCA1 is its cellular localization pattern. During S phase, BRCA1 is localized to punctate nuclear foci (35). Following DNA damage, however, these foci are disrupted, and BRCA1 is redistributed to foci thought to mark sites of DNA damage. These damage-dependent foci colocalize with γ-H2AX and DNA repair proteins, including Rad50 and Rad51, and offer further evidence for a direct role in DNA damage repair by BRCA1 (36–38). Additional immunofluorescence experiments have shown that these foci also contain ubiquitin polymers. The ubiquitin chains in DNA repair foci are linked via ubiquitin lysine 6, an unusual ubiquitin linkage that has been reported for BRCA1/BARD1 (28, 39–41). This suggests that the DNA repair and ubiquitination functions are concurrently active and interdependent.

BRCA1 function is tightly linked to its RING-binding partner, BARD1. BARD1 was identified in a screen for proteins that interact with the RING domain of BRCA1. Like BRCA1, BARD1 contains amino-terminal RING domains and a carboxyl-terminal BRCT repeats (26). Cellular levels of BRCA1 and BARD1 are reciprocally regulated and studies of BRCA1 complexes have failed to detect BRCA1 that is not associated with BARD1 (42, 43), suggesting that BRCA1 exists as an obligate heterodimer with BARD1. Therefore, the cellular processes in which BRCA1 has been implicated are also likely to involve BARD1.

Consistent with this, BARD1 mutations show many of the same phenotypes as BRCA1, including chromosome instability and defects in homology-directed DNA repair (44–46). In addition to its involvement in BRCA1-dependent ubiquitination, BARD1 has been shown to be required for proper localization of BRCA1, both in the absence of and following DNA damage. Binding to BARD1 masks a

Requests for reprints: Jeffrey D. Parvin, Department of Pathology, Brigham and Women's Hospital, 77 Avenue Louis Pasteur, Boston, MA 02115. Phone: 617-525-4401; Fax: 617-525-4422; E-mail: jparvin@rics.bwh.harvard.edu.

doi:10.1158/0008-5472.CAN-05-3296

DOI:10.1158/0008-5472.CAN-05-3296

nuclear export signal in BRCA1, making it essential for nuclear retention of BRCA1. BARD1 is also required for proper localization of BRCA1 to damaged induced foci. This relationship is reciprocal as BRCA1 is required for proper localization of BARD1 (47).

These data suggest that BARD1 and BRCA1 act together in a pathway promoting tumor suppression and that an accurate model of BRCA1 function must include BARD1. In this article, therefore, we examine the role BARD1 plays in the direct DNA-binding activities of BRCA1. Although there have been a number of studies examining BRCA1 and BARD1 ubiquitination activities, there has been little biochemical work done with respect to the involvement of BARD1 in the direct role of BRCA1 in DNA repair.

In the following experiments, we show that BARD1 enhances the DNA-binding activity of BRCA1, although it itself exhibits no DNA-binding activity. This enhancement is due to previously unidentified interactions between BRCA1 and BARD1 downstream of the RING domain. The presence of BARD1 also provides a secondary means to stabilize DNA-bound BRCA1 as autoubiquitinated BRCA1/BARD1 heterodimers display an even greater affinity for DNA than their unubiquitinated counterparts. Taken together, these data suggest that BARD1 stabilizes BRCA1 at sites of DNA damage and that the ubiquitination function of the heterodimers plays an important role in DNA repair.

Materials and Methods

Baculovirus constructs. Baculovirus expressing FLAG-tagged BRCA1, FLAG-tagged BRCA1 truncations, BARD1, or BARD1 truncations were constructed using the Bac-to-Bac system (Invitrogen, Carlsbad, CA). Full-length BRCA1 was either 5′ or 3′ FLAG tagged. BRCA1 truncations were 3′ FLAG tagged, and activities we measured were unaffected by the location of the tag. BARD1 was untagged. Truncations used in these experiments include the following: BRCA1 (amino acids 1-1,863), BRCA1 (1-1,527), BRCA1 (1-1,000), BRCA1 (1-500), ΔN-BRCA1 (303-1,863), and BARD (1-292).

DNA constructs. Four-stranded Holliday junction DNA was assembled from the complementary oligonucleotides as follows: HJ1, 5′-CGTTTTGGGTGAACCTGCAGGTGGGCAAAGATGTCCTAGCAATCCATTGTCTATGACGTCAAGCT; HJ2, 5′-CCGCTACCAGTGATCACCAATGGATTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCC; HJ3: 5′-TGCCGATATTGACAAGACGGCAAAGATGTCCTAGCAATCCATTGGTGATCACTGGTAGCGG; HJ4: 5′-GAGCTTGACGTCATAGACAATGGATTGCTAGGACATCTTTGCCGTCTTGTCAATATCGGC.

Oligonucleotides were annealed by heating to 95°C for 2 minutes followed by incubation at 65°C, 37°C, 25°C, and 4°C for 10 minutes each. Holliday junctions used as gel mobility shift probes were end-labeled with [^{32}P]dATP using the Klenow fragment of DNA polymerase I. DNA junctions were gel purified by nondenaturing PAGE and eluted in TE buffer.

Protein purification. Hi-5 insect cells were infected with baculovirus expressing FLAG-tagged BRCA1 or BRCA1 truncations or coinfected with BRCA1 and BARD1-expressing baculovirus. Cells were lysed 48 to 72 hours postinfection using a tight-pestled Dounce homogenizer. Lysates were cleared through centrifugation and proteins were immunoprecipitated using M2 anti-flag agarose (Sigma Chemical Co., St. Louis, MO). Proteins were eluted in 20 mmol/L Tris-HCl (pH 7.9), 150 mmol/L NaCl, 2 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L DTT, 10 mmol/L β-glycerophosphate, 15% glycerol, 0.01% NP40, 0.2 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L benzamidine-HCl, 2 μg/mL leupeptin, 1 μg/mL aprotinin, 0.2 mg/mL FLAG peptide, and 0.5 mg/mL lysozyme. Protein was quantitated by comparison of an ovalbumin standard with the intensity of the stained band representing the expected-length protein on a Coomassie-stained polyacrylamide gel.

His-BRCA1-398-1097 (BRCA1 CBD) was overexpressed in BL21 cells. Histidine-tagged BRCA1-CBD was purified from crude bacterial lysate by chromatography using Ni-NTA matrix. Fractions containing BRCA1-CBD were pooled and purified using a BioScale S column (Bio-Rad, Hercules, CA). Fractions were eluted in a linear gradient containing buffer A [10 mmol/L HEPES (pH 7.9), 1 mmol/L EDTA, 10% glycerol, and 3 mmol/L DTT] and 0.1 mol/L KCl to buffer A plus 1.0 mol/L KCl. Fractions containing protein were pooled, diluted to buffer A plus 0.1 mol/L KCl, and purified using a BioScale Q column. Fractions were collected over a linear gradient of buffer A plus 0.1 mol/L KCl to buffer A plus 1.0 mol/L KCl. Fractions containing His-BRCA1-CBD were pooled and dialyzed in buffer A plus 0.1 mol/L KCl.

6×His-E1 ubiquitin ligase and 6×His-UbcH5c were purified as previously described (48).

The BRCT region of BRCA1 was purified as previously described (49).

Electrophoretic mobility shift assay. BRCA1, BRCA1/BARD1 heterodimers, BRCA1 CBD, or BRCT as indicated were incubated with 1 to 5 nmol/L Holliday junction for 25 minutes at 22°C in 7.4 mmol/L Tris-HCl (pH 7.4), 60 to 80 mmol/L KCl, 4 mmol/L DTT, 0.1 mg/mL bovine serum albumin, and 0.1% Triton X-100. Complex formation was followed by electrophoresis on 4% to 12% polyacrylamide gradient Tris-borate EDTA gels (Invitrogen) and visualization using autoradiography. Results were quantitated using the Molecular Dynamics Storm phosphorimager, followed by analysis with ImageQuant. Results were similar in the presence or absence of 0.2% glutaraldehyde.

Autoubiquitination experiments. BRCA1/BARD1 was immunopurified from baculovirus-infected cell lysates as described above, but before elution from the M2 agarose BRCA1/BARD1 heterodimers were autoubiquitinated. Protein-bound beads were washed and resuspended in 0.5× buffer A [10 mmol/L HEPES (pH 7.9), 0.5 mmol/L EDTA, and 2.5% glycerol] containing 5 mmol/L $MgCl_2$, 60 mmol/L KCl, 0.5 mmol/L ATP, 16 nmol/L 6×His-E1 ubiquitin ligase, and 0.8 μmol/L 6×His-UbcH5c. One half of the BRCA1/BARD1 bound to M2 agarose was incubated with 0.1 mg/mL bovine ubiquitin (Sigma Chemical) and one half of the BRCA1/BARD1 was incubated without ubiquitin for 45 minutes at 37°C. The beads were washed and protein was eluted as described above.

Results

BARD1 enhances DNA binding by BRCA1. To determine the effect of BARD1 on BRCA1-dependent DNA binding, we first purified BRCA1 and BRCA1/BARD1 heterodimers. Hi5 insect cells were infected with baculovirus expressing a FLAG-tagged BRCA1 or coinfected with the FLAG-BRCA1 and an untagged BARD1. BRCA1 and BRCA1/BARD1 heterodimers were immunoaffinity purified (Fig. 1*A*). Full-length BRCA1 (200 kDa) and BARD1 (90 kDa) are the major bands present in the purified protein. The minor bands (50-65 kDa) also present are detected by antibodies against BRCA1 by immunoblotting (data not shown) and are likely to be BRCA1 breakdown products. As will be shown below, these breakdown products are unlikely to bind DNA with significant affinity.

These purified recombinant proteins were then subjected to an electrophoretic mobility shift assay (EMSA). As BRCA1 was previously shown to bind with structure specificity to four-stranded Holliday junction DNA, BRCA1 and BRCA1/BARD1 heterodimers were incubated with a ^{32}P-labeled synthetic Holliday junction (Fig. 1*B*). As seen in lanes 2 to 5 of Fig. 1*B*, and consistent with published results (33), BRCA1-bound probe exhibited a complex banding pattern in the EMSA. As increasing amounts of BRCA1 were added, the resulting complex slowed in migration. Therefore, it is likely that the protein-DNA complexes contain multiple BRCA1 molecules.

Like BRCA1 alone, BRCA1/BARD1 yielded a complex shift in the EMSA (Fig. 1*B*, *lanes 7-10*). However, BRCA1/BARD1 bound to the DNA at a lower concentration than BRCA1 alone, indicating a higher affinity for DNA. Because the heterogeneity of the observed complexes prevented direct calculation of the dissociation constant, affinity of BRCA1 and BRCA1/BARD1 for DNA was estimated by comparing the concentration at which 50% of the

DOI:10.1158/0008-5472.CAN-05-3296

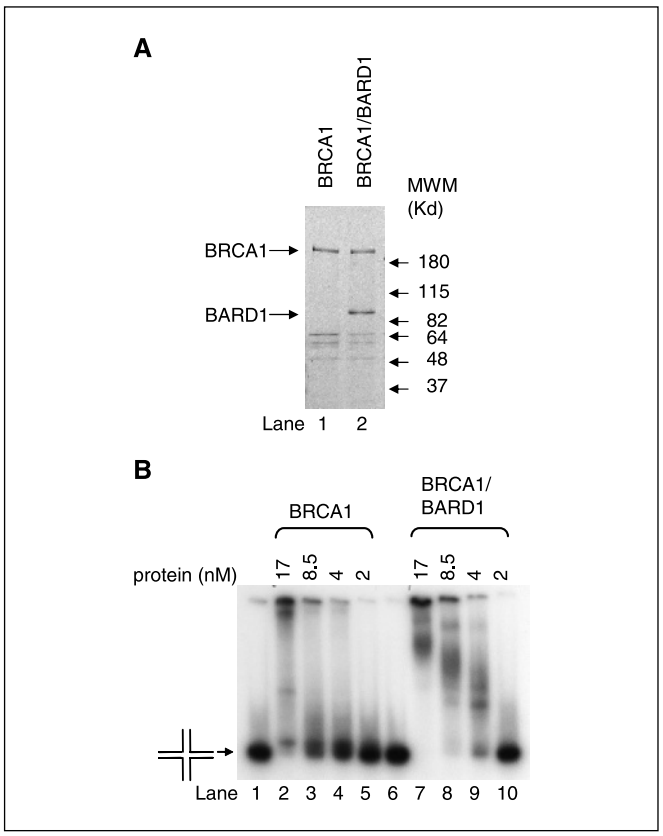

Figure 1. BRCA1/BARD1 heterodimers bind Holliday junction DNA with higher affinity than BRCA1 alone. *A,* FLAG-tagged BRCA1 was coexpressed with untagged BARD1 in insect cells and immunoaffinity purified via the FLAG tag. Analysis is shown of Coomassie-stained SDS-PAGE of the purified BRCA1 (*lane 1*) and BRCA1/BARD1 (*lane 2*), and the migration positions of a molecular weight marker (*MWM*) are indicated. *B,* BRCA1 and BRCA1/BARD1 heterodimers were incubated with ^{32}P-labeled cruciform DNA at the indicated concentrations followed by EMSA. Migration of the free probe is indicated.

probe was bound. At 4 nmol/L of BRCA1/BARD1, ⋙50% of the probe was bound (Fig. 1*B*, *lane 9*), whereas a comparable level of binding was not observed until between 8.5 and 17 nmol/L BRCA1 alone (Fig. 1*B*, *lanes 2-3*).

BARD1 does not directly bind DNA. In comparing the EMSA banding pattern of BRCA1/BARD1 and BRCA1 alone, we observed that the BRCA1 DNA complexes and the BRCA1/BARD1 DNA complexes did not comigrate (Fig. 1*B*, *lanes 2-5* versus *7-10*). We interpret this to mean that BARD1 is a component of the DNA-protein complex, rather than merely facilitating the loading of BRCA1 onto the DNA. Therefore, we examined whether BARD1 could directly bind DNA.

BARD1 proved to be unstable in the absence of BRCA1 (data not shown) but was stable when copurified with carboxyl-terminal truncations of BRCA1. Surprisingly, even a small (336 amino acids) truncation of BRCA1, which retained the central DNA-binding domain previously identified, exhibited greatly decreased DNA-binding activity. BRCA1 amino acids 1 to 1,527, BRCA1 amino acids 1 to 1,000 (Fig. 2*A*), and BRCA1 at amino acids 1 to 500 (Fig. 2*B*) all displayed diminished or undetectable DNA-binding activity. Coexpressing BARD1 with truncations of BRCA1 allowed BARD1 to be purified in the presence of BRCA1 without DNA-binding activity. We found that BRCA1 (1-500)/BARD1 heterodimers did not exhibit DNA-binding activity at these concentrations; thus, BARD1 does not directly bind DNA at these concentrations. Therefore, the enhanced DNA-binding activity observed with full-length BRCA1/BARD1 is due to an effect of BARD1 on BRCA1.

BARD1 interacts with BRCA1 distal to the RING domain. To answer the question of how BARD1 enhanced the DNA-binding activity of BRCA1, we further examined the interaction between BRCA1 and BARD1 and the effect of this interaction on the DNA-binding domains of BRCA1. Two domains of BRCA1 have DNA-binding activity. One is a central region of BRCA1 roughly encompassing amino acids 400 to 1,100 (33). The other is the carboxyl terminus of BRCA1 (34), which contains tandem BRCT repeats, a motif found in many DNA damage repair proteins.

The two DNA-binding domains of BRCA1 were separately expressed and purified from bacteria. When used in an EMSA, it was determined that the individual DNA-binding domains have significantly lower affinity for DNA than the full-length protein (Fig. 3). Although the full-length BRCA1 bound 50% of the labeled probe between 8.5 and 17 nmol/L, the central DNA-binding domain and the BRCT region individually bound 50% of the probe at 900 and 2,000 nmol/L protein, respectively. Together with the data from Fig. 2*A*, which show that carboxyl-terminal truncations of BRCA1 have reduced DNA-binding activity, this suggests that the two low-affinity DNA-binding domains act in concert to provide the full-length BRCA1 with high-affinity DNA-binding activity.

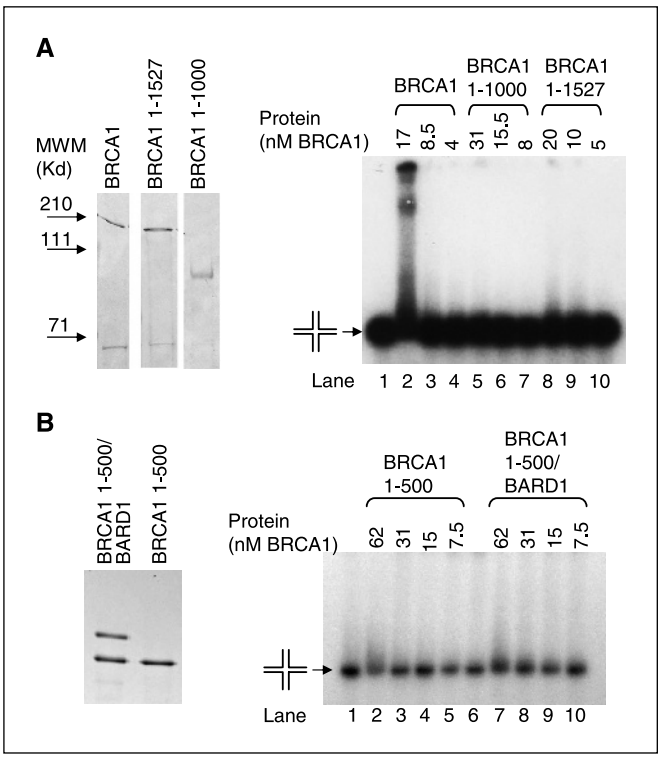

Figure 2. BARD1 does not directly bind DNA. *A,* BRCA1 at amino acids 1 to 1,000 and BRCA1 at amino acids 1 to 1,527 were expressed and purified as with the full-length protein. These proteins were subjected to SDS-PAGE and were Coomassie stained (*left*). ^{32}P-labeled Holliday junction DNA was incubated with varying amounts of BRCA1 (*lanes 2-4*), BRCA1 at amino acids 1 to 1,000 (*lanes 5-7*), or BRCA1 at amino acids 1 to 1,527 (*lanes 8-10*) followed by EMSA. Migration of the free probe is indicated. *B,* BRCA1 (1-500) and BRCA1 (1-500)/BARD1 heterodimers were purified as with the full-length protein and analyzed by SDS-PAGE and staining with Coomassie dye. Varying amounts of BRCA1 (1-500) or BRCA1 (1-500)/BARD1 were tested for DNA binding using EMSA as above.

DOI:10.1158/0008-5472.CAN-05-3296

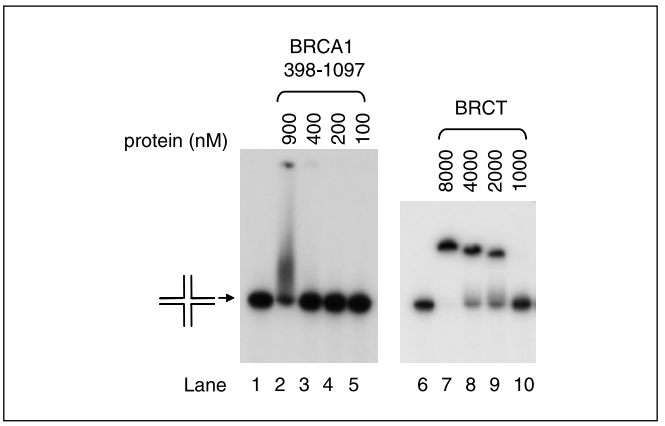

Figure 3. BRCA1 has two low-affinity DNA-binding domains. BRCA1 amino acids 398 to 1,097 and the BRCT domains of BRCA1 were incubated with ^{32}P-labeled Holliday junction DNA and subjected to EMSA. Migration of the free probe is indicated.

To determine whether BARD1 affected one domain more than another, we examined the differences in DNA-binding properties between the two domains. Using unlabeled competitor DNA in an EMSA, we confirmed that the two DNA-binding domains display differing specificity for DNA structure, with the central DNA-binding domain having higher affinity for branched Holliday junction DNA and the BRCT having higher affinity for DNA ends (data not shown). This is consistent with previously published observations (33, 34). The structure specificity of the full-length BRCA1 reflects the specificity of both domains, and BARD1 does not significantly change the structure specificity of BRCA1 (data not shown). Therefore, studies of the relative activities of the individual DNA-binding domains do not yield significant information about how BARD1 enhances the BRCA1 DNA-binding activity.

Because the DNA-binding domains of BRCA1 are distal to the RING dimerization domain, we hypothesized that BRCA1 and BARD1 might interact via other domains. BARD1 was identified in a screen for proteins that interact with the amino-terminal RING domain of BRCA1, but contact between the two proteins via other domains has not been described. To determine whether BRCA1 and BARD1 interact downstream of the RING domain, we coexpressed a FLAG-tagged amino-terminal deletion of BRCA1 (lacking the RING domain) with BARD1. ΔN-BRCA1/BARD1 complexes were immunoaffinity purified as was done with the full-length complexes. As with the full-length protein, purified ΔN-BRCA1 protein included several degradation products (Fig. 4*A*). Coomassie-stained SDS-PAGE reveals that ΔN-BRCA1 contains a degradation product that comigrates with BARD1. Nevertheless, it was readily apparent that the ΔN-BRCA1/BARD1 complexes were significantly enriched for a BARD1-sized band (Fig. 4*A*).

To confirm that this band did contain BARD1 and to quantitate the interaction between ΔN-BRCA1 and BARD1, 2-fold serial dilutions of BRCA1/BARD1 (Fig. 4*B*, *lanes 1-4*) and ΔN-BRCA1/BARD1 (Fig. 4*B*, *lanes 5-8*) were immunoblotted for both BRCA1 and BARD1. In Fig. 4*B*, lanes 1 and 5 contained approximately equal amounts of BRCA1 full-length and ΔN-BRCA1, respectively. Figure 4*B* (*lane 5*) also confirms the presence of BARD1 in the purified ΔN-BRCA1/BARD1. However, the amount of BARD1 in this lane is equivalent to the amount of BARD1 present in a 4-fold dilution of the full-length BRCA1/BARD1 heterodimers. The RING-distal interactions between BRCA1 and BARD1, therefore, have about one quarter the affinity of the sum of the interactions between the two proteins. This is consistent with removing a major interaction domain between the two proteins as would be expected in eliminating the RING domain of BRCA1.

As a negative control, we tested nonspecific interaction with the antibody-coupled beads. Untagged BARD1 was expressed alone in insect cells. Lysates of these cells were then subjected to the same immunopurification procedure used for purifying BRCA1 and

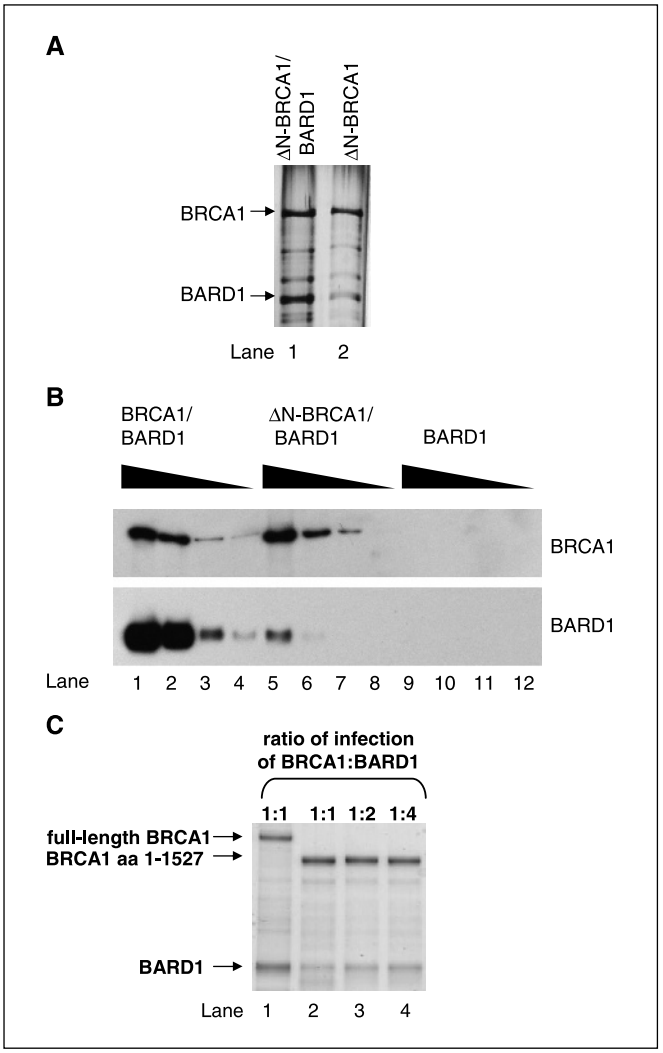

Figure 4. BRCA1 interacts with BARD1 downstream of the RING domain. *A,* a FLAG-tagged amino-terminal truncation of BRCA1 that lacks the RING domain (ΔN-BRCA1) was coexpressed with untagged BARD1. Complexes were immunoaffinity purified via the FLAG tag and subjected to gel electrophoresis followed by Coomassie staining. *B,* insect cells were infected with FLAG-BRCA1 and BARD1, FLAG-ΔN-BRCA1 and BARD1, or BARD1 alone. Complexes were immunoaffinity purified using anti-FLAG antibody and were subjected to Western blotting for BRCA1 and BARD1. Samples from each of the three preparations were serially diluted in 2-fold steps, allowing a determination of the relative concentrations of the polypeptides in each preparation. A 4-fold dilution of the BRCA1/BARD1 (*lane 3*) had approximately equal concentration of BARD1 as did the highest concentration of ΔN-BRCA1/BARD1 tested (*lane 5*). The concentration of BRCA1 in each preparation was approximately equal. *C,* the BARD1 content of BRCA1/BARD1 (*lane 1*) was compared with BRCA1 (1-1,527)/BARD1 (*lane 2*). To exclude the possibility that poor BARD1 expression was responsible for low yields of BARD1 when coexpressed with the BRCA1(1-1,527), the baculovirus encoding BARD1 expression was infected into cells at 2-fold (lane 3) and 4-fold (lane 4) higher levels, and the proteins purified via the epitope-tagged BRCA1 were analyzed by silver-stained protein gel.

DOI:10.1158/0008-5472.CAN-05-3296

heterodimers. No BARD1 could be detected in the control purification (Fig. 4*B*, *lanes 9-12*).

An examination of carboxyl-terminal truncated BRCA1/BARD1 heterodimers revealed that removal of the BRCT domains of BRCA1 also decreased the amount of BARD1 that copurified with BRCA1. Full-length BRCA1/BARD1 and BRCA1 (1-1,527)/BARD1 heterodimers were analyzed by SDS-PAGE and were silver stained (Fig. 4*C*, *lanes 1* and *2*). BRCA1 (1-1,527)/BARD1 contained significantly less BARD1 than the full-length BRCA1 heterodimers. To ensure that this was not due to differences in baculovirus titer, we increased the ratio of infection of BARD1/BRCA1 (1-1,527) virus. Increasing up to 4-fold the amount of BARD1 baculovirus used for infection could not drive the copurification of BARD1 at the same levels as seen with the full-length BRCA1 (Fig. 4*C*, *lanes 3* and *4*). This result suggested that the BRCT of BRCA1 interacts with BARD1 and offers additional confirmation that there are significant interactions between the two proteins exclusive of the RING domain.

Interactions between BRCA1 and BARD1 distal to the RING domain are responsible for enhanced DNA binding by BRCA1. To distinguish whether the RING-dependent or RING-independent interactions between BRCA1 and BARD1 were responsible for the enhanced DNA-binding activity of BRCA1, we coexpressed FLAG-tagged full-length BRCA1 with a truncation of BARD1 containing the RING domain (amino acids 1-292). Heterodimers were immunoaffinity purified as described (Fig. 5*A*).

The DNA-binding activities of BRCA1, BRCA1/BARD, and BRCA1/BARD (1-292) were assessed in an EMSA with a four-stranded Holliday junction DNA probe (Fig. 5*B*). In Fig. 5*C*, the results of Fig. 5*B* were quantitated using a phosphorimager to estimate the K_d of BRCA1 for DNA. The complex nature of the shift prevents direct calculation of the K_d; however, we estimated the K_d from the amount of BRCA1 at which half of the probe was bound. From Fig. 5*C*, we estimated the K_d of BRCA1 alone for DNA to be $\sim$10 nmol/L, the K_d of BRCA1/BARD1 to be $\sim$3 nmol/L, and the K_d of BRCA1/BARD1 (1-292) to be $\sim$11 nmol/L. Therefore, BARD1 (1-292) must lack the necessary RING-independent interaction domains required to enhance DNA binding by BRCA1.

BRCA1/BARD1 autoubiquitination further enhances DNA binding. The heterodimerization of BRCA1 and BARD1 via their RING domains forms a functional E3 ubiquitin ligase, which is capable of autoubiquitination. Although BARD1 enhancement of BRCA1 DNA-binding activity requires RING-independent interaction between the two proteins, we examined whether the RING-dependent autoubiquitination of BRCA1/BARD1 might have a secondary effect on DNA binding.

BRCA1/BARD1 heterodimers were autoubiquitinated *in vitro* by the following approach. BRCA1/BARD1 heterodimers coexpressed in baculovirus-infected insect cells were immunoaffinity purified via the FLAG tag on BRCA1. Before elution from the FLAG-tag–specific agarose beads, the beads were divided in two equal portions and either autoubiquitinated or subjected to a mock ubiquitination reaction containing all necessary factors but ubiquitin. The bound proteins were then purified away from additional components of the ubiquitination reaction and eluted from the beads (Fig. 6*A*).

Quantitation of the autoubiquitinated BRCA1/BARD1 was complicated because the ubiquitinated polypeptides did not migrate as a single focused band but rather as a ladder. In addition, the 76-amino-acid ubiquitin contributed to the mass of the protein. Because a single BRCA1/BARD1 preparation was divided equally,

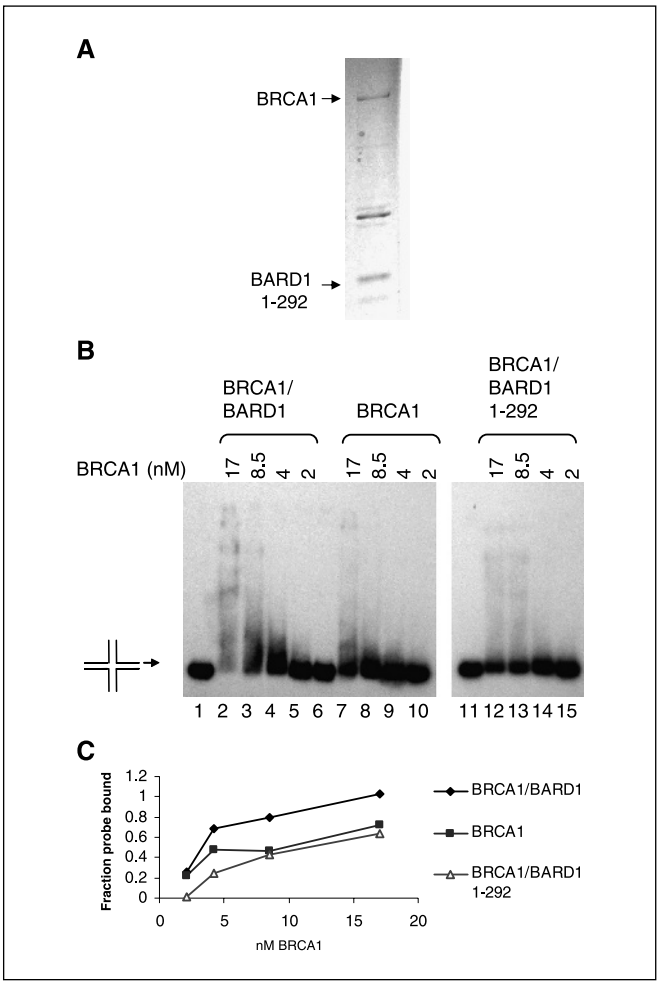

Figure 5. BARD1 RING domain is not sufficient for enhanced DNA binding by BRCA1. *A,* FLAG-tagged BRCA1 was coexpressed with the RING domain of BARD1 (amino acids 1-292) and purified as above. *B,* BRCA1/BARD1 (*lanes 1-5*), BRCA1 alone (*lanes 6-10*), or BRCA1/BARD1 at amino acids 1 to 292 (*lanes 11-15*) were incubated with Holliday junction DNA and subjected to EMSA. Migration of free probe is indicated. *C,* the results from (*B*) were quantitated using a phosphorimager and the percentage of free probe bound by each BRCA1/BARD1 preparation was measured.

we set the eluted ubiquitinated BRCA1/BARD1 at the same concentration as the unmodified protein prepared in parallel. That the concentrations were equivalent was supported by experiments in which these two preparations had equal transcription activity.[4]

These ubiquitinated heterodimers were then used in an EMSA to determine DNA-binding activity. As before, ^{32}P-labeled four-stranded Holliday junction DNA was used as a probe. Surprisingly, ubiquitinated BRCA1/BARD1 heterodimers (Fig. 6*B*, *lanes 7-10*) displayed a higher affinity for DNA than the unubiquitinated control (Fig. 6*B*, *lanes 2-5*). The binding was quantitated in Fig. 6*C*. Ubiquitinated heterodimers display nearly a 3-fold higher affinity for DNA than unubiquitinated BRCA1/BARD1, binding 50% of the free probe at $\sim$0.9 nmol/L. In comparison, this represents a 12-fold increase over BRCA1 alone (Figs. 1 and 5).

[4] A.A. Horwitz and J.D. Parvin, unpublished observations.

DOI:10.1158/0008-5472.CAN-05-3296

Discussion

Because nuclear BRCA1 is associated with BARD1, we analyzed the effect that BARD1 had on the DNA-binding properties of BRCA1. BARD1 and BRCA1 were known to interact via the amino-terminal RING domains of both proteins (26, 50). The RING domain is separate from both known DNA-binding domains of BRCA1, but BARD1 nevertheless had the effect of increasing the affinity of BRCA1 for DNA. In this study, we found that (*a*) the two characterized low-affinity DNA-binding domains of BRCA1 act synergistically to create high-affinity DNA binding by the full-length protein. (*b*) Binding of BRCA1 to BARD1 increased the affinity of BRCA1 for Holliday junction DNA probe 3-fold. (*c*) The effect of BARD1 on BRCA1 affinity for DNA revealed that BARD1 and BRCA1 make contacts via the carboxyl-terminal 336 amino acid residues of BRCA1. (*d*) Autoubiquitination of BRCA1/BARD1 increases DNA-binding activity 3-fold.

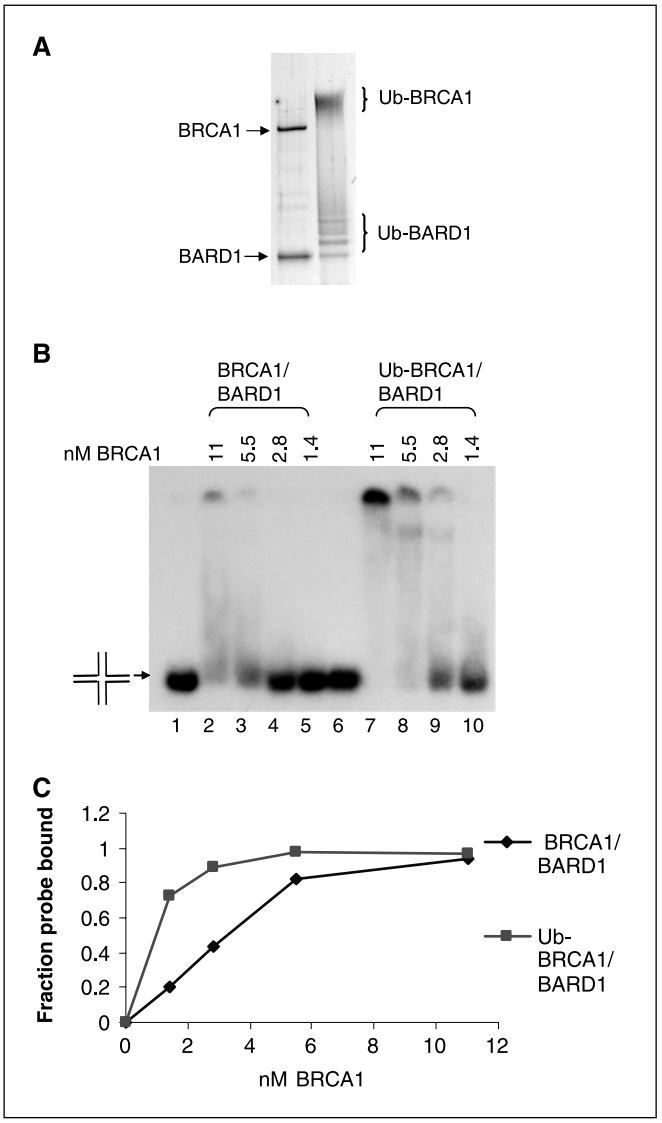

Figure 6. Autoubiquitination of BRCA1/BARD1 further enhances DNA-binding activity. *A,* BRCA1/BARD1 autoubiquitinated *in vitro* (*lane 2*) and a control reaction lacking ubiquitin (*lane 1*) were analyzed by SDS-PAGE and were silver stained. *B,* BRCA1/BARD1 (*lanes 2-5*) and autoubiquitinated BRCA1/BARD1 (*lanes 7-10*) were incubated with ^{32}P-labeled Holliday junction and analyzed by EMSA. Migration of free probe is indicated. *C,* results from (*B*) for DNA binding, determined as the percent of free probe bound by the BRCA1/BARD1 complex, were quantitated using a phosphorimager.

Two separate, low-affinity DNA-binding domains of BRCA1 had been characterized: the central DNA-binding domain encompassing residues 452 to 1,097 and the BRCT domain encompassing residues 1,641 to 1,863 (33, 34). Although our EMSA methods differed slightly from those previously published, our observations regarding the individual DNA-binding domains are consistent with the previously published results. We found that combining the two domains in a single BRCA1 protein increased the affinity for DNA ~50-fold. Consistent with this effect of linking the two DNA-binding domains, the ΔN-BRCA1 bound to the DNA probe with similar affinity as full-length BRCA1 alone (data not shown). Binding to BARD1 contributes an additional 3-fold increase in DNA binding, and autoubiquitination of BRCA1/BARD1 contributes another 3-fold increase.

The effect of BARD1 on DNA binding led to the realization that BRCA1 and BARD1 make contacts outside of the amino terminus. BARD1 was originally isolated via binding to the BRCA1 amino-terminal RING domain but it had never been shown that other domains do not stabilize this heterodimerization (26, 50, 51). If contacts between BRCA1 and BARD1 were limited to the amino-terminal 100 amino acids of each protein, then BARD1 would be predicted to have no effect on BRCA1 DNA-binding activity. The observed increase in DNA-binding activity could therefore be explained by two possibilities: One is that BARD1 makes additional contacts with BRCA1 and the other is that heterodimerization changes the conformation of the entire protein. Although we have shown evidence for the first possibility, we cannot rule out the second.

Our data strongly suggest that the additional interactions between BRCA1 and BARD1 are within the BRCT domain of BRCA1. Appropriate relocalization of BRCA1 and BARD1 in cells after DNA damage depends on both the amino and carboxyl termini of the proteins (52). Because relocalization correlates with the response to DNA damage, we suggest that following relocalization to DNA damage–induced foci, the compound DNA-binding domains stably associate with the genome. Further, autoubiquitinated BRCA1 has been detected at these foci (40). This suggests that the increase in affinity of BRCA1 for DNA upon autoubiquitination, discovered in this study, is a necessary function for DNA damage response *in vivo*.

Structural studies of the interaction between BRCA1 and BARD1 have been limited to the RING domain (50, 51, 53). The recent discovery of a splice variant of BARD1 that fails to interact with BRCA1 adds further support to the biological relevance of the RING-independent interactions between BRCA1 and BARD1. This splice variant retains both the RING and BRCT domains of BARD1 but lacks a significant central region; thus, the RING domain is not sufficient for heterodimerization with BRCA1 (54). That this splice variant fails to interact with BRCA1 suggests that either it lacks the additional BRCA1 interaction domain or that proper alignment of the RING and BRCT domains is necessary for proper association of BRCA1 and BARD1.

The interaction of BARD1 with the BRCA1 BRCT raises interesting questions about clinically relevant mutations in the BRCA1 carboxyl terminus. Many of these mutations seem to disrupt proper folding of BRCA1 (55). We speculate that improper folding of the BRCA1 BRCTs may destabilize binding of BRCA1 to BARD1, thus inhibiting DNA binding and/or ubiquitination.

DOI:10.1158/0008-5472.CAN-05-3296

Using recombinantly purified proteins, the interaction between BRCA1 and BARD1 was characterized in light of the effect of BARD1 on the DNA-binding properties of BRCA1. Although we found no evidence of direct DNA binding by BARD1, we did observe that the presence of BARD1 enhanced DNA binding by BRCA1. The RING domain interaction of these proteins was not sufficient for this enhancement; rather, we show that the RING-independent interactions between BRCA1 and BARD1 are responsible for increasing the affinity of BRCA1 for DNA. The RING domain interactions of BRCA1 and BARD1 do, however, have a secondary effect on DNA binding. RING-dependent autoubiquitination of BRCA1/BARD1 heterodimers results in an even higher affinity of BRCA1 for DNA. Together, the net effect of the presence of BARD1 is an ~12-fold increase in the affinity of BRCA1 for DNA. We conclude that the ubiquitination function of BRCA1 and BARD1 is likely to play an important role in DNA damage response by BRCA1.

Acknowledgments

Received 9/14/2005; revised 10/24/2005; accepted 12/8/2005.

Grant support: Predoctoral fellowship from the National Science Foundation (A.M. Simons), predoctoral fellowships from the Department of Defense Breast Cancer Research Program (L.M. Starita and A.A. Horwitz), and National Cancer Institute research grant CA90281 (J.D. Parvin).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Monteiro AN, August A, Hanafusa H. Evidence for a transcriptional activation function of BRCA1 C-terminal region. Proc Natl Acad Sci U S A 1996;93:13595–9.
2. Chiba N, Parvin JD. The BRCA1 and BARD1 association with the RNA polymerase II holoenzyme. Cancer Res 2002;62:4222–8.
3. Lu M, Arrick BA. Transactivation of the p21 promoter by BRCA1 splice variants in mammary epithelial cells: evidence for both common and distinct activities of wildtype and mutant forms. Oncogene 2000;19:6351–60.
4. Scully R, Anderson SF, Chao DM, et al. BRCA1 is a component of the RNA polymerase II holoenzyme. Proc Natl Acad Sci U S A 1997;94:5605–10.
5. Moynahan ME, Cui TY, Jasin M. Homology-directed DNA repair, mitomycin-c resistance, and chromosome stability is restored with correction of a Brca1 mutation. Cancer Res 2001;61:4842–50.
6. Moynahan ME, Chiu JW, Koller BH, Jasin M. Brca1 controls homology-directed DNA repair. Mol Cell 1999;4:511–8.
7. Johnson RD, Jasin M. Double-strand-break-induced homologous recombination in mammalian cells. Biochem Soc Trans 2001;29:196–201.
8. Jasin M. Homologous repair of DNA damage and tumorigenesis: the BRCA connection. Oncogene 2002;21:8981–93.
9. Huber LJ, Yang TW, Sarkisian CJ, Master SR, Deng CX, Chodosh LA. Impaired DNA damage response in cells expressing an exon 11-deleted murine Brca1 variant that localizes to nuclear foci. Mol Cell Biol 2001;21:4005–15.
10. Tibbetts RS, Cortez D, Brumbaugh KM, et al. Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. Genes Dev 2000;14:2989–3002.
11. Yarden RI, Pardo-Reoyo S, Sgagias M, Cowan KH, Brody LC. BRCA1 regulates the G_2/M checkpoint by activating Chk1 kinase upon DNA damage. Nat Genet 2002;30:285–9.
12. Somasundaram K, Zhang H, Zeng YX, et al. Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. Nature 1997;389:187–90.
13. Starita LM, Machida Y, Sankaran S, et al. BRCA1-dependent ubiquitination of γ-tubulin regulates centrosome number. Mol Cell Biol 2004;24:8457–66.
14. Lotti LV, Ottini L, D'Amico C, et al. Subcellular localization of the BRCA1 gene product in mitotic cells. Genes Chromosomes Cancer 2002;35:193–203.
15. Deng CX. Roles of BRCA1 in centrosome duplication. Oncogene 2002;21:6222–7.
16. Sankaran S, Starita LM, Groen AC, Ko MJ, Parvin JD. Centrosomal microtubule nucleation activity is inhibited by BRCA1-dependent ubiquitination. Mol Cell Biol 2005;25:8656–68.
17. Ko MJ, Murata K, Hwang DS, Parvin JD. Inhibition of BRCA1 in breast cell lines causes the centrosome duplication cycle to be disconnected from the cell cycle. Oncogene. Epub 2005 Sep 19.
18. Koonin EV, Altschul SF, Bork P. BRCA1 protein products. Functional motifs. Nat Genet 1996;13:266–8.
19. Yu X, Chini CC, He M, Mer G, Chen J. The BRCT domain is a phospho-protein binding domain. Science 2003;302:639–42.
20. Williams RS, Lee MS, Hau DD, Glover JN. Structural basis of phosphopeptide recognition by the BRCT domain of BRCA1. Nat Struct Mol Biol 2004;11:519–25.
21. Glover JN, Williams RS, Lee MS. Interactions between BRCT repeats and phosphoproteins: tangled up in two. Trends Biochem Sci 2004;29:579–85.
22. Manke IA, Lowery DM, Nguyen A, Yaffe MB. BRCT repeats as phosphopeptide-binding modules involved in protein targeting. Science 2003;302:636–9.
23. Couch FJ, Weber BL. Mutations and polymorphisms in the familial early-onset breast cancer (BRCA1) gene. Breast Cancer Information Core. Hum Mutat 1996;8:8–18.
24. Mirkovic N, Marti-Renom MA, Weber BL, Sali A, Monteiro AN. Structure-based assessment of missense mutations in human BRCA1: implications for breast and ovarian cancer predisposition. Cancer Res 2004;64:3790–7.
25. Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM. RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. Proc Natl Acad Sci U S A 1999;96:11364–9.
26. Wu LC, Wang ZW, Tsan JT, et al. Identification of a RING protein that can interact *in vivo* with the BRCA1 gene product. Nat Genet 1996;14:430–40.
27. Hashizume R, Fukuda M, Maeda I, et al. The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. J Biol Chem 2001;276:14537–40.
28. Chen A, Kleiman FE, Manley JL, Ouchi T, Pan ZQ. Autoubiquitination of the BRCA1*BARD1 RING ubiquitin ligase. J Biol Chem 2002;277:22085–92.
29. Ouchi T, Monteiro AN, August A, Aaronson SA, Hanafusa H. BRCA1 regulates p53-dependent gene expression. Proc Natl Acad Sci U S A 1998;95:2302–6.
30. Krum SA, Miranda GA, Lin C, Lane TF. BRCA1 associates with processive RNA polymerase II. J Biol Chem 2003;278:52012–20.
31. El-Deiry WS. Transactivation of repair genes by BRCA1. Cancer Biol Ther 2002;1:490–1.
32. Wang Y, Cortez D, Yazdi P, Neff N, Elledge SJ, Qin J. BASC, a supercomplex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. Genes Dev 2000;14:927–39.
33. Paull TT, Cortez D, Bowers B, Elledge SJ, Gellert M. Direct DNA binding by Brca1. Proc Natl Acad Sci U S A 2001;98:6086–91.
34. Yamane K, Katayama E, Tsuruo T. The BRCT regions of tumor suppressor BRCA1 and of XRCC1 show DNA end binding activity with a multimerizing feature. Biochem Biophys Res Commun 2000;279:678–84.
35. Jin Y, Xu XL, Yang MC, et al. Cell cycle-dependent colocalization of BARD1 and BRCA1 proteins in discrete nuclear domains. Proc Natl Acad Sci U S A 1997;94:12075–80.
36. Zhong Q, Chen CF, Li S, et al. Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. Science 1999;285:747–50.
37. Scully R, Chen J, Ochs RL, et al. Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. Cell 1997;90:425–35.
38. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol 2000;10:886–95.
39. Choudhury AD, Xu H, Baer R. Ubiquitination and proteasomal degradation of the BRCA1 tumor suppressor is regulated during cell cycle progression. J Biol Chem 2004;279:33909–18.
40. Morris JR, Solomon E. BRCA1: BARD1 induces the formation of conjugated ubiquitin structures, dependent on K6 of ubiquitin, in cells during DNA replication and repair. Hum Mol Genet 2004;13:807–17.
41. Nishikawa H, Ooka S, Sato K, et al. Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase. J Biol Chem 2004;279:3916–24.
42. Joukov V, Chen J, Fox EA, Green JB, Livingston DM. Functional communication between endogenous BRCA1 and its partner, BARD1, during *Xenopus laevis* development. Proc Natl Acad Sci U S A 2001;98:12078–83.
43. Chiba N, Parvin JD. Redistribution of BRCA1 among four different protein complexes following replication blockage. J Biol Chem 2001;276:38549–54.
44. Irminger-Finger I, Soriano JV, Vaudan G, Montesano R, Sappino AP. *In vitro* repression of Brca1-associated RING domain gene, Bard1, induces phenotypic changes in mammary epithelial cells. J Cell Biol 1998;143:1329–39.
45. McCarthy EE, Celebi JT, Baer R, Ludwig T. Loss of Bard1, the heterodimeric partner of the Brca1 tumor suppressor, results in early embryonic lethality and chromosomal instability. Mol Cell Biol 2003;23:5056–63.
46. Westermark UK, Reyngold M, Olshen AB, Baer R, Jasin M, Moynahan ME. BARD1 participates with BRCA1 in homology-directed repair of chromosome breaks. Mol Cell Biol 2003;23:7926–36.
47. Rodriguez JA, Schuchner S, Au WW, Fabbro M, Henderson BR. Nuclear-cytoplasmic shuttling of BARD1 contributes to its proapoptotic activity and is regulated by dimerization with BRCA1. Oncogene 2004;23:1809–20.
48. Starita LM, Horwitz AA, Keogh MC, Ishioka C, Parvin JD, Chiba N. BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II. J Biol Chem 2005;280:24498–505.
49. Williams RS, Green R, Glover JN. Crystal structure of the BRCT repeat region from the breast cancer-associated protein BRCA1. Nat Struct Biol 2001;8:838–42.
50. Meza JE, Brzovic PS, King MC, Klevit RE. Mapping the functional domains of BRCA1. Interaction of the ring finger domains of BRCA1 and BARD1. J Biol Chem 1999;274:5659–65.
51. Brzovic PS, Rajagopal P, Hoyt DW, King MC, Klevit RE. Structure of a BRCA1-BARD1 heterodimeric RING-RING complex. Nat Struct Biol 2001;8:833–7.
52. Au WW, Henderson BR. The BRCA1 RING and BRCT domains cooperate in targeting BRCA1 to ionizing radiation-induced nuclear foci. J Biol Chem 2005;280:6993–7001.
53. Morris JR, Keep NH, Solomon E. Identification of residues required for the interaction of BARD1 with BRCA1. J Biol Chem 2002;277:9382–6.
54. Tsuzuki M, Wu W, Nishikawa H, et al. A truncated splice variant of human BARD1 that lacks the RING finger and ankyrin repeats. Cancer Lett. Epub 2005 May 3.
55. Williams RS, Chasman DI, Hau DD, Hui B, Lau AY, Glover JN. Detection of protein folding defects caused by BRCA1-BRCT truncation and missense mutations. J Biol Chem 2003;278:53007–16.