

Identification of Domains of BRCA1 Critical for the Ubiquitin-Dependent Inhibition of Centrosome Function

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Abstract

The breast and ovarian cancer specific tumor suppressor BRCA1, bound to BARD1, has multiple functions aimed at maintaining genomic stability in the cell. We have shown earlier that the BRCA1/BARD1 E3 ubiquitin ligase activity regulates centrosome-dependent microtubule nucleation. In this study, we tested which domains of BRCA1 and BARD1 were required to control the centrosome function. In the present study, (a) we confirmed that the ubiquitination activity of BRCA1 regulates centrosome number and function in Hs578T breast cancer cells; (b) we observed that both the amino and carboxyl termini of BRCA1 are required for regulation of centrosome function *in vitro*; (c) an internal domain (770-1,290) is dispensable for centrosome regulation; (d) BARD1 is required for regulation of centrosome function and protein sequences within the terminal 485 amino acids are necessary for activity; and (e) BARD1 is localized at the centrosome throughout the cell cycle. We conclude that the BRCA1-dependent E3 ubiquitin ligase functions to restrain centrosomes in mammary cells, and loss of BRCA1 in the precancerous breast cell leads to centrosomal hypertrophy, a phenotype commonly observed in incipient breast cancer. (Cancer Res 2006; 66(8): 4100-7)

Introduction

The breast and ovarian cancer specific tumor suppressor BRCA1 is a multifunctional phosphoprotein that maintains genomic stability in the cell by participating in cell cycle regulation, DNA repair, and transcription (1). Composed of 1,863 amino acid residues, the amino-terminal 109 residues of BRCA1 contain a RING domain, a motif that mediates ubiquitin ligation, initially shown using the isolated RING domain (2). Subsequently, it was found that BRCA1 ubiquitin ligase activity is markedly enhanced when it is in complex with another RING finger protein, BARD1 (3, 4).

The only other domain of BRCA1 with a recognizable motif is the carboxyl terminus, which encodes two tandem BRCT repeats (BRCA1 carboxyl-terminal domain) found in proteins involved in DNA binding and repair (5). The BRCT domain has been shown to mediate the transcription function of BRCA1 (6, 7) and is a phosphopeptide binding domain (8–10) that may be involved in signal cascades in response to binding to phosphorylated proteins. Apart from these two conserved domains, the central region of BRCA1 encompassing ~1,500 residues does not have any identifiable domains.

Mutations in BRCA1 have been found in ~40% of familial breast cancer cases and in 80% of both familial breast and ovarian cancers (11–13). Most cancer-predisposing mutations have been mapped either to the RING or the BRCT domain, indicating the importance of these two domains in the tumor suppressor function of BRCA1. Cancer-predisposing mutations within the RING domain of BRCA1 have been shown to correlate with the lack of ubiquitin ligase function of BRCA1 (14). Cancer-associated mutations, and not benign polymorphisms, in the BRCT domain of BRCA1 lead to loss of its transcriptional activation function (15) and loss of binding to RNA polymerase II (16). Mutations in the BRCT domains of BRCA1 can also prevent it from being transported to the nucleus for DNA damage repair (17).

BARD1 encodes a 777-residue protein and is structurally similar to BRCA1, in that it has a RING domain (residues 46-90) at the amino terminus and two tandem BRCT repeats (residues 616-653 and 743-777) at the carboxyl terminus. BARD1 interacts with BRCA1 through the RING domains of the two proteins, although there is evidence from our lab that these two proteins have additional stabilizing interactions in domains distal to the RING fingers (18). Unlike BRCA1, BARD1 mutations in breast cancer are much less frequent (19, 20). Most of the functions of BARD1 are associated with BRCA1. Independent functions of BARD1 involve its roles in S-phase progression, contact inhibition nuclear division (21), and apoptotic response to genotoxic stress (22).

In addition to its multiple nuclear functions, BRCA1 has been shown to regulate centrosome function and duplication (23, 24). Centrosomes are the microtubule nucleation centers of the cell. Microtubules are an important component of cell cytoskeleton affecting the basic functions of the cell: shape and polarity, intracellular trafficking, motility, and cell division. Centrosome abnormalities are common in many tumors including breast cancer. Centrosomes in tumor cells exhibit hyperactivity; i.e., they are larger and nucleate more microtubules than their normal counterparts (25) and these centrosomes undergo numerical amplification leading to abnormal mitoses and defective chromosomal segregation (26–28). In breast cells, the regulation of centrosome number and function depends on BRCA1 (23, 24). We have shown that BRCA1 can ubiquitinate centrosomal proteins and this covalent modification renders the centrosome less active. We identified γ -tubulin as one of the centrosomal targets ubiquitinated by BRCA1. γ -Tubulin is a critical component of the γ -tubulin ring complex, which initiates microtubule nucleation.

In this study, we expanded prior results by showing that expression of a stable but enzymatically inert mutant of the BRCA1 E3 ubiquitin ligase resulted in the same phenotype as BRCA1 knockdown in cells: centrosome amplification and hyperactivity. We then mapped, using *in vitro* assays, the domains of BRCA1 and BARD1, which are necessary to inhibit the microtubule nucleation function of purified centrosomes. We reveal that both full-length BRCA1 and BARD1 are required to regulate centrosome

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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microtubule nucleation activity although the truncated versions of BRCA1 and BARD1 were active ubiquitin ligases on another substrate. The requirement for extra domains of BRCA1 and BARD1, besides their respective RING domains, indicates that the ubiquitination of centrosomes by the E3 ubiquitin ligase activity of BRCA1/BARD1 is specific and dependent on multiple interaction domains. Because our data showed that BARD1 was required along with BRCA1 to regulate the centrosomes, we tested whether BARD1 localized to centrosomes. We found that endogenous BARD1 protein is present at the centrosomes throughout the cell cycle. These findings reveal the functional domains of BRCA1 and BARD1 in centrosome regulation.

Materials and Methods

Plasmids. BRCA1 (I26A) was made from the wild-type (wt) plasmid in pcDNA3 (16) by using the Stratagene Quick Change Kit (Stratagene, La Jolla, CA), incorporating the substituted amino acid using the primer sequence 5'-CTTAGAGTGTCCCGCTGTCTGGAGTTGATCAAGG-3' primer. Green fluorescent protein (GFP)-centrin was a kind gift from Dr. Michel Bornens (Institut Curie/UMR 144 du Centre National de la Recherche Scientifique, Paris, France). BRCA1-Flag (full-length and all deletion constructs) and BARD1 baculoviruses were constructed according to FastBac system (Invitrogen, Carlsbad, CA) as previously described (23).

Antibodies. Anti- γ -tubulin and anti- α -tubulin (Sigma, St. Louis, MO) were used at 1:1,000 dilution for both immunoblots as well as immunofluorescence. Anti-BRCA1 (Ab-1; Oncogene, San Diego, CA) was used for immunoblots at 1:100 dilution. BRCA1 and ubiquitin antibodies were raised in rabbit and used at 1:1,000 for immunoblots. Anti-BARD1 antibody was kindly provided by Dr. J. Harb (Institut National de la Sante et de la Recherche Medicale, Nantes, France).

Cell culture and transfection. Hs578T (ATCC cell line HTB-126) and HeLa S3 cells were cultured according to American Type Culture Collection recommendations. Transfections with various plasmid DNAs were carried out using Lipofectamine 2000 (Invitrogen) and protein expression and phenotypic changes were observed 2 days posttransfection unless mentioned otherwise. The GFP-centrin-encoding plasmid was cotransfected to mark transfected cell centrosomes. Small interfering RNA (siRNA) transfection was done using oligofectamine (Invitrogen). siRNA oligo targeting BRCA1 was designed from the 3' untranslated region (UTR) as described earlier (24). The control GL2 oligonucleotide targets luciferase mRNA.

Microtubule regrowth assay. Cells were treated for 40 minutes with chilled medium containing 25 μ M nocodazole to depolymerize microtubules and then asters in cells were allowed to regrow in warm medium without nocodazole for 2 to 15 minutes, as described earlier (24).

Immunofluorescence. Cells were treated with 200 μ g/mL saponin at room temperature for 2 minutes and fixed in cold methanol before staining with primary antibodies diluted 1:1,000 in 1 \times PBS containing 3% bovine serum albumin and 0.1% Triton X-100.

For BARD1 staining, cells were fixed in methanol and the primary antibody was used at 1:100 dilution. Cells were washed with PBS + 0.1% Triton X-100 and stained with secondary antibodies. Images were viewed using the 100 \times objective lens with a Nikon Eclipse TE2000-S microscope and captured using a model 2.3.1 SPOT digital camera. Images were processed using advanced SPOT software.

Protein purification. The BRCA1 and BARD1 baculoviruses were coinfecting in SF9 or Hi-Five insect cells using standard protocols and purified using M2-agarose (Sigma) as described earlier (23). Protein concentrations were determined after SDS-PAGE analysis by comparing the intensities of Coomassie-stained polypeptides of BRCA1 and BARD1 with known protein standards.

Centrosome fractions were prepared from HeLa S3 cells according to previously described protocol (29). *Xenopus* extract was prepared as described earlier (30, 31).

In vitro ubiquitination and microtubule nucleation assays. Reactions contained 0.5 \times BRB80 (80 mmol/L K-PIPES, 1 mmol/L MgCl₂, 1 mmol/L

EGTA), 2 mmol/L ATP, 4 mmol/L MgCl₂, 1 mmol/L ubiquitin, 200 nmol/L E1, 5 μ M UbcH5c-his, and 5 to 60 nmol/L BRCA1/BARD1. Centrosome fraction (~500-1,000 ng) was added to the reaction and incubated at 37 $^{\circ}$ C for 30 minutes. The reactions were transferred to ice and 10 μ L of *Xenopus* extract (20 mg/mL) were added and incubated at 23 $^{\circ}$ C for 20 minutes to allow aster formation. The asters were fixed with 1% glutaraldehyde and spun through a 40% glycerol cushion onto glass coverslips precoated with poly-L-lysine (<http://mitchison.med.harvard.edu/protocols/spindown.html>). The asters were then fixed with cold methanol and visualized by immunostaining with anti- α -tubulin and anti- γ -tubulin antibodies as described in Materials and Methods (Immunofluorescence).

For densitometric quantification of the microtubule content of asters, asters were photographed at \times 1,000 magnification, all using the same exposure variables. Using the histogram tool in Adobe Photoshop, the microtubule content of the asters was measured as the product of the mean intensity and the number of pixels. The average values (\pm SE) were plotted for 20 asters from each condition.

Results

An active BRCA1 E3 ubiquitin ligase is required to regulate centrosomes in cells. Reducing BRCA1 protein levels by transfecting cells with BRCA1-specific siRNA caused centrosome hyperactivity (24). To study if the hyperactivity of centrosomes could be corrected by providing exogenous BRCA1, Hs578T breast cancer cells were transfected with a plasmid encoding wt BRCA1 or vector alone. A GFP-centrin plasmid was cotransfected to mark the centrosomes of transfected cells. Twenty-four hours later, these cells were transfected with either the control siRNA or the siRNA designed to target the 3'UTR of BRCA1 (BRCA1c; ref. 24). The transfected cells were subjected to microtubule regrowth assay (described in ref. 24) in which microtubules were transiently depolymerized by treating cells with nocodazole. Following replacement of medium to remove the nocodazole, microtubules regrew for 2 minutes, followed by fixing and staining for α -tubulin (to mark microtubules). Cells were examined by fluorescence microscopy and scored for the percentage cells with large asters or those containing supernumerary (more than two) centrosomes. Representative examples of cells containing small (Fig. 1Aa) or large asters (Fig. 1Ab), two centrosomes (Fig. 1Ac), or more than two centrosomes (Fig. 1Ad) are shown. Knockdown of BRCA1 expression resulted in a 3-fold increase in the fraction of cells with large asters (Fig. 1B, top) and a 2-fold increase in the fraction of cells with more than two centrosomes (Fig. 1B, bottom) as compared with control siRNA-transfected cells. On expression of exogenous BRCA1, which is resistant to the 3'UTR-specific siRNA, the centrosome microtubule nucleation activity and centrosome number in cells transfected with BRCA1 siRNA were comparable to those in the control cells (Fig. 1B). To test the levels of BRCA1 protein, lysates of cells transfected with siRNAs and plasmids, as described above, were probed with BRCA1-specific antibody. BRCA1 levels in the control siRNA- and vector-transfected cells (Fig. 1C, lane 2) were identical to those in cells transfected with neither (Fig. 1C, lane 1). BRCA1 siRNA transfection significantly decreased the levels of the protein (Fig. 1C, lane 3). Expression of exogenous BRCA1 increased the levels of the protein (Fig. 1C, lanes 4 and 5). These results indicated that the effects of siRNA transfection on centrosome number and function in Hs578T cells were indeed due to BRCA1 protein levels and not due to an unknown off-target protein.

We tested whether expression of a stable, yet enzymatically inert, BRCA1 E3 ubiquitin ligase would affect centrosome phenotype. Hs578T cells were transfected with increasing amounts of BRCA1-encoding plasmid or a plasmid encoding a mutant of BRCA1 (I26A)

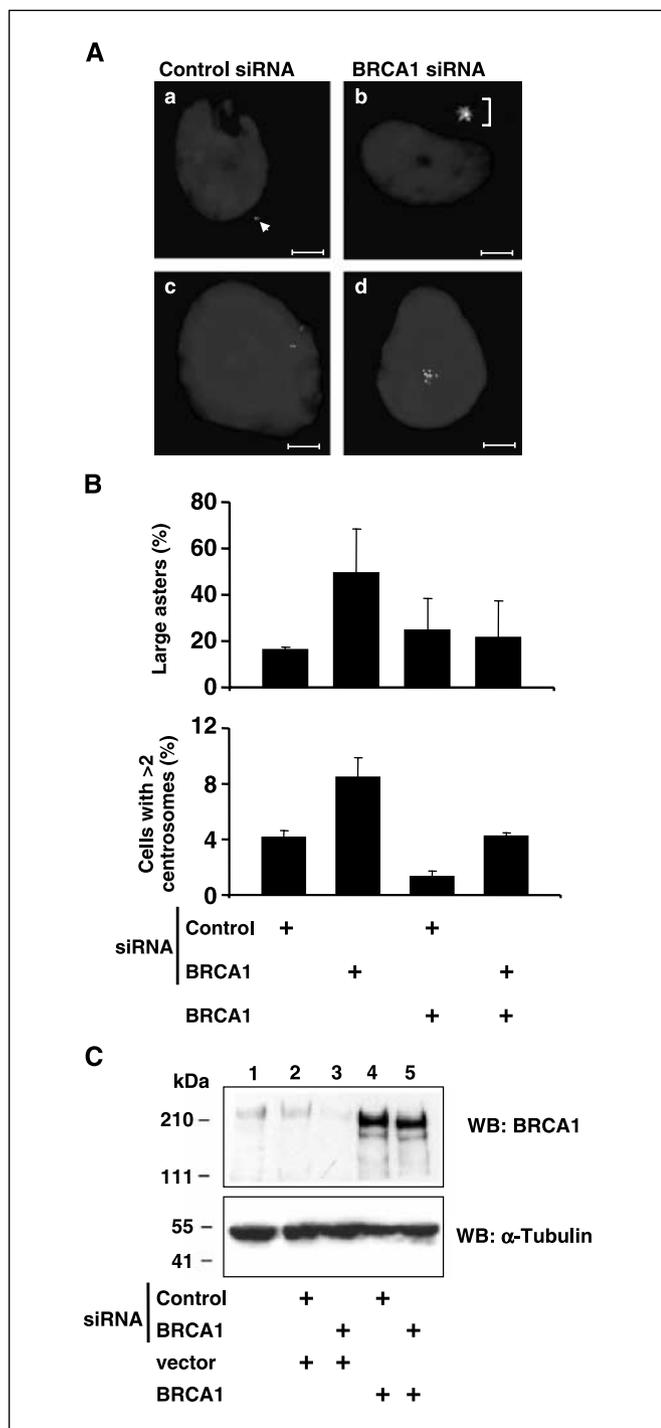


Figure 1. BRCA1 protein is required for *in vivo* regulation of centrosome function and number. *A*, Hs578T cells were transfected with either the control siRNA or BRCA1 3'UTR-specific siRNA with or without a cotransfected plasmid expressing BRCA1. A GFP-centrin-expressing plasmid was included to mark transfected cell centrosomes. Forty-eight hours posttransfection, cells were subjected to the microtubule regrowth assay. Microtubules were detected by staining for α -tubulin. *A*, *a* to *d*, bar, 10 μ m. *B*, the percentage of cells with either small asters (*Aa*) or large asters (*Ab*) was scored for 100 to 150 cells. Histogram of the percentage of cells with large asters (*top*) and, taken from the same slide, histogram of the percentage of cells with more than two centrosomes (*bottom*). *Columns*, mean of two independent experiments; *bars*, SE. *C*, immunoblots of total cell lysates from cells transfected with the control siRNA (*lanes 2* and *4*), BRCA1 siRNA (*lanes 3* and *5*), 4 μ g of either vector (*lanes 2* and *3*) or plasmid encoding BRCA1 (*lanes 4* and *5*), and no siRNA and plasmid DNA (*lane 1*) were probed for BRCA1. α -Tubulin levels served as loading control.

that lacked the ubiquitin ligase activity (32). A GFP-centrin expression plasmid was used to mark transfected cell centrosomes. Interestingly, expression of BRCA1 (I26A) profoundly affected the number of cells with large asters. Vector-transfected (4 μ g pcDNA3 vector, 0 μ g pcDNA3-BRCA1 plasmid) and wt BRCA1-transfected cells all had ~20% to 25% of the cells with large asters. By contrast, transfection with BRCA1 (I26A) caused about half of the cells to have hyperactive asters (Fig. 2*A*). When we scored in the same experiment for cells with more than two centrosomes, BRCA1 (I26A) transfection caused a 6-fold increase in centrosome amplification as compared with cells transfected with wt BRCA1 (Fig. 2*B*). The amounts of BRCA1 wt or I26A protein in lysates for the same amount of plasmid used for transfections were comparable, as seen by Western blot analysis (Fig. 2*C*). These results clearly indicate that the BRCA1 E3 ubiquitin ligase activity is important for maintaining centrosome number and function in Hs578T cells growing in tissue culture.

The role of the BRCA1 carboxyl terminus in centrosome regulation. We investigated whether other domains of BRCA1, besides the RING domain, are important for the centrosome regulation function of BRCA1. To avoid complications resulting

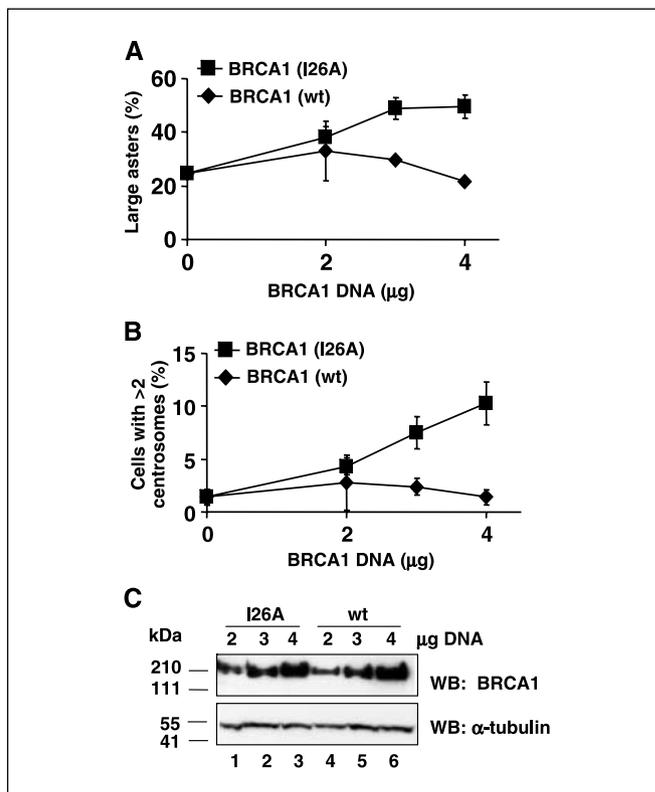


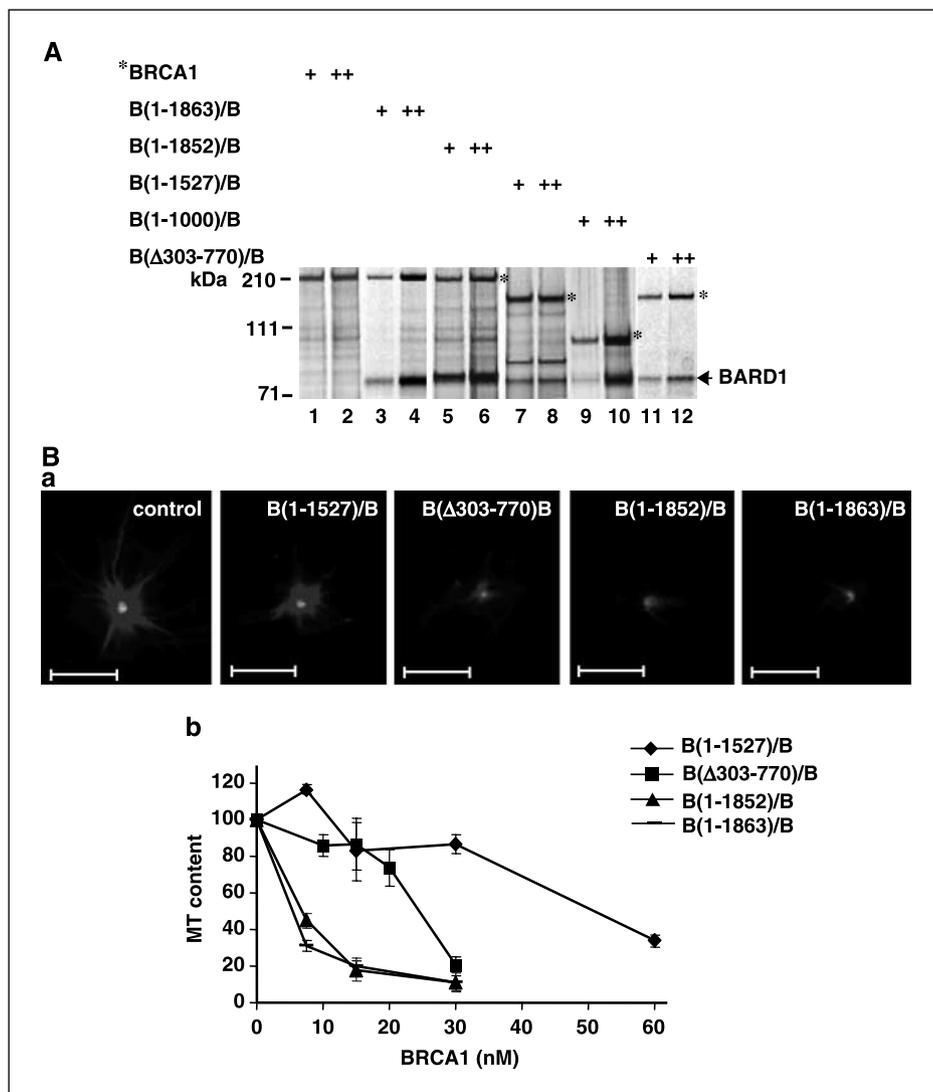
Figure 2. BRCA1-dependent ubiquitin ligase activity is required for *in vivo* regulation of centrosome function and duplication. *A*, Hs578T cells were transfected with 2, 3, or 4 μ g of plasmid expressing either BRCA1 (wt) or enzymatically inert BRCA1 (I26A). GFP-centrin was used as a transfection and centrosome marker as in Fig. 1. Forty-eight hours posttransfection, cells were subjected to the microtubule regrowth assay and microtubules were detected by staining for α -tubulin as above. The percentage of cells containing large asters was scored for 100 to 150 cells. *Points*, mean of two independent experiments; *bars*, SE. *B*, using the same cells as in (*A*), the percentage of cells with more than two centrosomes was scored. *C*, immunoblots of total cell lysates from cells transfected with the indicated quantity of plasmid expressing either BRCA1 (wt) (*lanes 4-6*) or BRCA1 (I26A) (*lanes 1-3*) were probed for BRCA1 and also for α -tubulin levels as loading control.

from unequal expression of deletion mutants *in vivo*, we tested these mutant proteins *in vitro* in reactions where the concentration of a given protein is rigorously controlled. We purified various deletions of Flag-tagged BRCA1 in heterodimeric complex with BARD1 from insect cells. Full-length Flag-tagged BRCA1(1-1,863), BRCA1(1-1,852), BRCA1(1-1,527), BRCA1(1-1,000), BRCA1(Δ 303-770), and BRCA1(Δ 770-1,290) were each coexpressed with untagged BARD1 and purified via the epitope tag. These protein preparations were balanced for their BRCA1 content (Figs. 3A and 4A) and used in the *in vitro* aster formation assay (described in ref. 24). Centrosomes purified from HeLa S3 cells were incubated in the presence of varying quantities of the different BRCA1 deletions in complex with BARD1, along with ubiquitin activating enzyme (E1), conjugating enzyme (UbcH5c/E2), and ubiquitin. The ubiquitinated centrosomes were then assayed for aster formation function by incubation with *Xenopus* extract. The asters were fixed and stained for microtubule (using an anti- α -tubulin antibody) and centrosomes (using anti- γ -tubulin antibody) and the microtubule content of 20 randomly selected asters was quantitated in each reaction. Representative asters from the control reaction (without any BRCA1) and reactions

containing 30 nmol/L of the various BRCA1/BARD1 preparations are shown in Fig. 3Ba.

All carboxyl-terminal deletion preparations of BRCA1 in association with BARD1 had approximately equal *in vitro* ubiquitin ligase activities when using the phosphorylated RNA polymerase II as a substrate (23) or in polymerizing ubiquitin chains (data not shown), but the following results indicate that deletions in BRCA1 affect the regulation of the centrosome. BRCA1(1-1,852)/BARD1 lacks 11 amino acids from the carboxyl terminus and was as effective as the full-length protein in inhibiting centrosome function (Fig. 3Bb). BRCA1(1-1,527)/BARD1, lacking the carboxyl-terminal 336 amino acids, was 4-fold less effective compared with the full-length protein at 30 nmol/L, although at 60 nmol/L BRCA1(1-1,527)/BARD1, the inhibition of aster formation was reduced to 40% of the control reaction (Fig. 3Bb). At 60 nmol/L concentration of BRCA1(1-1,000)/BARD1, the centrosome aster microtubule content was reduced to 60% of the control reaction (data not shown). This level of inhibition was similar to that observed with BRCA1(1-500)/BARD1 at the same concentration of enzyme (24), and we judge this low level of inhibition to be nonspecific. Taken together, these results clearly indicate that the

Figure 3. Domains of BRCA1 critical for inhibiting centrosome function *in vitro*. **A**, BRCA1 protein lacking different regions of the protein as indicated were coexpressed with BARD1 in insect cells and purified via the 5'-Flag tag present on BRCA1. Silver-stained gels containing either 0.5 (+) or 1 (++) pmol of the various protein preparations as indicated. The BRCA1 band was marked with an asterisk. **B**, all of the protein preparations were normalized for BRCA1 content and used in an *in vitro* microtubule nucleation assay using centrosomes purified from HeLa S3 cells. Purified centrosomes were ubiquitinated with E1, E2 (UbcH5c), and varying concentrations of BRCA1/BARD1 (B/B) protein preparations. 7.5, 15, and 30 nmol/L proteins were used for B(1-1,863)/B, B(1-1,852)/B, and B(1-1,527)/B, and 10, 15, 20, and 30 nmol/L proteins were used for B(Δ 303-770)/B. The ubiquitinated centrosomes were allowed to grow microtubule by addition of *Xenopus* extract, and the resulting asters were centrifuged onto coverslips and stained for γ -tubulin and α -tubulin to mark centrosomes and microtubule, respectively. Although two colors were used in immunofluorescence microscopy, these black and white images faithfully show the centrosomes as bright centers in the microtubule asters. **a**, representative fields containing asters from reactions containing 30 nmol/L of the different BRCA1 deletions; bar, 10 μ m. Twenty asters were photographed under same exposure and the microtubule content determined. The mean values obtained for each condition were normalized with the control reaction containing centrosomes and ubiquitination factors but no BRCA1. **b**, points, normalized mean for each condition (expressed as microtubule content); bars, SE.



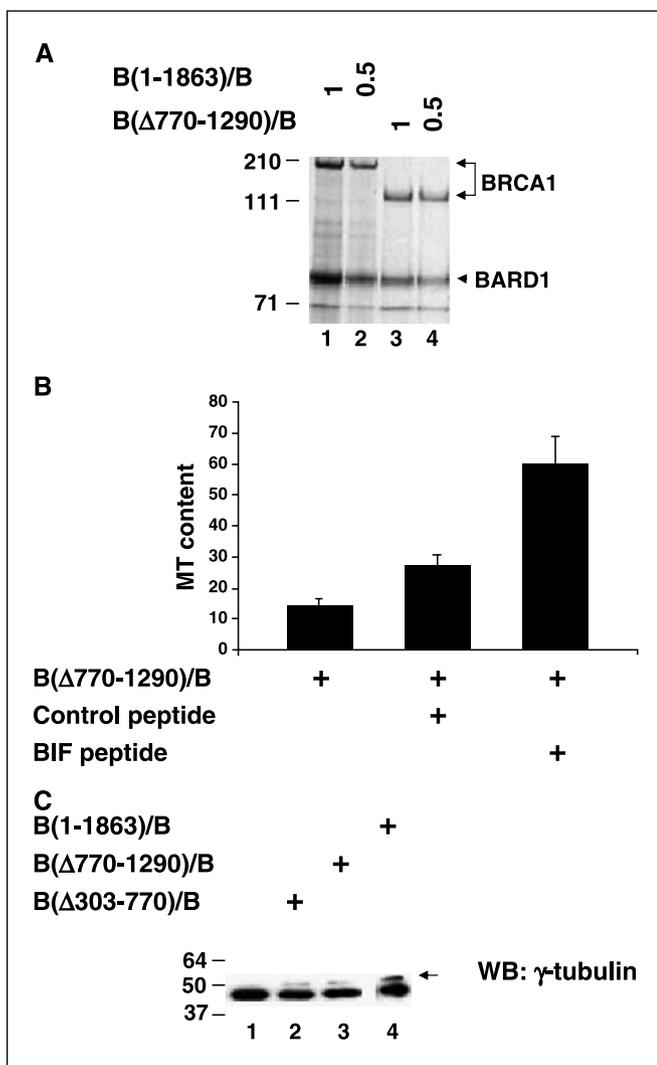


Figure 4. An internal domain of BRCA1 is dispensable for centrosome regulatory function. *A*, BRCA1(Δ 770-1,290)/BARD1 was coexpressed with BARD1 in insect cells and copurified. 0.5 pmol (lane 4) and 1 pmol (lane 3) of this protein were loaded along with 0.5 pmol (lane 2) and 1 pmol (lane 1) BRCA1(1-1,863)/BARD1 and detected by silver staining. *B*, 7.5 nmol/L BRCA1(Δ 770-1,290)/BARD1 was tested in the *in vitro* microtubule nucleation assay with purified centrosomes, E1, E2, and ubiquitin. The BRCA1 inhibitory fragment or the control peptide, at 3.5 μ mol/L, was added to the reaction as indicated. The normalized microtubule content for 20 asters was calculated and plotted for each reaction condition. *C*, centrosomes purified from HeLa cells were incubated with ubiquitination factors and different BRCA1/BARD1 proteins at 30 nmol/L concentration, and proteins were analyzed by immunoblot analysis using a monoclonal antibody specific for human γ -tubulin. Arrow, ubiquitinated γ -tubulin.

carboxyl-terminal 336 amino acids of BRCA1 are important for the inhibition of centrosome function.

Internal domains of BRCA1 and the regulation of centrosome function. BRCA1 has been shown to bind γ -tubulin via amino acid residues 504-803 and the amino-terminal portion of this peptide fragment (residues 510-622) was required for binding to γ -tubulin (33). We tested whether this reported γ -tubulin binding domain affected centrosome function. We made two internal deletions of BRCA1, BRCA1(Δ 303-770) that removes the required portion of the γ -tubulin binding domain and BRCA1(Δ 770-1,290) that deletes the region downstream to the γ -tubulin binding domain. We expressed these internal deletion

mutants along with BARD1, as above, and purified the mutant BRCA1/BARD1 heterodimers.

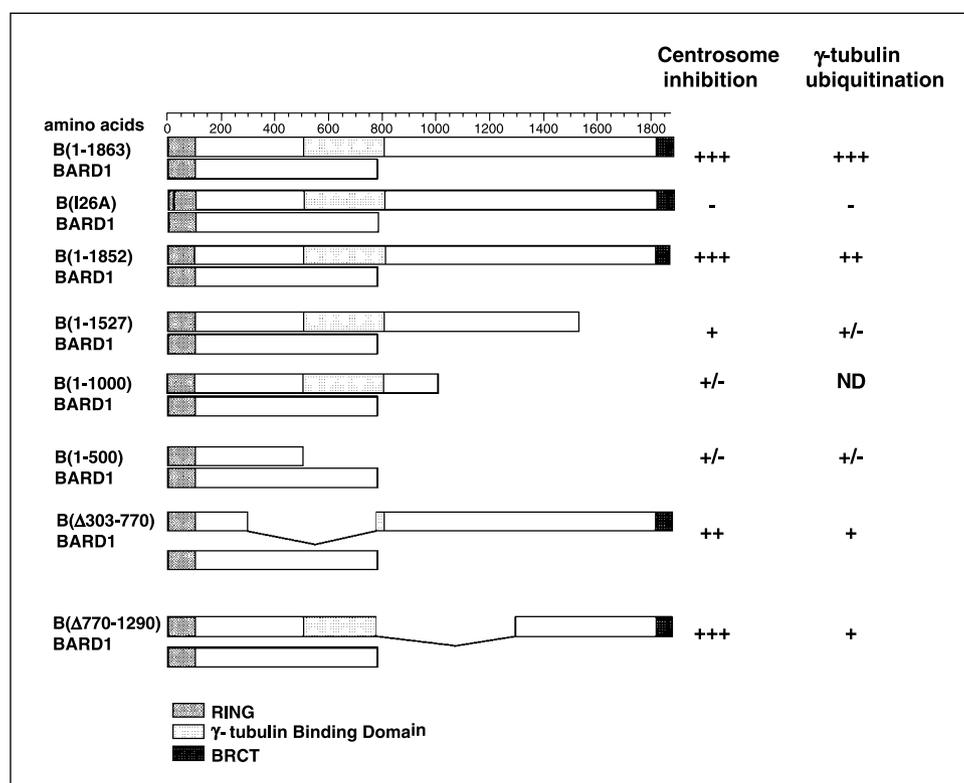
BRCA1(Δ 303-770)/BARD1 had only a marginal effect at concentrations lower than 20 nmol/L, but at 30 nmol/L BRCA1(Δ 303-770)/BARD1, inhibition of aster formation was as effective as observed with the full-length protein (Fig. 3*Bb*). This result indicates that the reported γ -tubulin binding domain is stimulatory, but not essential, for BRCA1/BARD1 regulation of centrosomes.

At low concentration (7.5 nmol/L), BRCA1(Δ 770-1,290)/BARD1 caused an 85% reduction in microtubule content compared with control (Fig. 4*B*). At higher concentrations, this BRCA1(Δ 770-1,290)/BARD1 apparently aggregated (as observed from two independent preparations; data not shown), but this deletion was clearly as active as the wt protein. The effect of this internal deletion mutant was dependent on the presence of ubiquitin in the reaction (data not shown), emphasizing the role of the E3 ligase activity in causing the inhibition to centrosome activity. To reconfirm that the inhibition was specific, we included in reactions a peptide fragment of RNA helicase A, which we call the BRCA1 inhibitory fragment. BRCA1 inhibitory fragment is a fragment of the RNA helicase A protein (residues 89-344) that interacts with BRCA1 and functionally inhibits it (34). This polypeptide was shown earlier to inhibit the BRCA1/BARD1-mediated ubiquitination of γ -tubulin (23) and to reverse the inhibition of centrosome aster formation *in vitro* by BRCA1/BARD1 (24). The control peptide contains an overlapping fragment of RNA helicase A (residues 1-250) that lacks the BRCA1 binding domain. Inclusion in reactions of BRCA1 inhibitory fragment polypeptide blocked the inhibition of centrosome function by BRCA1(Δ 770-1,290)/BARD1. Inclusion in reactions of the control polypeptide only had a minor effect on aster formation. Thus, the decrease in microtubule content of asters by BRCA1(Δ 770-1,290)/BARD1, along with other ubiquitination factors, was specific and was partially reversed by adding BRCA1 inhibitory fragment polypeptide to the reaction.

From these results, we concluded that sequences of BRCA1 from residues 770-1,290 were dispensable with regard to centrosome regulation, but these residues contributed to the overall solution stability of BRCA1. Results for the various BRCA1 deletion proteins are summarized in Fig. 5. Only full-length (residues 1-1,863), an 11-amino-acid truncation (residues 1-1,852), and the 520-amino-acid internal deletion (Δ 770-1,290) had maximum inhibition of centrosome function. With the exception of the enzymatically inert I26A mutation, all of these proteins had approximately equal nonspecific ubiquitin ligase activity (ref. 35 and data not shown). We compared all of these protein preparations for the specific monoubiquitination of γ -tubulin. In an earlier study (23), we had shown that at 15 nmol/L BRCA1/BARD1, only full-length BRCA1/BARD1 had high γ -tubulin ubiquitination activity, and the BRCA1(1-1,852)/BARD1 protein had lower levels of this specific activity. At higher concentrations of protein, very low levels of ubiquitination of γ -tubulin were detectable by all of these deletion mutants (ref. 24 and data not shown). The two internal deletions of BRCA1 also weakly modified γ -tubulin (at 30 nmol/L; Fig. 4*C*, lanes 2 and 3). The results of centrosome function inhibition roughly correlate with the extent of monoubiquitination of γ -tubulin for each protein. These results are consistent with a key role of γ -tubulin modification in this reaction, but certainly, other unknown substrates within the centrosome may also be important.

The carboxyl-terminal 485 amino acids of BARD1 are required for inhibition of centrosome function by BRCA1. Because all the BRCA1 protein preparations tested in the *in vitro*

Figure 5. BRCA1 domains required for efficient inhibition of centrosome function and for ubiquitination of γ -tubulin. Data are summarized in this schematic of different BRCA1 constructs used in the study in association with BARD1. Effects of the different proteins on inhibition of centrosome microtubule nucleation and on γ -tubulin monoubiquitination. With regard to centrosome inhibition: +++, 70% inhibition at BRCA1/BARD1 concentrations of 15 nmol/L or less; ++, >70% inhibition at 30 nmol/L; +, >50% inhibition at 60 nmol/L; +/-, <50% inhibition at 60 nmol/L. With regard to γ -tubulin ubiquitination tested at 30 nmol/L BRCA1/BARD1: +++, most efficient ubiquitination as seen for full-length BRCA1 and BARD1; ++, ~50% of the efficiency of the full-length protein; +, <50%.



aster formation assay contained BARD1, we tested if BARD1 was essential. BRCA1 alone can ubiquitinate target proteins, although far less strongly than when associated with BARD1 (2, 3, 14). BARD1 by itself does not exhibit E3 ubiquitin ligase activity *in vitro* (4). When 30 nmol/L BRCA1 alone was used in the *in vitro* assay, it failed to inhibit centrosome aster formation (Fig. 6B). Flag-tagged BRCA1 was coexpressed with a truncated BARD1 protein [BARD1(1-292)] that had the RING domain but lacked the 485 amino acid residues from the carboxyl terminus. BRCA1(1-1,863)/BARD1(1-292) was balanced for BRCA1 content (Fig. 6A) and tested in the *in vitro* aster formation assay. BRCA1(1-1,863)/BARD1(1-292) is a functional ubiquitin ligase and ubiquitinates Npm1, a known target for BRCA1 (data not shown); synthesizes ubiquitin polymers in the absence of target proteins (data not shown); and weakly ubiquitinates γ -tubulin (Fig. 6C). BRCA1(1-1,863)/BARD1(1-292) at 30 nmol/L only partially inhibited centrosome microtubule nucleation (60%) relative to the control reaction (Fig. 5B). These results reveal that full-length BARD1, along with BRCA1, has the highest activity in inhibiting the microtubule nucleation of centrosomes.

BARD1 is localized to centrosomes at all phases of the cell cycle. BARD1 has been shown to localize predominantly to the nucleus throughout the cell cycle (36). We had found that although BRCA1 has highest concentrations during S phase, and that most of the S and G₂ phase BRCA1 is nuclear, there is a relatively constant amount of BRCA1 associated with the centrosome throughout the cell cycle (24). Because BARD1 is required for the BRCA1-dependent regulation of centrosome function, we examined whether BARD1 was localized at centrosomes *in vivo*. For this purpose, Hs578T cells were treated with nocodazole to depolymerize microtubules. This ensured that only proteins strongly associated with centrosomes were detected.

Extraction with saponin reduced the background stain due to the presence of cytosolic proteins. The cells were fixed with methanol and stained with γ -tubulin, to mark centrosomes, and with BARD1 antibodies. From the asynchronous cell population, cells from different cell cycle phases were identified based on the position of centrosomes. It was observed that BARD1 colocalized with centrosomes during the G₁-S, S, and M phases of the cell cycle (Supplementary Fig. S1). During S phase, BARD1 was predominantly nuclear and formed speckles similar to BRCA1. At G₁-S, BARD1 was not detected in the nucleus and was predominantly centrosomal. From these immunofluorescence data, it was clear that BARD1 was present along with BRCA1 at the centrosome to regulate its function.

Discussion

For several years, it has been known that BRCA1 localizes to centrosomes during mitosis and disruption of BRCA1 in murine cells caused centrosome amplification (33, 37, 38), but a mechanistic explanation for how BRCA1 regulates centrosomes was unknown. In prior work, we found that transient inhibition of BRCA1 caused centrosome amplification in human breast cell lines but not in cell lines derived from other tissues (23). We found that BRCA1-dependent ubiquitination activity could modify several centrosome proteins, including γ -tubulin, and that the γ -tubulin modification was important for centrosome regulation (23). We recently applied the established *in vitro* functional assay of microtubule nucleation to reveal regulation by the BRCA1/BARD1 E3 ubiquitin ligase (24). In the present study, using an *in vitro* functional assay dependent on the BRCA1/BARD1 ubiquitin ligase activity, we mapped domains of each protein that are necessary to regulate centrosome function. Although

carboxyl-terminal truncations of each BRCA1 and BARD1 could produce active E3 ubiquitin ligases, only heterodimers of the full-length BRCA1 and BARD1 regulate centrosome function. An internal deletion of BRCA1, removing amino acids 770-1,290, was equivalent in centrosome regulating activity when compared with the full-length protein, but all other deletions of BRCA1 or BARD1 tested were diminished in activity. Thus, a large amount of the BRCA1 protein sequence is necessary for the regulation of centrosomes.

The expression in cells of the BRCA1 (I26A) mutant, which lacks E3 ubiquitin ligase activity, had a dominant-negative phenotype of centrosome amplification and centrosome hyperactivity (Fig. 2). This result is the first proof that the *in vitro* functions we previously revealed (24) apply also to BRCA1

function in the cell. We had also observed that the expression of an amino-terminal deletion mutant of BRCA1 (containing residues 301-1,863) disrupted centrosome regulation in a breast cell line (39), but in that case BRCA1 would not stably interact with BARD1. In the experiment in Fig. 2B, the BRCA1 (I26A) is stable and dimerizes fully with BARD1, but it cannot bind to the E2 factor (32) and is thus inactive as E3 ligase. That expression of this mutant dominantly causes centrosome disruption indicates that BRCA1 function at this organelle requires its ubiquitin ligase activity.

Nearly the full-length of BRCA1 is required for full regulation of centrosome microtubule nucleation function. The requirement for the carboxyl terminus likely is dependent on specificity for binding to substrates on the centrosome. Because BARD1 is required for centrosome regulation, another possibility is that the carboxyl terminus of BRCA1 is required to stabilize BARD1 in the heterodimer. We have recently found that the BRCA1 carboxyl terminus stabilizes BARD1 heterodimerization. Without the BRCA1 carboxyl terminus, the quantity of BARD1 binding to BRCA1 drops significantly (18). However, when we renormalized the data in Fig. 3 according to the BARD1 concentrations of each BRCA1 mutant/BARD1 heterodimer, we found that the effects on centrosome microtubule nucleation did not significantly correlate with the amount of BARD1 present in the reaction (data not shown). Thus, we infer that the requirement for the carboxyl terminus of BRCA1 in the ubiquitination-dependent regulation of centrosome function is, in fact, due to substrate binding.

We have observed that multiple centrosome proteins are ubiquitination substrates of BRCA1/BARD1 *in vitro*, and one of these is γ -tubulin (23). The ubiquitination of γ -tubulin is clearly important because expression of mutant γ -tubulin molecules that can no longer be ubiquitinated by BRCA1/BARD1 results in the dominant phenotype of centrosome amplification and centrosome hyperactivity (23, 24). The amount of γ -tubulin ubiquitination in these reactions is low, and it is unclear whether the activity of the centrosomes in the *in vitro* reaction is, in fact, regulated entirely by γ -tubulin ubiquitination. On the other hand, the analysis of the deletion mutants of BRCA1 and BARD1 in this study reveals that the level of γ -tubulin ubiquitination *in vitro* roughly correlates with effectiveness in inhibiting microtubule nucleation. The ubiquitination of γ -tubulin may serve as a surrogate marker for efficiency of ubiquitinating centrosome proteins in general. However, the BRCA1(Δ 303-770)/BARD1 construct ubiquitinates γ -tubulin weakly, and yet that deletion mutant of BRCA1 strongly inhibits centrosome function *in vitro*. Summarizing these observations, it is evident that there are other ubiquitination candidates at the centrosome modified by BRCA1/BARD1. Whereas the regulation of centrosome function by the BRCA1/BARD1 E3 ubiquitin ligase is complex, loss of this activity in breast cells mimics the pathogenesis of breast cancer.

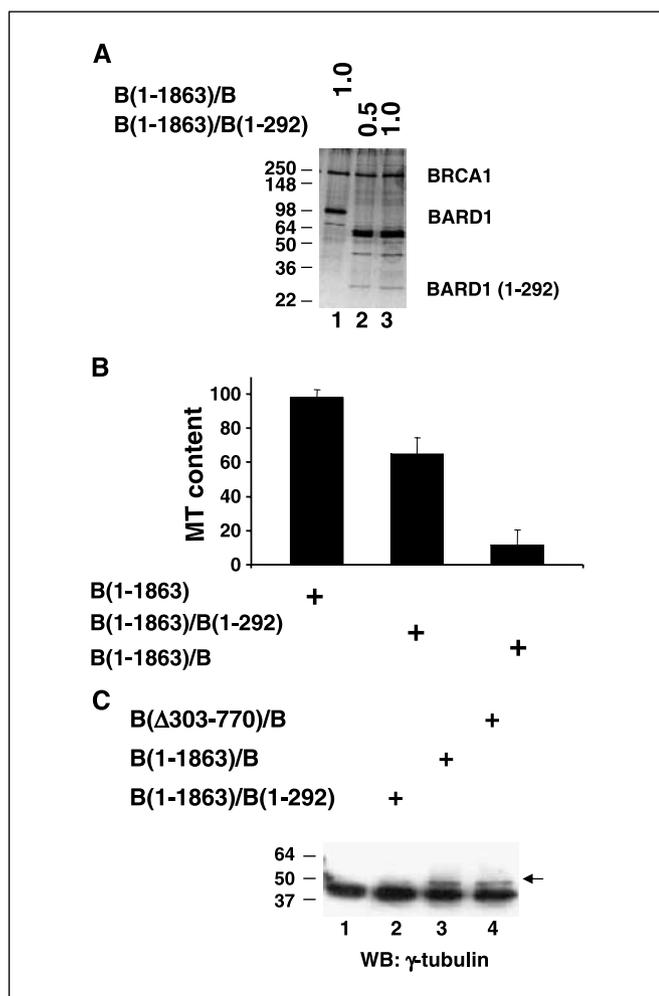


Figure 6. Full-length BARD1 is required for BRCA1-mediated inhibition of centrosome microtubule nucleation *in vitro*. **A**, BARD1 lacking its carboxyl-terminal 485 amino acids, BARD1(1-292), was coexpressed with 5'-Flag-tagged BRCA1 in insect cells and purified. Silver-stained gel containing 1 pmol BRCA1(1-1,863)/BARD1(1-770) (lane 1) and BRCA1(1-1,863)/BARD1(1-292) at 0.5 pmol (lane 2) and 1 pmol (lane 3). **B**, the effect of BARD1 on the *in vitro* microtubule nucleation assay was tested using 30 nmol/L BRCA1 alone, 30 nmol/L BRCA1/BARD1(1-770), or 30 nmol/L BRCA1/BARD1(1-292). As in other experiments, the microtubule content of 20 asters was determined and normalized with respect to the control sample that did not contain BRCA1/BARD1. **Columns**, normalized mean; **bars**, SE. **C**, centrosomes were incubated with ubiquitination factors and different BRCA1/BARD1 proteins at 30 nmol/L and were analyzed by immunoblot analysis using an antibody specific for human γ -tubulin.

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Identification of Domains of BRCA1 Critical for the Ubiquitin-Dependent Inhibition of Centrosome Function

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