Structural Requirements for the BARD1 Tumor Suppressor in Chromosomal Stability and Homology-directed DNA Repair*

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The BRCA1 tumor suppressor exists as a heterodimeric complex with BARD1, and this complex is thought to mediate many of the functions ascribed to BRCA1, including its role in tumor suppression. The two proteins share a common structural organization that features an N-terminal RING domain and two C-terminal BRCT motifs, whereas BARD1 alone also contains three tandem ankyrin repeats. In normal cells, the BRCA1/BARD1 heterodimer is believed to enhance chromosome stability by promoting homology-directed repair (HDR) of double strand DNA breaks. Here we have investigated the structural requirements for BARD1 in this process by complementation of Bard1-null mouse mammary carcinoma cells. Our results demonstrate that the ankyrin and BRCT motifs of BARD1 are each essential for both chromosome stability and HDR. Tandem BRCT motifs, including those found at the C terminus of BARD1, are known to form a phosphoprotein recognition module. Nonetheless, the HDR function of BARD1 was not perturbed by synthetic mutations predicted to ablate the phospho-recognition activity of its BRCT sequences, suggesting that some functions of the BRCT domains are not dependent on their ability to bind phosphorylated ligands. Also, cancer-associated missense mutations in the BRCT domains of BARD1 (e.g. C557S, Q564H, V695L, and S761N) have been observed in patients with breast, ovarian, and endometrial tumors. However, none of these was found to affect the HDR activity of BARD1, suggesting that any increased cancer risk conferred by these mutations is not because of defects in this repair mechanism.

Inactivating mutations in the *BRCA1* tumor suppressor gene are found in a large proportion of hereditary breast and ovarian cancers, underscoring the importance of the BRCA1 pathway in modulating tumor susceptibility (1, 2). BRCA1 promotes genomic stability through a number of cellular processes, including DNA double strand break (DSB)[2] repair and cell cycle checkpoint control (reviewed in Refs. 3–5). As such, cells deficient in BRCA1 accumulate chromosome abnormalities and display hypersensitivity to a variety of genotoxic agents. Although the genome maintenance functions of BRCA1 are believed to be a major determinant of its tumor suppression activity, the specific molecular mechanisms by which BRCA1 affects genome stability remain elusive.

The *BRCA1* gene encodes a 1863-amino acid protein with an N-terminal RING domain and two tandem C-terminal BRCT repeats (1, 6). *In vivo*, BRCA1 forms a stable heterodimer with BARD1, a structurally related 777-amino acid protein that also contains an N-terminal RING domain and two C-terminal BRCT repeats (7). In addition, the central region of BARD1 harbors three tandem ankyrin repeats of unknown function. The interaction between BRCA1 and BARD1 is mediated primarily by N-terminal sequences encompassing their respective RING domains (7, 8), and the resulting heterodimer functions as a potent E3 ubiquitin ligase (9). As the enzymatic activity of the BRCA1/BARD1 heterodimer is substantially higher than that of the isolated polypeptides, the heterodimer is likely to represent the physiologic mediator of BRCA1 and BARD1 functions. In support of this notion, *Brca1-* and *Bard1*-null mice suffer embryonic lethality with phenotypes that are essentially indistinguishable (10). Because embryonic cells from these mice exhibit aneuploidy and accumulate chromosome abnormalities, BARD1, like BRCA1, is likely to function in the maintenance of genomic stability.

Given the intimate relationship between BRCA1 and BARD1, lesions of the *BARD1* gene might also be expected to promote oncogenesis, and indeed, tumor-specific missense mutations of *BARD1* have been found in patients with breast, ovarian, and endometrial carcinomas (11–13). Moreover, these mutations are often accompanied by tumor-specific loss of the normal *BARD1* allele (11). Thus, although cancer-associated BARD1 mutations are uncommon, their existence implies that BARD1 can serve as a target for genetic lesions that disrupt BRCA1-mediated tumor suppression. Experimental support for this possibility has recently emerged from studies of mice that undergo cre-mediated inactivation of the *Bard1* gene in their mammary epithelial cells. Remarkably, these animals

[2] The abbreviations used are: DSB, double strand break; GFP, green fluorescent protein; E3, ubiquitin-protein isopeptide ligase; IR, ionizing radiation; HDR, homology-directed repair; MMC, mitomycin C.

develop breast tumors with an incidence, latency, and phenotype similar to those of mice undergoing mammary-specific inactivation of *Brca1*.[3] Thus, BARD1 appears to function *in vivo* as a tumor suppressor in a manner reminiscent of BRCA1.

Recent studies have shown that two tandem BRCT motifs, such as those arrayed at the C termini of BRCA1 and BARD1, can form a structural domain that specifically binds phosphorylated peptide sequences (14, 15). In this manner, the BRCT motifs of BRCA1 can control distinct cell cycle checkpoints through phospho-dependent interactions with different cellular proteins. For example, the transient G_2/M checkpoint induced by ionizing radiation (IR) is dependent on recognition of Ser-327-phosphorylated isoforms of the CtIP protein by the BRCT motifs of BRCA1 (16). Conversely, an interaction between these same motifs and the Ser-990-phosphorylated BACH1/BRIP1/FancJ protein is required for activation of the G_2 accumulation checkpoint (15). Although cellular proteins that associate with the BRCT motifs of BARD1 have not yet been identified, these motifs can also bind peptides in a phospho-dependent manner (17).

BRCA1 is required for homology-directed repair (HDR) of DSBs (18, 19). In a previous study we showed that overexpression of a truncated BARD1 polypeptide interferes with HDR in mouse embryonic stem cells, presumably through dominant-negative inhibition of the endogenous Brca1/Bard1 heterodimer (20). More recently, the plant ortholog of BARD1 was shown to participate in homology-driven DNA recombination in somatic cells (21). Because HDR is a major pathway for DSB repair in mammals (22), the HDR defects associated with BARD1 dysfunction may contribute to genomic instability. Therefore, in the current study, we have investigated the structural requirements for BARD1 function in this process by complementation of Bard1-null mouse mammary carcinoma cell lines. Our results demonstrate that the ankyrin and BRCT motifs of BARD1 are both essential for chromosomal stability and HDR-mediated repair of an induced chromosomal break. However, the HDR function of BARD1 was not perturbed by synthetic mutations of the BRCT sequences predicted to ablate phosphoprotein recognition. Thus, although the BRCT motifs of BARD1 are critical for HDR, their ability to bind phosphorylated ligands does not appear to be required for this process. Interestingly, although several cancer-associated missense mutations of BARD1 target the BRCT domains, none of these was found to affect HDR function, suggesting that the increased cancer risk conferred by these variants is not because of defects in HDR.

EXPERIMENTAL PROCEDURES

Cell Lines—Bard-null mouse mammary carcinoma cell lines 10-05 and 18-09 were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin/streptomycin, and 2 mM L-glutamine. To generate stably transformed subclones of these lines, 10-05 and 18-09 cells were transfected using FuGENE 6 (Roche Applied Science) with either the empty pIRESpuro vector (Clontech) or

with the corresponding vector encoding human BARD1 with either a single (FLAG-BARD1/pIRESpuro) or three tandem (3xFLAG-BARD1/pIRESpuro) N-terminal FLAG epitopes. Following 3 weeks of selection with 5 μg/ml puromycin, individual clones were screened for BARD1 expression by immunoblotting with BARD1- or FLAG-specific antibodies. To generate HDR reporter lines, Bard1-null 18-09 cells were transfected with the DR-GFPhygro reporter construct (23), and transformants harboring randomly integrated copies of the reporter were selected with 0.25 mg/ml hygromycin B. To identify subclones bearing a single intact copy of the reporter, the genomic DNAs of individual subclones were digested with restriction endonucleases (BglII, HindIII, PstI, and XbaI) and analyzed by Southern hybridization with the 800-bp HindIII fragment of the DR-GFPhygro plasmid. Two independently derived subclones with a single integrated copy of the DR-GFP reporter (18-09/DR-GFP-2 and 18-09/DR-GFP-4) were used for all HDR experiments.

Antibodies—The BARD1-specific polyclonal antiserum has been described (7). A monoclonal antibody (GH118) specific for mouse Brca1 was kindly provided by Shridar Ganesan and David Livingston (24). Other antibodies used included anti-FLAG (M2, Sigma), anti-NuMa (Ab-1, Calbiochem), and anti-α-tubulin (Ab-1, Oncogene Science Inc.).

Cytogenetic Analysis—Cells in log phase of growth were treated with colcemid for 2 h, trypsinized, washed with PBS, and subjected to hypotonic treatment with 0.38% KCl at 37 °C for 20 min. After fixing in 3:1 methanol/glacial acetic acid, the cells were placed on glass slides and stained with Giemsa (Karyomax, Invitrogen). Metaphase spreads were examined for the presence of chromatid-type aberrations, including breaks, gaps, and exchanges. Statistical analysis was performed using the Simple Interactive Statistical Analysis website. Fisher's exact test was used for analysis of cytogenetic data. Multicolor spectral karyotype analysis was performed on metaphase preparations from Bard1-null carcinoma cells grown in culture using standard procedures as recommended by the manufacturer (Applied Spectral Imaging, Vista, CA).

HDR Assays—To measure repair of an I-SceI-induced DSB in the stably reconstituted 18-09/DR-GFP-2 reporter subclones (*e.g.* Fig. 3), $\sim4 \times 10^5$ cells/well were seeded in 2 ml of nonselective medium on 6-well plates. After 20–24 h (at $\sim70\%$ confluency), the cells were transfected with 0.5 μg of an I-SceI expression vector (pCBASce) (26) or the corresponding empty vector (pCAGGS) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 18 h after transfection, the cells were washed and replated on 60-mm dishes in nonselective medium. Flow cytometric analysis to quantify the presence of GFP-positive cells was performed 2 days later on a FACSCalibur using Cellquest software (BD Biosciences). For each sample, 50,000–100,000 cells were assessed, and the percentage of GFP-positive cells was calculated and displayed in Figs. 3–5. An independent transfection with a GFP-encoding vector was performed concurrently to provide a control for transfection efficiency. To measure HDR in transiently reconstituted 18-09/DR-GFP-2 and -4 subclones (*e.g.* Figs. 4 and 5), 5×10^5 reporter cells were seeded on 60-mm plates, allowed to attach for 20–24 h, and co-transfected with 1 μg of pCBASce or the

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[3] R. Shakya, M. Szabolcs, E. E. McCarthy, E. Ospina, K. Basso, S. V. Nandula, V. V. Murty, R. Baer, and T. Ludwig, unpublished data.
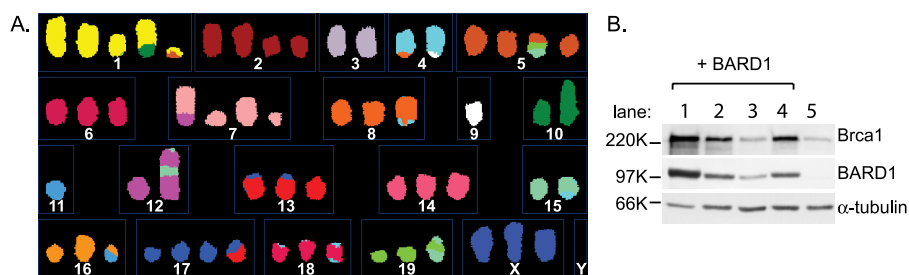
FIGURE 1. **Reconstitution of Bard-null cells with human BARD1.** *A,* representative spectral karyotype of a Bard1-null carcinoma cell line (10-05) showing aneuploidy and structural chromosome aberrations. *B,* subclones of Bard1-null 10-05 cells stably transformed with an expression vector encoding human BARD1 (*lanes 1–4*) or the empty vector (*lane 5*) were analyzed by immunoblotting with antibodies specific for mouse Brca1, human BARD1, or α-tubulin. The endogenous levels of mouse Brca1 (*lane 5*) are increased upon stable complementation with human BARD1 (*lanes 1–4*).

TABLE 1

The chromosomal instability of Bard1-null mammary carcinoma cells is reduced by reconstitution with human BARD1

	Subclones of Bard1-null 10-05 cells	No. of metaphases scored	% metaphases with the indicated no. of aberrations[a]			
			≥1	≥2	≥3	≥4
−MMC	Empty vector[b]	100	26	6	1	0
	BARD1	95	11	1	1	0
	BARD1-ΔAnk[c]	100	30	9	4	1
	BARD1-ΔBRCT[d]	100	33	9	1	1
+MMC	Empty vector[e]	100	70	44	25	10
	BARD1	100	53	17	5	2
	BARD1-ΔAnk[f]	100	78	56	32	14
	BARD1-ΔBRCT[g]	106	71	39	23	16

[a] The percentage of metaphases containing one or more chromatid-type aberrations (breaks, gaps, and/or exchanges) are shown. The difference in the percentage of abnormal metaphases between subclones reconstituted with full-length BARD1 and either empty vector, BARD1-ΔAnk, or BARD1-ΔBRCT is statistically significant. Pairwise comparison using the Fisher's exact test (25) on number of metaphases with ≥1 aberration(s) is shown below.
[b] Values are $p = 3.0 \times 10^{-3}$.
[c] Values are $p = 4.5 \times 10^{-4}$.
[d] Values are $p = 9.1 \times 10^{-5}$.
[e] Values are $p = 5.5 \times 10^{-3}$.
[f] Values are $p = 1.1 \times 10^{-4}$.
[g] Values are $p = 3.7 \times 10^{-3}$.

corresponding empty vector (pCAGGS) and 2 μg of pIRESpuro expression vector encoding 3xFLAG-BARD1 or the corresponding empty vector. At 18 h after transfection, the cells were replated on 100-mm dishes. All other steps were performed as above.

RESULTS

The Ongoing Chromosomal Instability of Bard1-null Tumor Cells Is Ameliorated by Exogenous BARD1—To study the contribution of BARD1 to genomic stability, we examined BARD1 function in mouse mammary tumor cell lines that lack endogenous Bard1 expression. These lines were derived from tumors that developed in mice harboring a conditional *Bard1* allele that was specifically inactivated in mammary epithelial cells by cre-mediated recombination.[3] Cytogenetic analysis of these lines, including the two used in this study (10-05 and 18-09), revealed aneuploid karyotypes exhibiting highly complex chromosomal rearrangements (see Fig. 1*A* for a spectral karyotype of the 10-05 tumor line). Giemsa-stained metaphase spreads of each line also displayed a high frequency of chromatid-type aberrations, indicative of ongoing chromosomal instability (data not shown). To determine whether Bard1 inactivation was respon-

sible for the ongoing chromosomal instability, we asked whether complementation with exogenous BARD1 would ameliorate this phenotype and correct the chromosomal instability phenotype. Therefore, isogenic subclones of the 10-05 Bard1-null tumor line were generated by stable transfection with an empty mammalian expression vector or a vector that encodes the human BARD1 polypeptide with an N-terminal FLAG epitope (FLAG-BARD1). As shown in Fig. 1*B*, expression of exogenous BARD1 resulted in a proportional increase in endogenous Brca1 levels, indicating that human BARD1 can interact with and stabilize the mouse Brca1 polypeptide. Karyotype analysis of two independently derived BARD1-complemented subclones and two empty vector control subclones revealed a marked reduction in the frequency of chromatid-type aberrations in cells reconstituted with BARD1. Thus, the percentage of metaphases with one or more of these abnormalities was much lower in the BARD1-complemented subclones (11%) than in the empty vector controls (26%) (Table 1).

To assess whether complementation with BARD1 would also reduce chromosomal instability induced by genotoxic stress, cells were exposed to the DNA cross-linking agent mitomycin C (MMC). MMC treatment resulted in increased levels of chromatid-type aberrations in both the BARD1-complemented subclones and the control subclones (Table 1). Significantly, however, the percentage of MMC-treated cells displaying abnormal metaphases was lower for the BARD1-complemented subclones (53%) than for the control subclones (70%). The difference was more pronounced when considering the number of metaphases displaying multiple aberrations; for example, although MMC-treated metaphases harboring three or more chromatid-type aberrations were rarely found in BARD1-reconstituted subclones (5%), these were clearly more prevalent in the control subclones (25%). These results indicate that the chromosomal instability phenotype of Bard1-null tumor cells is caused, at least in part, by loss of Bard1.

The Ankyrin and BRCT Motifs of BARD1 Are Necessary for Maintenance of Chromosomal Stability—We previously showed that a truncated form of BARD1 lacking the C-terminal 575 amino acids, including the three ankyrin and the two BRCT repeats, exerts a dominant-negative effect on HDR of DSBs (20). This suggests that one or both of these functional domains is important for maintenance of chromosomal stability. To assess the structural requirements for BARD1 in this process, isogenic subclones of 10-05 cells were generated by stable transformation with vectors encoding FLAG-tagged human BARD1 polypeptides that lack either the three ankyrin repeats encoded by amino acids 426–521 (BARD1-ΔAnk) or the two BRCT repeats encoded by amino acids 602–777 (BARD1-ΔBRCT) (Fig. 2*A*). As shown in Fig. 2*B*, the range of BARD1 protein expression in these subclones was comparable with that observed for the subclones complemented with full-length
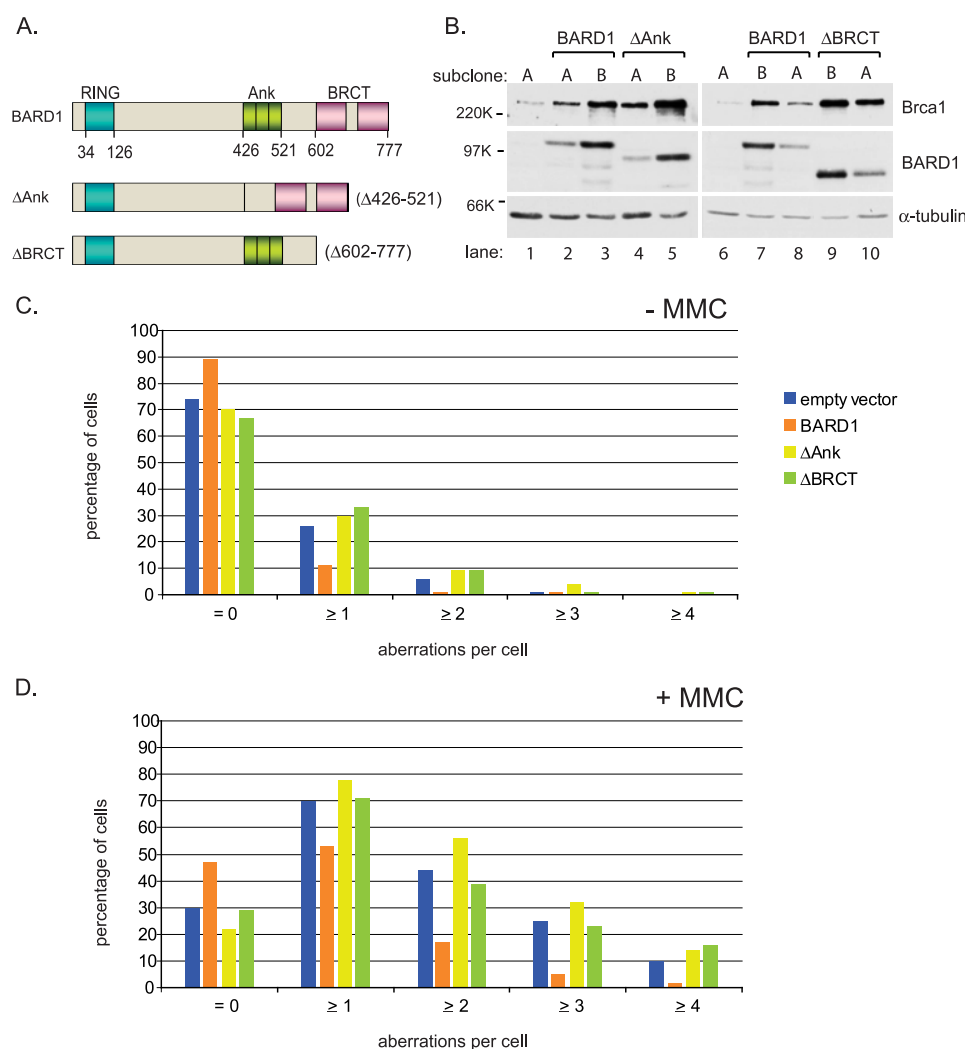
A.

B.

C.

D.

FIGURE 2. **The ankyrin and BRCT domains of BARD1 are necessary for chromosomal stability.** *A,* schematic representations of the full-length BARD1 polypeptide, the BARD1-ΔAnk polypeptide lacking the three ankyrin domains (amino acids 426–521), and the BARD1-ΔBRCT polypeptide lacking the two BRCT repeats (amino acids 602–777). *B,* Bard1-null 10-05 cells stably transformed with full-length BARD1 (*lanes 2, 3, 7,* and *8*), BARD1-ΔBRCT (*lanes 9* and *10*), BARD1-ΔAnk (*lanes 4* and *5*), or the empty expression vector (*lanes 1* and *6*) were analyzed by immunoblotting with antibodies specific for mouse Brca1, human BARD1, or α-tubulin. The endogenous levels of mouse Brca1 (*lanes 1* and *6*) are increased to a similar extent upon stable complementation with either full-length BARD1 (*lanes 2, 3, 7,* and *8*), BARD1-ΔBRCT (*lanes 9* and *10*), or BARD1-ΔAnk (*lanes 4* and *5*). *C* and *D,* subclones complemented with full-length BARD1, BARD1-ΔAnk, BARD1-ΔBRCT, or the empty expression vector were cultured in the presence or absence of MMC. One hundred metaphase spreads of each type of complemented subclone (50 each from two independent subclones shown in *B*) were analyzed for the presence of *de novo* chromatid breaks, gaps, and exchanges. *C,* the percentage of untreated cells displaying these chromatid-type aberrations. *D,* the percentage of MMC-treated cells displaying these aberrations.

BARD1. Also, expression of either BARD1-ΔAnk or BARD1-ΔBRCT increased endogenous Brca1 levels to a similar extent as full-length BARD1, indicating that the ankyrin and BRCT deletions do not impair the ability of these polypeptides to heterodimerize with and stabilize mouse Brca1.

Two subclones expressing each of the BARD1 deletion mutants, matched by expression level to the full-length BARD1-complemented subclones, were then subjected to cytogenetic analysis. In contrast to full-length BARD1, stable expression of the mutant BARD1 polypeptides failed to restore chromosomal stability (Fig. 2*C* and Table 1). Thus, the proportion of metaphases bearing spontaneous *de novo* chromatid-type aberrations in the BARD1-ΔAnk (30%) and BARD1-ΔBRCT (33%) subclones was comparable with that of the control subclones (26%) and significantly higher than that of subclones reconstituted with full-length BARD1 (11%). Whereas MMC exposure increased the levels of chromatid-type aberrations in all subclones, the proportion of abnormal metaphases was greater in the control (70%), BARD1-ΔAnk (78%), and BARD1-ΔBRCT (71%) subclones than in those reconstituted with full-length BARD1 (53%). Again, this effect was more pronounced when considering metaphases that harbor multiple aberrations; thus, the number of metaphase spreads displaying three or more chromatid-type defects in the BARD1-ΔAnk (32%) and BARD1-ΔBRCT (23%) subclones was similar to that in the control subclones (25%) and significantly greater than that in subclones expressing full-length BARD1 (5%). These data indicate that both the ankyrin and BRCT motifs of BARD1 are necessary for suppression of both spontaneous and MMC-induced chromosomal instability.

The RING, Ankyrin, and BRCT Motifs of BARD1 Are Each Necessary for Homology-directed Repair—To gain insight into the mechanism of BARD1-mediated chromosomal stability, we examined the structural requirements for BARD1 in the HDR of DSBs. HDR function can be examined *in vivo* using a DR-GFP reporter system that measures recombinational repair of an induced chromosomal DSB (27). Briefly, the reporter harbors two distinct nonfunctional copies of the GFP gene (Fig. 3*A*). The first copy (SceGFP) is disrupted by an 18-bp insertion bearing the recognition site for the rare-cutting endonuclease I-SceI, whereas the second copy (*i*GFP) encodes only an internal region of the GFP gene lacking the N- and C-terminal coding sequences. However, a functional GFP gene can be generated when a DNA break triggered by I-SceI cleavage of SceGFP is repaired by HDR using *i*GFP as the template, and such events can be accurately quantified using flow cytometry (27).

To evaluate the effect of BARD1 on HDR, we generated Bard1-null cells containing a chromosomal DR-GFP reporter by transfecting the Bard1-null mammary carcinoma line 18-09 with the linearized DR-GFPhygro construct (23). Following selection with hygromycin B, individual subclones were isolated and screened by Southern analysis to identify those pos-
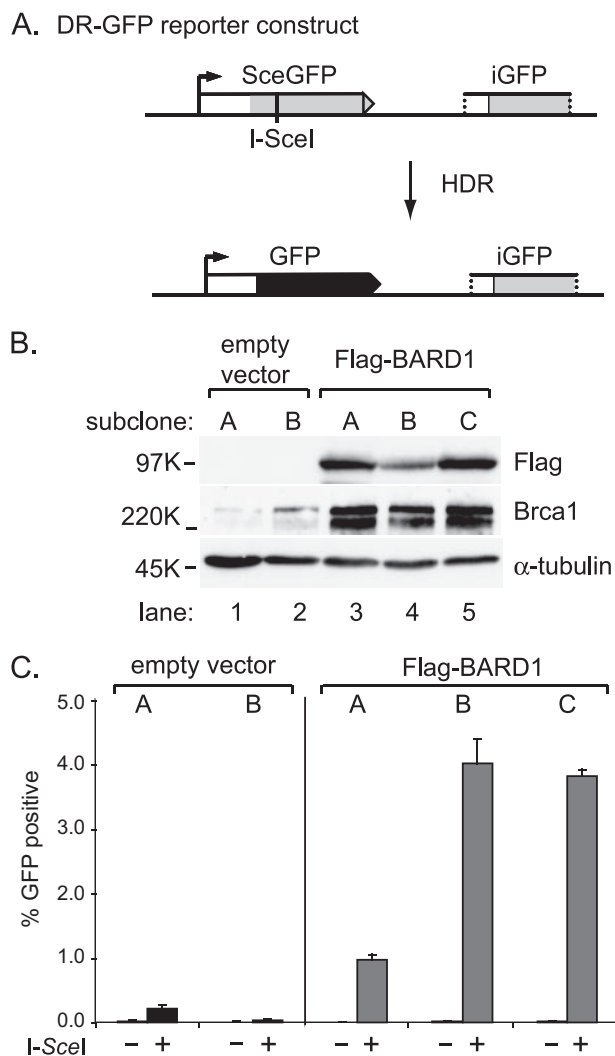
A. DR-GFP reporter construct

B.

C.

FIGURE 3. **HDR defect of Bard1-null cells can be rescued by complementation with BARD1.** *A,* schematic of the DR-GFP reporter construct used to measure HDR. The reporter contains two nonfunctional copies of the GFP gene: Sce*GFP* and *i*GFP. A functional copy of the GFP gene can be generated when an I-SceI-induced chromosomal break in Sce*GFP* is repaired by homology-directed repair using *i*GFP as the template, and the efficiency of HDR can be estimated by the percentage of GFP-positive cells (as measured by flow cytometry) (27). *B,* subclones of Bard-null DR-GFP reporter cells stably transformed with an expression vector encoding human BARD1 (*lanes 3–5*) or the empty vector (*lanes 1* and *2*) were analyzed by immunoblotting with antibodies specific for the FLAG epitope (to verify expression of the exogenous 3xFLAG-BARD1 polypeptide), mouse Brca1, and α-tubulin. *C,* HDR efficiency in clones stably complemented with exogenous BARD1. Three subclones expressing 3xFLAG-BARD1 and two empty vector controls were transfected with either I-SceI or the empty expression vector and assayed for the percentage of GFP positive cells. A representative experiment performed in triplicate is shown. The values were normalized for transfection efficiency using independent transfections with an enhanced GFP expression vector, and the *error bars* indicate S.D.

sessing a single integrated copy of the intact reporter (supplemental Fig. 1). One of the DR-GFP subclones of 18-09 (18-09/DR-GFP-2) was then transfected with an empty expression vector or the same vector encoding a FLAG-tagged full-length BARD1 polypeptide (3xFLAG-BARD1), and stably transformed subclones were selected with puromycin. As expected, BARD1 expression in the resultant subclones increased the steady-state levels of endogenous Brca1 (Fig. 3*B*). To measure HDR of an induced chromosomal DSB, the BARD1-comple-

mented and control subclones were evaluated for the appearance of GFP-positive cells at 72 h after transient transfection with an expression vector encoding the I-SceI endonuclease or the empty control vector. To control for variability in transfection efficiency among the different subclones, independent transfections with an expression vector encoding eGFP were performed in parallel and used to normalize the results. In the absence of I-SceI expression, very few GFP-positive cells were generated in either the BARD1-complemented or control subclones (0–0.01%) (Fig. 3*C*). These rare GFP-positive cells are thought to result from spontaneous gene conversion events (27). As expected, I-SceI expression increased the number of GFP-positive cells in all samples, consistent with its ability to induce HDR by producing chromosomal DSBs within the integrated DR-GFP locus. Notably, the number of GFP-positive cells generated was much greater for the BARD1-expressing subclones (0.96, 3.99, and 3.81% for subclones A, B, and C, respectively) than for the control subclones (0.21 and 0.04% for subclones A and B). This observation indicates that exogenous BARD1 expression up-regulates HDR in Bard1-null mammary carcinoma cells, consistent with our previous observation that Bard1 functions with Brca1 to promote HDR in mouse embryonic stem cells (20). Moreover, it indicates that reconstitution of Bard-null DR-GFP reporter cells can serve as an assay to investigate the structural requirements for BARD1 in HDR.

To determine whether the RING, ankyrin, or BRCT domains of BARD1 are necessary for HDR, we evaluated the ability of BARD1 polypeptides lacking these motifs to promote repair of an induced chromosomal break. For these experiments, BARD1 complementation of Bard1-null DR-GFP cells was achieved by transient transfection with BARD1 expression vectors (reporter cells of subclone 18-09/DR-GFP-4 were used in Figs. 4 and 5, but the same results were also observed with other subclones). As shown in Fig. 4*A*, when a vector encoding wild-type (*i.e.* full-length) BARD1 was transiently co-transfected with I-SceI, the measured HDR efficiency of 18-09/DR-GFP-4 cells (1.08% GFP-positive cells) was much greater than the base-line level observed when the corresponding empty vector was introduced with I-SceI (0.19% GFP-positive cells). This represents a 5.0-fold increase in HDR efficiency, which is comparable with, albeit in the lower range of the spectrum, that observed in 18-09/DR-GFP-2 cells stably complemented with wild-type BARD1 (Fig. 3*C*). In contrast, expression of BARD1-ΔAnk or BARD1-ΔBRCT along with I-SceI increased HDR by only 1.9- and 1.5-fold, respectively (0.36 and 0.30% GFP-positive cells), which is significantly lower than the increase produced by wild-type BARD1 (5.0-fold), despite the fact that the expression levels of BARD1-ΔAnk and BARD1-ΔBRCT were comparable with that of wild-type BARD1 (Fig. 4*B*). Thus, loss of either structural motif greatly impairs the ability of BARD1 to promote HDR. Likewise, a RING-only mutant that lacks both the ankyrin and BRCT sequences (B202; residues 1–202) also induced HDR to levels (1.2-fold) significantly lower that wild-type BARD1 (5.0-fold). These data indicate that the ankyrin and BRCT domains are required for the full HDR activity of BARD1 (Fig. 4).

We also evaluated the HDR function of BARD1-ΔRING, a FLAG-tagged derivative of human BARD1 that retains the

ankyrin and BRCT motifs but lacks residues 34–126, including the RING domain and the two flanking α-helices that mediate its interaction with BRCA1. When expressed in Bard1-null 18-09/DR-GFP-4 cells along with I-SceI, the BARD1-ΔRING polypeptide failed to increase HDR above the background level observed with the empty expression vector (Fig. 4). Thus, the RING domain and its flanking helices are absolutely required for any level of BARD1-mediated HDR activity.

Cancer-associated Missense Mutations Do Not Affect the HDR Function of BARD1—Missense mutations of BARD1 have been identified in sporadic cases of breast, ovarian, and uterine carcinoma, as well as in patients with familial breast or ovarian cancer (11–13). Intriguingly, all known cancer variants of BARD1 are missense mutations, and most cluster within or adjacent to the BRCT motifs. To evaluate the impact of these lesions on BARD1-mediated HDR, we examined three BARD1 missense mutations (Q564H, V695L, and S761N) that were identified in a screen of 150 sporadic tumors (11). At least two of these mutations were accompanied by tumor-specific loss of the other BARD1 allele

(Q564H and V695L) (11). The V695L and S761N mutations were not present in normal cells of the patients, indicating that they arose somatically during tumor development. The other variant, Q564H, was a germ line alteration in a patient who had developed primary carcinomas of the breast, ovary, and endometrium. Because Q564H was not found in >300 normal individuals investigated in the original study (11) and has not been reported in the cancer patients or corresponding controls of subsequent BARD1 mutation studies, it may represent a rare disease-causing germ line alteration. In addition to these rare tumor mutations, we analyzed a common polymorphic variant of BARD1 (C557S) that also targets sequences adjacent to the BRCT motifs (11, 12, 28–31). Because several studies have shown a higher frequency of the C557S allele in patients with breast cancer than in control populations, this variant of BARD1 may confer cancer susceptibility at a low penetrance (12, 28, 29, 31).

To investigate whether these mutations affect the HDR function of BARD1, we tested the ability of mutant BARD1 polypeptides to promote repair at the DR-GFP locus of Bard-null 18-09/DR-GFP-4 cells (Fig. 5). When co-expressed with I-SceI, each of the FLAG-tagged mutant BARD1 polypeptides significantly increased the number of GFP-positive cells (C557S, 1.22%; Q564H, 1.42%; V695L, 1.22%; and S761N, 1.41%) over the base-line level observed in the absence of BARD1 expression (0.19%). These increases are statistically indistinguishable from those obtained with wild-type BARD1 (1.33%), and the expression level of each mutant polypeptide was nearly identical to that of wild-type BARD1 (Fig. 5). These results suggest that the heightened cancer risk associated with the germ line BARD1 variants (C557S and Q564H) and the tumorigenicity of
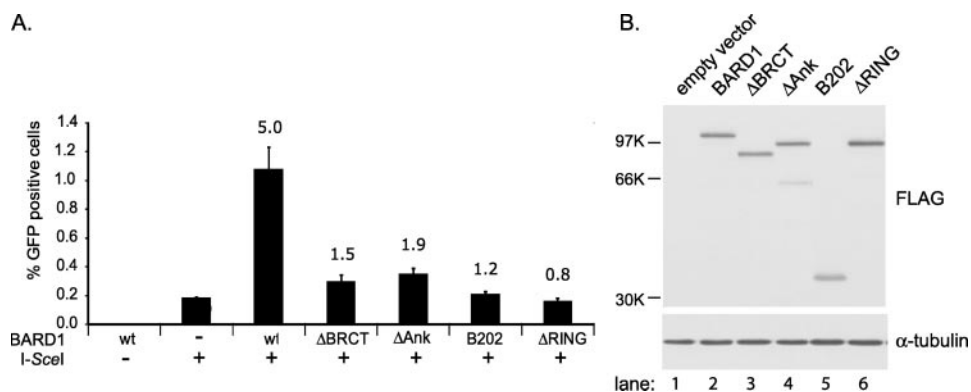
FIGURE 4. **Loss of the RING, ankyrin, or BRCT motifs decreases the efficiency of BARD1-mediated HDR.** *A*, Bard1-null DR-GFP reporter cells (subclone 18-09/DR-GFP-4) were co-transfected with I-SceI endonuclease along with a vector encoding wild-type (*i.e.* full-length) BARD1, BARD1-ΔAnk, BARD1-ΔBRCT, BARD1-B202, BARD1-ΔRING, or the corresponding empty expression vector (pIRESpuro). Flow cytometry was performed 72 h post-transfection to quantify the number of GFP-positive cells. Average values from a representative experiment performed in triplicate are shown, and the *error bars* indicate S.D. The efficiency of HDR relative to the background level observed in the samples transfected with the empty expression vector is indicated *above* each bar. *B*, verification of BARD1 protein expression in the transfected samples was performed by immunoblotting with antibodies against the FLAG epitope.
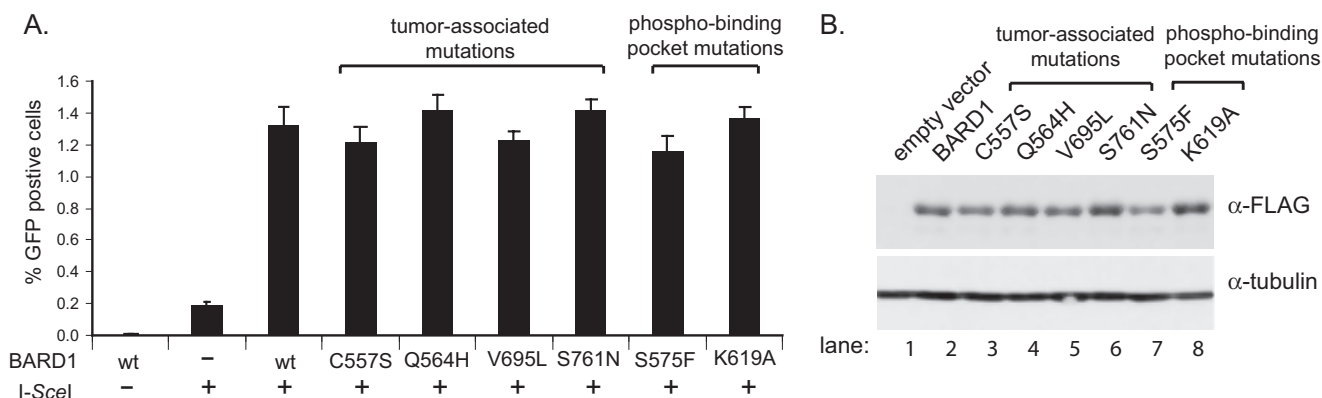

FIGURE 5. **Cancer-associated missense mutations or phospho-binding pocket mutations do not decrease the efficiency of BARD1-dependent HDR.** *A*, Bard1-null DR-GFP reporter cells (subclone 18-09/DR-GFP-4) were co-transfected with I-SceI endonuclease along with a vector encoding wild-type BARD1 or the indicated BARD1 mutant or the corresponding expression vector (pIRESpuro). Flow cytometry was performed 72 h post-transfection to quantify the number of GFP-positive cells. Average values from a representative experiment performed in triplicate are shown. The *error bars* indicate S.D. *B*, comparison of BARD1 protein expression in transfected samples.

the somatic BARD1 mutations (V695L and S761N) are unlikely to result from defects in HDR function.

The Phosphopeptide Binding Potential of the Tandem BRCT Repeats of BARD1 Is Not Essential for HDR—The results in Fig. 4 indicate that the BRCT sequences of BARD1 are required for full HDR activity. Because tandem BRCT repeats can form a phosphopeptide-binding module (14, 15, 17), we asked whether this property underlies the functional significance of this domain for BARD1-mediated HDR. Structural analyses of tandem BRCT repeats bound to their cognate phosphorylated ligands have now been performed for two BRCT proteins as follows: BRCA1 in complex with phosphopeptides derived from either BACH1 or CtIP (32–35), and MDC1 in complex with a phosphopeptide from γH2AX (36). These studies identified the structural determinants underlying phosphopeptide binding and showed that the amino acids responsible for phosphate recognition are also present in other BRCT proteins, including BARD1. Indeed, the crystal structure of the BARD1 BRCT domains harbors a phosphoserine binding pocket that is strikingly similar to those of BRCA1 and MDC1 (37). Moreover, the predicted phosphoserine contact residues in the BRCT domains of human BARD1 (Ser-575 and Lys-619) are phylogenetically conserved in its rat, mouse, and frog orthologs. To address whether the phosphopeptide binding activity of the BRCT repeats of BARD1 is important for HDR, we asked whether mutating these residues affects the ability of BARD1 to promote this type of repair. To this end we generated BARD1 expression vectors bearing either an S575F or K619A missense mutation. Mutation of the corresponding residues of BRCA1 (Ser-1655 and Lys-1702) has already been shown to disrupt BRCT-mediated binding to Ser-990-phosphorylated BACH1 (32, 34). Therefore, Bard-null carcinoma cells with a single chromosomally integrated DR-GFP reporter (18-09/DR-GFP-4) were co-transfected with I-SceI endonuclease together with an expression vector encoding either the wild-type or mutant forms of BARD1. As shown in Fig. 5, the BARD1-S575F and BARD1-K619A mutants induced HDR repair at levels (1.16 and 1.37% GFP-positive cells, respectively) indistinguishable from that obtained with wild-type BARD1 (1.33%). These results suggest that phospho-specific binding of the BRCT repeats of BARD1 to its ligands is not essential for HDR of an induced chromosomal break.

DISCUSSION

The tumor suppressor activity of BRCA1 is thought to derive, at least in part, from its ability to promote genome integrity (3–5, 38). The data presented here establish that BARD1, the heterodimeric partner of BRCA1, is required for maintenance of chromosomal stability and HDR of DSBs. Specifically, we show that complementation of Bard1-null mouse carcinoma cells with exogenous BARD1 results in suppression of both spontaneous and MMC-induced *de novo* chromatid-type aberrations. In contrast, BARD1 polypeptides lacking the three ankyrin repeats or the two C-terminal BRCT motifs fail to suppress formation of *de novo* chromatid-type abnormalities, indicating that both domains are required for maintenance of chromosomal stability by BARD1. Because chromatid-type aberrations are likely to be initiated by DSBs, these results

imply a role for the ankyrin and BRCT sequences of BARD1 in the cellular response to DSBs. A C-terminal BARD1 segment encompassing these sequences was previously implicated in homology-directed repair of DSBs, suggesting a potential mechanism by which one or both of these domains could contribute to chromosomal stability (20). Here we exploited the availability of Bard1-null mouse mammary carcinoma cell lines to develop a convenient assay to measure the HDR function of BARD1, and we have used this system to establish that the ankyrin and the BRCT motifs of BARD1 are both necessary for HDR.

At present, there a few clues as to the mechanisms by which the ankyrin repeats of BARD1 contribute to HDR or chromosomal stability. However, it may be possible to draw insights about the BRCT domains of BARD1 by considering the known functions of the corresponding motifs in BRCA1. For example, the BRCT sequences of BRCA1 are required for its recruitment to IR-induced foci (41, 42), the presumptive sites of DNA damage in cells exposed to ionizing radiation, and a similar role in IR-induced foci recruitment has also been proposed for the BRCT sequences of other repair proteins, including NBS1 and MDC1 (43, 44). In addition, the G_2/M checkpoint functions of BRCA1 are effected through phosphorylation-dependent binding of its BRCT motifs to either the BACH1 or CtIP polypeptides (15, 16). Indeed, a recent study shows that the BRCA1/BARD1 heterodimer forms at least two different protein complexes with distinct cell cycle checkpoint functions, one containing BACH1 and one containing CtIP (45).

Although BARD1 derivatives lacking either the ankyrin or BRCT sequences were clearly defective in HDR function relative to full-length BARD1, each polypeptide does restore a modest level of HDR activity to Bard1-null cells that is reproducible and statistically significant (Fig. 4). In contrast, deletion of the N-terminal RING domain completely abrogates the ability of BARD1 to rescue the repair defect of Bard-null cells. This presumably reflects the fact that the deleted region encompasses sequences of BARD1 that mediate its interaction with BRCA1 (7, 8). As such, the deletion is expected to profoundly affect both the subcellular localization of BRCA1 (42, 46) and the level of BRCA1/BARD1-associated E3 ubiquitin ligase activity (9). The importance of this enzymatic activity for tumor suppression is underscored by the fact that BRCA1 mutations that inhibit ubiquitin transfer are among the most common BRCA1 missense mutations associated with familial breast and ovarian cancer (9, 47, 48). Recently, the enzymatic activity of BRCA1/BARD1 was shown to regulate the G_2/M checkpoint by catalyzing damage-induced polyubiquitination of CtIP (49). This finding illustrates the interplay between the E3 ubiquitin ligase activity of the BRCA1/BARD1 heterodimer and the phospho-recognition function of its BRCT motifs. In light of our data, it is also possible that BRCA1/BARD1 promotes HDR by ubiquitinating other repair factors that interact with the ankyrin or BRCT sequences of BARD1. The notion that BRCA1/BARD1 coordinates the cellular response to genotoxic stress by catalyzing the ubiquitination of multiple repair factors is consistent with the observation that polyubiquitin chains accumulate at DNA repair foci in a BRCA1/BARD1-dependent manner (50, 51).

Because the BRCT sequences of BARD1 exhibit phosphopeptide binding activity (17) and form a phosphoserine binding pocket (37), we anticipate that some downstream functions of BRCA1/BARD1 may be mediated by phospho-dependent interactions of BARD1 with other repair proteins. Structural studies have identified specific BRCT residues of BRCA1 and MDC1 that bind the phosphate group of their respective phosphopeptide ligands (32–34, 44). These major contact residues are also present in human BARD1 (Ser-575 and Lys-619) and conserved phylogenetically in many BARD1 orthologs. This evidence, together with its ability to bind model phosphopeptides *in vitro* (17), suggests that the BRCT domain of BARD1 has the potential for phosphoprotein recognition. Therefore, we asked whether the same property underlies the functional requirement for the BRCT sequences of BARD1 in HDR. To interfere with phospho-recognition by BARD1, we substituted phospho-contact residue Ser-575 with a phenylalanine (S575F), thus mimicking a naturally occurring tumor-associated BRCA1 variant (S1655F), and we changed phospho-contact residue Lys-619 to alanine (K619A). Corresponding mutations in the BRCT repeats of BRCA1 not only abrogate phosphopeptide binding *in vitro* (32, 34) but also disrupt formation of IR-induced nuclear foci (32) and ablate G_2/M checkpoint activation by the BACH1 phospholigand (25). Nevertheless, neither mutation affected the ability of BARD1 to participate in HDR, suggesting that the phosphopeptide binding potential of its BRCT domains is dispensable for BARD1-dependent HDR (Fig. 5). This result is surprising in light of the fact that BARD1 polypeptides without BRCT sequences (BARD1-ΔBRCT) are clearly defective in HDR activity (Fig. 4). Perhaps the BRCT sequences of BARD1 can support distinct classes of protein-protein interactions that include not only "canonical" phospho-dependent interactions comparable with those mediated by the tandem BRCT motifs of BRCA1 (15, 16) but also noncanonical BRCT interactions that may or may not require ligand phosphorylation. Although the canonical phospho-dependent interactions of BARD1 appear to be dispensable for HDR, they are probably important for other BARD1-dependent functions, and perhaps it is these functions that are targeted by some of the tumor-associated BARD1 mutations studied here. In any case, the identification of proteins that interact with the ankyrin and the BRCT motifs of BARD1 should provide important insights into the mechanism of BARD1-mediated HDR.

Because BARD1 appears to function as a tumor suppressor, it is intriguing to consider whether the HDR function of BARD1 is responsible, at least in part, for its tumor suppression activity and whether disease-associated lesions of BARD1 affect HDR function. Surprisingly, however, none of the four tumor-associated mutations tested here had an appreciable effect on the HDR activity of BARD1 (Fig. 5). It is possible that some of the BARD1 variants analyzed here do not represent true cancer-causing alterations. In particular, there have been conflicting reports as to whether the common germ line variant C557S represents a benign polymorphism or a deleterious allele based on epidemiological data collected from distinct, but ethnically related, populations (28, 30). However, a large collaborative analysis of 2906 breast and ovarian patients and 3591 controls of Nordic ancestry revealed that the C557S variant is more frequently observed in familial breast cancer patients with no known mutations in the BRCA1/BRCA2 tumor suppressors (6.8%) than in healthy controls (2.8%) or BRCA1/2 mutation-positive families (2.2%), supporting the contention that this mutation does confer increased cancer risk (31). In addition, because the S761N alteration originally identified as a somatic mutation accompanied by loss-of-heterozygosity in an endometrial carcinosarcoma (11) was subsequently observed in a breast tumor with no evidence of loss-of-heterozygosity (13), we cannot rule out the possibility that S761N represents a nonconsequential genetic change. Nevertheless, when BARD1 missense alterations were evaluated using growth-suppressive and apoptotic properties of ectopic BARD1 expression as a surrogate assay, all four of the mutations we examined (C557S, Q564H, S761N, and V695L) exhibited altered activity and, as such, were designated as deleterious BARD1 variants (13). In any case, a possible interpretation of our data is that these BARD1 mutations represent separation-of-function alleles that leave the HDR function of the BRCA1/BARD1 heterodimer intact but disrupt a distinct aspect of its tumor suppression activity. Indeed, at least one of these mutations (Q564H) is known to disrupt the interaction of BRCA1/BARD1 with the 50-kDa subunit of the mRNA polyadenylation factor CstF, leading to de-repression of mRNA processing following exposure to genotoxic stress (39). Because BRCA1/BARD1 is also thought to promote genome stability and tumor suppression through its involvement in other cellular processes, such as mitotic spindle assembly (40) and cell cycle checkpoint control (3), further studies of the tumor-associated BARD1 mutations should address their effect on these aspects of BRCA1/BARD1 function.

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