BARD1 Induces BRCA1 Intranuclear Foci Formation by Increasing RING-dependent BRCA1 Nuclear Import and Inhibiting BRCA1 Nuclear Export*

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BRCA1 is a tumor suppressor with several important nuclear functions. BRCA1 has no known cytoplasmic functions. We show here that the two previously identified nuclear localization signals (NLSs) are insufficient for nuclear localization of BRCA1 due to the opposing action of an NH2-terminal nuclear export signal. In transfected breast cancer cells, BRCA1 nuclear localization requires both the NLSs and NH2-terminal RING domain region; mutating either of these sequences shifts BRCA1 to the cytoplasm. The BRCA1 RING element mediates nuclear import via association with BARD1, and this is not affected by cancer-associated RING mutations. Moreover, BARD1 directly masks the BRCA1 nuclear export signal, and the resulting block to nuclear export is requisite for efficient import and nuclear localization of ectopic and endogenous BRCA1. Our results explain why BRCA1 exon 11 splice variants, which lack the NLSs but retain the RING domain, are frequently detected in the nucleus and in nuclear foci in vivo. In fact, co-expression of BARD1 promoted formation of DNA damage-induced nuclear foci comprising ectopic wild-type or NLS-deficient BRCA1, implicating BARD1 in nuclear targeting of BRCA1 for DNA repair. Our identification of BARD1 as a BRCA1 nuclear chaperone has regulatory implications for its reported effects on BRCA1 protein stability, ubiquitin ligase activity, and DNA repair.

The tumor suppressor, BRCA1, was the first susceptibility gene linked to breast and ovarian cancer (1). Germ-line mutations of BRCA1 are found in ~50% of patients with inherited breast cancer and up to 90% of families with breast and ovarian cancer susceptibility (1, 2). The role of BRCA1 as a tumor suppressor is not fully defined, although accumulated evidence suggests that BRCA1 plays a role in transcriptional regulation (3), cell cycle control (4, 5), and cell survival responses to DNA damage (6–8).

BRCA1 is a large gene of 24 exons that encodes a 1,863-amino acid protein (1). The BRCA1 protein contains several protein-interaction domains: an NH2-terminal RING domain common to many regulatory proteins (1), two tandem copies of the BRCT (BRCA1 carboxyl terminus) motif at the COOH terminus (9), and both nuclear import (10, 11) and export signals (12). The BRCT domain is found in a variety of proteins, including 53BP1, RAD9, RAD4, Crb2, and RAP1, all of which are associated with cell cycle regulation and DNA repair (13). The BRCT motifs of BRCA1 appear to be critical for its transcription activation function (3, 14), and cancer mutations in this COOH-terminal region impair transcriptional activity (3, 15). This is likely due to altered association with specific proteins, such as the RNA polymerase II holoenzyme, which normally interacts with the COOH terminus of BRCA1 (16).

The NH2-terminal RING domain of BRCA1 mediates association with proteins including BARD1 (17) and BAP1 (18). BARD1 is similar in primary structure to BRCA1, in that it also contains an NH2-terminal RING finger and two COOH-terminal BRCT domains (17). BRCA1 and BARD1 interact via their RING domains (19), and co-localize in discrete nuclear dots during S-phase of the cell cycle (20), and in DNA damage-inducible nuclear foci thought to be involved in DNA repair/replication (6). The BRCA1-BARD1 complex has recently been shown to exhibit ubiquitin ligase activity that is disrupted by BRCA1 breast cancer-associated RING finger mutations (21–23), implicating BARD1 as a regulator of BRCA1 function and tumor suppressor activity.

In recent years, the subcellular localization of BRCA1 has been controversial (24–26), due in part to variable specificity of BRCA1 antibodies. BRCA1 is now generally regarded as a nuclear protein (4, 26) that accumulates in discrete nuclear foci in epithelial cell lines (6, 25, 26), in particular those derived from breast tumors. The known tumor suppressor functions of BRCA1 also occur most in the nucleus (15, 27). BRCA1 contains two SV40-like nuclear localization signals (NLSs1; 503KRKRRP508 and 606PKKNRLRRKS615) that facilitate nuclear import by the importin α/β receptor pathway (10, 11). Although BRCA1 locates predominantly to the nucleus, it contains an NH2-terminal nuclear export signal (NES) and can shuttle between nucleus and cytoplasm (12). The BRCA1 splice variants, BRCA1 Δ11b (28) and BRCA1 A672–4095 (11), are highly expressed in many cells, and yet even though they lack exon 11 which contains the two nuclear localization signals (28), several groups have detected BRCA1 Δ11b in the nucleus (28–30). In particular, Huber et al. (30) examined localization

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1 The abbreviations used are: NLS, nuclear localization signal; NES, nuclear export signal; MMS, methyl methanesulfonate; YFP, yellow fluorescent protein.
Chaperone role for BARD1 in promoting BRCA1 nuclear entry of the endogenous BRCA1 exon 11 splice variant expressed in mouse embryo fibroblasts, and showed that this NLS-deficient protein enters the nucleus and assembles into DNA damage-inducible nuclear foci almost identical to that of full-length BRCA1. Given the importance of nuclear targeting for BRCA1 function, we have searched for alternative BRCA1 nuclear import pathways that act independent of its nuclear import signals.

In this study, we show that transiently expressed BRCA1 splice variant and full-length forms of BRCA1 that lack an NLS can enter the nucleus. This novel NLS-independent import process is dependent on the NH2-terminal RING domain region of BRCA1, a sequence well conserved in all BRCA1 splice variants. The RING-mediated import pathway is facilitated by the BRCA1-binding partner, BARD1. BARD1 not only translocates BRCA1 into the nucleus, but retains it there by masking its nuclear export signal, which lies buried within the BARD1-BRCA1 binding interface (31). Our findings identify a key chaperone role for BARD1 in promoting BRCA1 nuclear entry and formation of DNA damage-inducible BRCA1 nuclear foci, and provide an explanation for the DNA damage response observed for cellular BRCA1 splice variants (30), which contains a RING domain but no NLS sequence. Our results also help resolve much of the controversy concerning BRCA1 subcellular localization, and shed light on a new and unexpected regulatory role of BARD1.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

MCF-7 and T47D human breast cancer cells, and HBL100 immortalized human breast epithelial cells, were maintained in Dulbecco's modified Eagle's media supplemented with 10% fetal calf serum. All cells were grown at 37 °C in a humidified 5% CO2 atmosphere. Cells were seeded onto sterile glass coverslips and transfected at 50–60% confluency with 1 to 2 g of plasmid DNA using LipofectAMINE Reagent (Invitrogen) according to the manufacturer's instructions. At 6 h post-transfection, the transfection mixture was removed and replaced with Dulbecco's modified Eagle's media containing 10% fetal calf serum. Cells were fixed and processed 30 h post-transfection for fluorescence microscopy. When required, transfected cells were treated with leptomycin B at a final concentration of 6 ng/ml for 4 h prior to fixation.

Plasmid Construction

Construction of the expression vectors pF-BRCA1, pYFP-BRCA1, pYFP-BRCA1(Δ306–1312), pBRCA1-NESm, pBRCA1(Δ1–70), and pYFP-CRM1 were described previously (12). The YFP cDNA was excised as a NotI fragment from the above YFP-fusion constructs to create untagged pBRCA1(Δ306–1312), pBRCA1(Δ1–70), and pBRCA1-NESm plasmids. To create pF-CRM1, the CRM1 cDNA was excised from the CRM1 pET1b6 plasmid (provided by Dr. M. Yoshida, Tokyo) and inserted into the pFlag-CMV2 vector (Eastman Kodak Co.) as a KpnI/BamHI fragment. We used a PCR strategy to introduce site-directed mutations into the two nuclear localization signals of BRCA1 cDNA.

Mutation of NLS (503–508)—The first PCR introduced mutations into NLS1 in wild-type BRCA1 using primers MF1 (forward) and MF2 (reverse; codons in bold represent the amino acids Lys606, Arg610, Lys687, and Arg692 that were mutated to alanine). The primer sequences are shown in Table I. A second overlapping PCR product was generated using primers MF6 (forward) and MF7 (reverse). The two DNA products (MF1/2 and MF6/7) were then annealed and amplified with primers MF1 and MF7, and the resulting PCR fragment was gel purified and inserted into wild-type BRCA1 as an AflI/BamHI fragment, to generate pBRCA1-NLS1m.

Mutation of NLS (606–615)—BRCA1-NLSm1 was used as template for two PCR reactions using primers MF3 (forward) and MF4 (reverse), where the codons in bold in the MF3 sequence represent amino acids Lys606, Arg610, Arg612, and Arg613 changed to alanine. A second PCR (primers MF1 and MF5) was annealed with the first and amplified with primers MF1 and MF7, and the resulting PCR fragment was gel purified and inserted into wild-type BRCA1 as an AflI/BamHI fragment, to generate pBRCA1-NLSm1.

A PCR-based strategy was also used to introduce the C61G mutation into the RING domain of BRCA1. Two PCR fragments were generated. First, we PCR amplified a fragment at the NH2 terminus of wild-type BRCA1, using primers BH986 (forward) and BH984 (reverse, see Table I). A second PCR product was amplified with primers BH986 (forward) and BH987 (reverse). The two PCR products were annealed and amplified with primers MH1 and MH2 to generate a fragment that was inserted into pCR-SCRIPT-BRCA1 (provided by Dr. J. Holt, Nashville) as a NotI/XhoI fragment. The NotI restriction site is underlined in the BH986 sequence (see Table I). A NotI/EcoRI fragment containing the C61G mutation was then subcloned into pF-BRCA1 to produce pBRCA1(Δ61G).

pBRCA1(Δ1–304) was made by replacing the EcoRI/ClaI fragment of BRCA1 cDNA with a 27-bp linker sequence, generated by annealing the two oligonucleotides MF17 and MF18. The primer sequences are shown in Table I. A second overlapping PCR product was generated using primers MF3 (forward) and MF7 (reverse), where the codons in bold in the MF3 sequence represent amino acids Lys505, Arg509, Arg510, and Arg511 that were mutated to alanine. A second PCR (primers MF1 and MF5) was annealed with the first and amplified with primers MF1 and MF7, and the resulting PCR fragment was gel purified and inserted into wild-type BRCA1 as an AflI/BamHI fragment, to generate pBRCA1-NLSm1.

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pBRCA1(Δ1–304) was made by replacing the EcoRI/ClaI fragment of BRCA1 cDNA with a 27-bp linker sequence, generated by annealing the two oligonucleotides MF17 and MF18. The EcoRI and ClaI restriction sites are underlined in the oligonucleotide sequences (see Table I). pBRCA1(NESmC) is a mutant in which the BRCA1 NES (two annealed sites are underlined in the oligonucleotide sequences (see Table I). A second overlapping PCR product was generated using primers BH986 (forward) and BH984 (reverse, see Table I). A NotI/EcoRI fragment containing the C61G mutation was then subcloned into pF-BRCA1 to produce pBRCA1(Δ61G).

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BARD1 Is a BRCA1 Nuclear Chaperone

BARD1 (Δ1–95) was created by replacing a NotI/Nhel DNA fragment from the BARD1 cDNA with a PCR fragment generated with the oligonucleotides, JR35 (forward primer) and BH9882 (reverse primer). The NotI restriction site is underlined in the JR35 sequence (Table I). YFP-tagged BRCA1-NLSm and CtIP expression vectors were generated by inserting the YFP cDNA as a NotI fragment (in-frame) at the 5’ end of the cDNA. The YFP gene was also inserted into the pFlag-CMV2 vector as a NotI fragment to generate the expression plasmid referred to as “YFP” in Fig. 3D. All plasmid mutations were confirmed by DNA sequencing.

Immunofluorescence Microscopy and Image Analysis

Immunostaining was carried out as described (12). Cells expressing YFP-tagged proteins were fixed in 3.7% formalin/phosphate-buffered saline for 15 min at room temperature, washed, and then mounted for direct detection of the autofluorescent protein. Untagged ectopic BRCA1 was detected by immunofluorescence using monoclonal antibodies Ab-1 and Ab-4 (Oncogene Research), which recognize an epitope in the amino terminus and the central portion of BRCA1, respectively. BRCA1-bound antibody was detected with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Sigma). BARD1, HA-c-Myc, p53, and HA-Rad51 were detected with polyclonal antibodies 699D (20) diluted 1:800, HA-probe Y-11 (Santa Cruz) was diluted 1:500, and HA-probe Y-11 (Santa Cruz) was diluted 1:500 in blocking solution, respectively. Rb was detected with the monoclonal antibody anti-Rb 14001A (Pharminogen) diluted 1:300 in blocking solution, respectively. Antibody bound to BARD1, c-Myc, p53, Rb, or Rad51 was detected with biotin-conjugated secondary antibodies (Santa Cruz) and Texas Red-avidin D (Vector Laboratories). Cells nuclei were counterstained with the chromosome dye Hoechst 33258 (Sigma). The subcellular localization of each ectopic protein was determined by scoring using an Olympus BX40 epifluorescence microscope. Confocal cross-sections were captured using an Optiscan confocal microscope, and image processing and quantification of nuclear fluorescence using the NIH Image software was carried out as previously described (32).

Scoring of BRCA1 Intranuclear Foci

Cells expressing YFP-BRCA1 and BARD1 proteins were detected by immunostaining as described above. One hour before fixation the appropriate samples were treated with 0.01% methyl methanesulfonate (MMS). YFP-BRCA1 transfected cells were assessed by immunofluorescence microscopy and scored as containing either no nuclear foci, <10, or ≥10 BRCA1 nuclear foci. At least 90 YFP-BRCA1 transfected cells were scored.

Cell Fractionation and Western Blotting

HBL100, MCF-7, and T47D cells were separated into nuclear and cytoplasmic fractions by using the NE-PER extraction kit (Pierce) according to the manufacturer’s instructions. Protein concentrations in nuclear and cytoplasmic fractions were determined using the bicinchoninic acid method (Pierce). The Western blot filters were blocked in blocking buffer (1% fetal calf serum, 5% dried milk in phosphate-buffered saline containing 0.1% Tween 20) and probed with the primary antibody. BRCA1 was detected with either the monoclonal antibody Ab-1 diluted 1:200 or Ab-4 diluted 1:200, followed by incubation with the horseradish peroxidase-conjugated secondary antibody (1:1000). BARD1 was detected with either the monoclonal antibody EE#6 (Bayer Lab) diluted 1:800 or the polyclonal antibody 42C (17) diluted 1:1000. Blotted proteins were visualized using the ECL detection system (Amersham Biosciences). Rainbow color markers (Amersham Biosciences) were used as molecular size standards.

RESULTS

NLS-independent Nuclear Import of BRCA1—Several groups have recently detected the NLS-deficient BRCA1 splice variant, BRCA1 Δ11b, in the nucleus of cells (26, 28–30). We confirmed by Western blot analysis that endogenous BRCA1 Δ11b is present in the nuclear fraction of HBL100 cells (see Fig. 1A). This prompted us to re-investigate the contribution of the BRCA1 NLS sequences in nuclear import. We first examined the subcellular localization of two NLS-deficient forms of BRCA1: a deletion mutant (Δ306–1312) that lacks exon 11 and full-length BRCA1 carrying site-directed mutations that inactive both NLSs (BRCA1-NLSm) (see Fig. 1B). In transfected breast epithelial cells, the localization of ectopic BRCA1 was scored as nuclear (N), nuclear/cytoplasmic (NC), or cytoplasmic (C). Wild-type BRCA1 consistently displayed a mixed nuclear/cytoplasmic distribution (≥80% NC) in different cell lines (see Table II and Fig. 1C). In contrast, BRCA1(Δ306–1312) was exclusively cytoplasmic in ≥90% of MCF-7 and HBL100 cells, but displayed partial nuclear staining in ≥40% of T47D cells (Table II). BRCA1-NLSm showed a similar distribution pattern to Δ306–1312, confirming that BRCA1 can enter the nucleus, albeit less efficiently, in the absence of an NLS.

To test for nuclear import of BRCA1 in the absence of opposing nuclear export activity, we introduced a site-directed NES mutation (L686A and I90A) (12) into each BRCA1 construct, and compared its effect on BRCA1 localization (see Fig. 1, B and C). Introduction of the NES mutation shifted wild-type BRCA1 to the nucleus in transfected MCF-7 cells (Fig. 1C), as previously reported (12). More dramatic was the effect of the NES mutation on BRCA1-NLSm, which was no longer restricted to the cytoplasm, but displayed at least some nuclear staining in >90% of transfected cells (Fig. 1C). BRCA1(Δ306–1312) also revealed a strong increase in nuclear staining (>40% of cells) following NES mutation. Similar results were obtained in HBL100 and T47D cells (Table II). These results provide incontrovertible evidence that BRCA1(Δ306–1312) and BRCA1-NLSm can enter the nucleus by an NLS-independent pathway, and their additional ability to exit the nucleus indicate that they are also capable of nuclear-cytoplasmic shuttling.

BARD1 Mediates Nuclear Import of BRCA1—What factor mediates NLS-independent BRCA1 nuclear import? BRCA1 is known to bind to many different cellular proteins. Therefore we
RING-finger domain characterized by the C2HC4 motif (1, 17), forms of BRCA1 also accumulated in the nucleus after NES mutation, although to a lesser extent than wild-type BRCA1.

The NH2-terminal binding partners BARD1 (17), c-Myc (34), retinoblastoma protein (Rb) (35), and the estrogen receptor (36), and the COOH-terminal binding partners, p53 (29), CtIP (37), the androgen receptor (38) and the histone deacetylase HDAC1 (39). p53 also binds within exon 11 (40), as does the androgen receptor (38) and Rad51 (7). Expression plasmids encoding these candidate escorts were co-transfected into MCF-7 cells with pBRCA1wt, pBRCA1-NLSm, or pBRCA1(Δ306–1312). As expected, wild-type BRCA1 alone displayed a nuclear-cytoplasmic distribution in most cells, while BRCA1-NLSm and BRCA1(Δ306–1312) were exclusively cytoplasmic in >95% of transfected MCF-7 cells (Fig. 2B).

Cotransfection of BARD1 induced a dramatic shift to the nucleus for each of the BRCA1 constructs (see Figs. 2B and 3A). Quantitative fluorescence imaging of transfected cells revealed a 2-fold increase in average nuclear fluorescence of wild-type BRCA1 and an 11-fold increase in average nuclear fluorescence of BRCA1-NLSm following the addition of BARD1 (see Fig. 3A). As previously reported, BARD1 and BRCA1 accumulated in the nucleus in >90% of co-transfected cells, and displayed frequent co-localization in nuclear ‘dots’ (20) (see confocal images in Fig. 3A). Similar results were obtained using yellow fluorescent protein fusions of BRCA1 (see below). This result is all the more remarkable given that none of the other BRCA1 binding partners tested (including Rad51, the estrogen receptor, and HDAC1, data not shown) induced nuclear localization of BRCA1 (see Figs. 2B and 3B). We conclude that BARD1 acts specifically to promote BRCA1 nuclear import.

Next we compared the localization of endogenous full-length BRCA1 in fractionated extracts from HBL100, MCF-7, and T47D breast cancer cells. Western blot analysis revealed that HBL100 and T47D cells express high levels of BARD1 and this correlated with a high nuclear/cytoplasmic ratio of endogenous BRCA1 compared with MCF-7 cells, which poorly express BARD1 (see Fig. 4). These data are consistent with our transfection experiments (see Figs. 2 and 3), and suggest a correlation between cellular BARD1 expression and BRCA1 nuclear accumulation.

**Table II**

| BRCA1        | HBL100 | MCF-7 | T47D |
|--------------|--------|-------|------|
| %N           | %NC    | %C    | %N   | %NC | %C   | %N  | %NC | %C  |
| Wild type    | 12.2   | 83.3  | 4.5  | 8.2 | 85.5 | 6.3 | 14.8| 82.5| 2.7 |
| NESm         | 64.5   | 35.5  | 0    | 76.1| 23.0 | 0.9 | 19.5| 77.9| 2.4 |
| NLSm         | 0      | 3.5   | 96.5 | 0   | 4.6  | 95.4| 4.6 | 33.5| 62.0|
| NLSm/NESm    | 1.2    | 93.3  | 5.5  | 0.2 | 92.9 | 6.9 | 1.6 | 94.6| 3.8 |
| Δ306–1312    | 0      | 4.5   | 95.5 | 0   | 7.7  | 92.3| 0.8 | 41.4| 57.8|
| Δ306–1312/NESm| 0      | 43.8  | 56.2 | 0   | 43.5 | 56.5| 0   | 65.8| 34.2|

Values shown (mean of two or more experiments with <15% variation) represent % transfected cells displaying nuclear (N), nuclear and cytoplasmic (NC), or cytoplasmic (C) staining of BRCA1. More than 400 transfected cells in total were scored for each sample. Note that introduction of an NES mutation strongly shifts wild-type BRCA1 to the nucleus in HBL100 and MCF-7 cells, but less so in T47D. NLS-deficient forms of BRCA1 also accumulated in the nucleus after NES mutation, although to a lesser extent than wild-type BRCA1.

reasoned that a BRCA1 binding partner may promote BRCA1 nuclear import via a “piggyback” mechanism (33). Several BRCA1-binding partners were tested for their effects on BRCA1 nuclear localization (outlined in Fig. 2A). These comprised the NH2-terminal binding partners BARD1 (17), c-Myc (34), retinoblastoma protein (Rb) (35), and the estrogen receptor (36), and the COOH-terminal binding partners, p53 (29), CtIP (37), the androgen receptor (38) and the histone deacetylase HDAC1 (39). p53 also binds within exon 11 (40), as does the androgen receptor (38) and Rad51 (7). Expression plasmids encoding these candidate escorts were co-transfected into MCF-7 cells with pBRCA1wt, pBRCA1-NLSm, or pBRCA1(Δ306–1312). As expected, wild-type BRCA1 alone displayed a nuclear-cytoplasmic distribution in most cells, while BRCA1-NLSm and BRCA1(Δ306–1312) were exclusively cytoplasmic in >95% of transfected MCF-7 cells (Fig. 2B).

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**BARD1 Nuclear Import of BRCA1 Is RING Domain-dependent**—The NH2 terminus of both BRCA1 and BARD1 contain a RING-finger domain characterized by the C2HC4 motif (1, 17), and the two proteins associate through this common interaction site (17). To determine whether BARD1-dependent regulation of BRCA1 localization requires interaction between the two proteins, we generated a BRCA1 deletion mutant that lacks the RING domain region (Δ1–70) (Fig. 5A), and is therefore unable to bind BARD1.

Confirming the importance of the RING domain in BRCA1 nuclear import, we found that transiently expressed BRCA1(Δ1–70) was predominantly cytoplasmic in MCF-7 cells (Fig. 5B). This is a key finding, as it reveals that the two NLSs are not sufficient to localize BRCA1 to the nucleus in the absence of the RING domain, and therefore implies that both NLS and RING sequences are required for nuclear localization. Importantly, co-transfection with BARD1 did not alter BRCA1(Δ1–70) subcellular localization, indicating that BARD1-mediated import of BRCA1 requires the RING finger. Surprisingly, the cancer-associated RING-finger mutations C61G and C64G had no significant effect on BRCA1 localization, and BARD1 enhanced the nuclear localization of these mutants almost as efficiently as wild-type BRCA1 (Fig. 5B and data not shown).

To demonstrate that the BRCA1 RING fragment is sufficient for BARD1-mediated import, we tested a DNA fragment encoding the first 304 amino acids of BRCA1, which includes the RING domain and the NES (Fig. 6A). In transfected MCF-7 cells, BRCA1-(1–304) was found to stain both nucleus and cytoplasm, but was predominantly located in the cytoplasm.

**Fig. 2. BARD1 promotes nuclear localization of BRCA1.** A. map of BRCA1 showing binding sites for various known co-factors, and locations of RING/BRCT domains and transport signals. B, different BRCA1 constructs were transiently expressed in MCF-7 cells, either alone or with the co-factors BARD1, c-Myc, or Rb. Co-transfection of Rb and c-Myc protein did not affect BRCA1 localization, whereas BARD1 shifted all forms of BRCA1 exclusively to the nucleus in >90% of co-transfected cells. The distribution profile of BRCA1 in transfected cells is shown graphed (mean ± S.D. from at least three independent experiments). n, total number of transfected cells scored. *Rb was co-transfected with YFP-tagged wild-type BRCA1, NLSm, or Δ306–1312.
Fig. 3. BRCA1 and BARD1 co-localize in the nucleus. A, confocal microscopic cross-sections showing nuclear co-localization of BRCA1 and BARD1 in transfected MCF-7 cells. The localization of BRCA1 (Ab-1 primary monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse antibody) and BARD1 (rabbit polyclonal 699D and Texas Red-conjugated secondary antibody) was determined by immunostaining. The increase in average nuclear fluorescence ($F_{nuc}$; see “Experimental Procedures”) of BRCA1 following co-expression of BARD1 is shown ($>60$ BRCA1-transfected cells were quantified). B, to illustrate the specificity of the BARD1 response, the localization of ectopic BRCA1(D306–1312) in cells alone and co-transfected with other BRCA1 binding partners is shown (see “Experimental Procedures”). The percentage of cells displaying nuclear staining of BRCA1(D306–1312) is shown below the confocal cell images. nt, not tested.

Fig. 4. Nuclear localization of endogenous BRCA1 correlates with high levels of endogenous BARD1. HBL100, MCF-7, and T47D cells were separated into nuclear (N) and cytoplasmic (C) fractions and subjected to Western blot analysis with antibodies specific to either BRCA1 (Ab-1 and Ab-4) or BARD1 (antibody 42C; and confirmed with antibody EE96, data not shown). Minimal cross-contamination between nuclear and cytoplasmic fractions was confirmed by probing for nuclear topoisomerase II. Endogenous BARD1 levels differed between the cell lines and correlated with the amount of BRCA1 detected in the nuclear fraction. The nuclear/cytoplasmic ratio of endogenous BRCA1 is shown for each cell line.

(Fig. 6B). Inactivation of the NES (by inserting the mutation L86A and I90A) shifted BRCA1(1–304) partially to the nucleus, whereas co-expression of BARD1 induced a near-complete nuclear shift (Fig. 6B). To test whether the RING domain of BARD1 is also required for nuclear import of BRCA1, we cloned a deletion mutant of BARD1 that lacks the RING domain (Δ1–95), and showed that in co-transfected cells, BARD1(Δ1–95) did not alter the subcellular distribution of BRCA1(1–304) (see Fig. 6B). These results identify the BRCA1 RING domain sequence as a nuclear localization element, and indicate that BARD1 must interact directly with BRCA1 to shift it to the nucleus, implicating BARD1 as a BRCA1 chaperone.

Fig. 5. The RING-finger domain of BRCA1 is essential for BRCA1 nuclear translocation. A, construct maps. Wild-type BRCA1 and BARD1 each contain a RING domain at the NH2 terminus and two BRCT domains at the COOH terminus (1, 17). The RING domain of BRCA1 interacts with BARD1 (19), which has been deleted in the construct BRCA1(Δ1–70). The cancer derived RING-finger mutation (C61G) was introduced into wild-type BRCA1. B, MCF-7 cells were transfected with plasmids expressing wild-type BRCA1, BRCA1(Δ1–70), or BRCA1(C61G), alone or with BARD1. The distribution profiles (as in legend to Fig. 2B) show that ectopic BARD1 shifts wild-type BRCA1 to the nucleus, but does not alter the localization of BRCA1(Δ1–70). In contrast, cancer-associated mutations within the RING domain of BRCA1 do not prevent BARD1-mediated nuclear localization of BRCA1.
BARD1 Is a BRCA1 Nuclear Chaperone

Evidence That BARD1 Overexpression Promotes DNA Damage-induced BRCA1 Nuclear Foci Formation—Endogenous cellular BRCA1 localizes in discrete nuclear dots in S-phase (7, 20), and following DNA damage it redistributes into more dispersed nuclear foci that reflect involvement in DNA repair (6). We observed similar BRCA1 foci patterns following transient expression of YFP-BRCA1 in MCF-7 cells, before and after a 1-h exposure to the DNA damaging agent, MMS (Fig. 7). After DNA damage, 78% of YFP-BRCA1 transfected cells displayed >50 foci, whereas only 9% of cells transfected with the NLS-deficient forms of BRCA1 showed foci (Fig. 7, A and B). When co-expressed with BARD1, however, even the NLS-deficient forms of BRCA1 formed >50 foci in almost every transfected cell after MMS treatment (Fig. 7B). In all cases, BRCA1 displayed focal co-localization with BARD1. Similar results were obtained with untagged forms of BRCA1 (data not shown). This novel result reveals that BARD1 can import wild-type or splice variant forms of BRCA1 into the nucleus to form DNA damage-induced foci. This provides a clear explanation for the previously puzzling observation that endogenous splice variant forms of BRCA1 formed damage-induced foci in mouse embryo fibroblasts (30).

Titration of Cellular BARD1 by a BRCA1 RING Fragment Reduces Nuclear Staining and Foci Formation of Endogenous BRCA1—It is difficult to define the role of BARD1 in nuclear accumulation of endogenous BRCA1, since BRCA1 stains predominantly in the nucleus of all known cell lines, and no BARD1-null cell lines exist. We therefore performed a competition experiment, in which a YFP fusion of the RING-containing fragment, BRCA1(1–304), was transfected into T47D breast cancer cells to saturate and compete out endogenous BARD1. We had already shown that BRCA1(1–304) is regulated by ectopic BARD1 (Fig. 6B). Transfected cells were stained with monoclonal antibody Ab-4 (which targets BRCA1 exon 11) and a Texas Red secondary antibody, and should only detect endogenous BRCA1. As illustrated in Fig. 7C, overexpression of the BRCA1 RING domain caused a 10-fold reduction in the number of cells displaying strong nuclear BRCA1 staining with BRCA1 dots. In co-transfection experiments, short RING-containing fragments of BRCA1 did not alter localization of full-length BRCA1 (data not shown). This experiment supports a physiological role for BARD1 in nuclear import of BRCA1 and recruitment into nuclear foci.

BARD1 Blocks CRM1-dependent Nuclear Export of BRCA1 by Masking Its Nuclear Export Signal—The BARD1-binding site on BRCA1 encompasses both the RING domain and the nuclear export signal (12, 17, 31), and the recently published structure of the BRCA1–BARD1 heterodimer reveals that the NES constitutes one of the helical coils at the interface of these two proteins (Ref. 31; see Fig. 8A). We hypothesized that binding of BARD1 might mask the BRCA1 NES and prevent its association with the CRM1 export receptor (33). If this were true, complete removal of the RING domain should not only prevent BARD1 binding, but should increase BRCA1 nuclear export activity. Indeed, the RING mutant BRCA1(1–70) was predominantly cytoplasmic in transfected cells, but re-located to the nucleus as efficiently as wild-type BRCA1 following a short 4-h treatment with the nuclear export inhibitor, leptomycin B (see Fig. 8B). Therefore, deletion of the BRCA1 RING domain increases its rate of nuclear export, indicating that the RING motif negatively regulates BRCA1 nuclear export.

Next, to show that BARD1 binding masks the BRCA1 NES, we examined the effect of BARD1 on CRM1-mediated nuclear export of BRCA1 (summarized in Fig. 9A). In transient expression assays, co-transfection of YFP-CRM1 caused wild-type BRCA1 to re-localize from nucleus to cytoplasm (compare Fig. 9, B and C). However, co-expression of BARD1 completely blocked the ability of CRM1 to export BRCA1 to the cytoplasm (Fig. 9C). To demonstrate that BARD1 exerted this negative effect on export by masking the NES, and not solely by an indirect retention mechanism, we tested its ability to regulate localization of a BRCA1 mutant in which the NES was re-positioned to the COOH terminus (see Fig. 9A). As shown in Fig. 9, B and C, BRCA1(NESmC) displayed a similar subcellular distribution and response to YFP-CRM1 as wild-type BRCA1. However, unlike wild-type BRCA1, BRCA1(NESmC) was exported to the cytoplasm via the CRM1-dependent pathway even in the presence of BARD1 (Fig. 9C), revealing that down-regulation of BRCA1 export by BARD1 requires positioning of the nuclear export signal close to the RING domain. Our results provide strong evidence that BARD1 blocks CRM1-dependent nuclear export of BRCA1 by masking the NES, and thereby reveal an important functional consequence of the recently solved structure of this dimeric complex (31).

BARD1 Prevents Nuclear Export of Endogenous BRCA1—To demonstrate that BARD1 inhibits CRM1-dependent nuclear export of endogenous BRCA1, we transfected T47D cells with YFP, YFP-CRM1, or YFP-CRM1 + BARD1, and stained for endogenous BRCA1 with monoclonal antibody Ab-4 (and Texas Red secondary antibody). As illustrated in Table III, cellular BRCA1 displayed a reproducible shift to the cytoplasm in response to overexpression of YFP-CRM1, as previously reported (12). In contrast, co-expression of BARD1 prevented the CRM1-dependent relocation of endogenous BRCA1, supporting a physiological role for BARD1 in regulating nuclear BRCA1 accumulation by NES masking.

DISCUSSION

BRCA1 functions primarily in the nucleus, where it regulates gene transcription (3), cell-cycle progression (4, 5), and DNA repair (27). There is overwhelming evidence for involve-
ment of nuclear BRCA1 in the DNA damage response (27, 41), including roles in homology-directed repair of double-strand DNA breaks (41) and transcription-coupled repair of oxidative DNA damage (8). The involvement of BRCA1 in multiple checkpoints that control cell-cycle progression following DNA damage implicates BRCA1 as a global “caretaker” of the genome (27, 41). In this study, we demonstrate that nuclear targeting of BRCA1 requires not only the two NLSs, but also the NH2-terminal 70 amino acids of BRCA1 encompassing the RING domain, the deletion of which shifted BRCA1 to the cytoplasm. The RING element mediates BRCA1 nuclear import via association with BARD1, and this was not affected by cancer-associated point mutations (e.g. C61G) in the RING sequence. We further discovered that BARD1 retains BRCA1 in the nucleus by masking its nuclear export signal. The BARD1-mediated block to CRM1-dependent nuclear export is required for nuclear localization of ectopic and endogenous BRCA1. Moreover, co-expression of BARD1 promoted formation of DNA damage-induced nuclear foci comprising ectopic wild-type or NLS-deficient splice variant forms of BRCA1, implicating BARD1 in nuclear targeting of BRCA1 for DNA repair. These new findings (summarized in Fig. 10) identify BARD1 as a BRCA1 nuclear chaperone and a key regulator of BRCA1 subcellular localization.

Role of BARD1 in BRCA1 Nuclear Import—BRCA1 expression remains low until just prior to the G1/S phase transition (42). At S-phase, BRCA1 levels increase (4, 7, 20), BRCA1 becomes phosphorylated (4) and it co-localizes with BARD1 in discrete nuclear dots (7, 20). Given that more than 75% of all BRCA1 levels increase at S-phase, it rapidly associates with BARD1 in the cytoplasm and is imported into the nucleus. The NLS-importin pathway alone is insufficient for nuclear localization of BRCA1, because removal of amino acids 1–70 greatly reduced BRCA1 nuclear localization and foci formation, despite the presence and activity of the NLSs. Therefore, efficient nuclear import of BRCA1 requires two distinct pathways: 1) NLS interaction with the importin-α/β receptors (10), and 2) a piggyback mechanism involving interaction of the RING domain with BARD1. The ability of BARD1 to mediate BRCA1 nuclear entry was highly specific, and none of the other nine binding partners tested enhanced BRCA1 nuclear staining (Figs. 2 and 3). The co-expression of BARD1 completely re-localized BRCA1 to the nucleus and increased BRCA1 nuclear fluorescence. Moreover, saturation of cellular BARD1 with the BRCA1 RING fragment caused a reduction in nuclear staining of endogenous BRCA1. The NH2-terminal BRCA1 RING fragment therefore functions as an alternative nuclear localization element, and is likely to contribute to nuclear targeting of NLS-deficient BRCA1 splice variants that lack exon 11 (Fig. 3). In support of this, BRCA1 splice variants are predominantly nuclear in HBL100 cells, and these cells express very high levels of BARD1 (26) (Figs. 1 and 4). While the specific function of BRCA1 splice variants is unresolved, they are highly expressed in some breast tumors (28) and their ability to enter the nucleus and form DNA damage-inducible foci has been linked to a role in DNA repair (41).

BARD1 Retains BRCA1 in the Nucleus by Masking Its Nuclear Export Signal—The BRCA1 nuclear export signal lies within 20 amino acids of the RING domain (12, 17), and forms part of the BARD1 interaction site (31). By re-positioning the NES to the COOH terminus of BRCA1, we observed that CRM1-mediated BRCA1 nuclear export was no longer blocked by BARD1 (Fig. 9), consistent with a role for BARD1 in masking the BRCA1 export signal. In contrast, when the BRCA1 NES was re-positioned, BRCA1 actually exported BARD1 out of the nucleus in the presence of CRM1 (data not shown). From these experiments we conclude that NES masking is critical for

Fig. 7. BARD1 promotes formation of DNA damage-inducible BRCA1 nuclear foci. A, representative confocal images showing the localization of YFP-BRCA1wt and NLS-deficient forms of YFP-BRCA1 (NLSm and Δ306–1312) expressed either alone or with BARD1, before and after a 1-h treatment with the DNA damaging agent, MMS (0.01%). B, quantification of foci. The two NLS-deficient forms of BRCA1 did not form nuclear foci when expressed alone; however, all forms of BRCA1 located to intranuclear foci when co-expressed with BARD1, and following MMS treatment the proportion of BRCA1 + BARD1 foci-positive cells increased substantially. Values shown are from one representative experiment. Similar results were obtained in at least two other experiments. C, in transfected T47D cells, the nuclear staining of endogenous BRCA1 decreased in cells expressing YFP-BRCA1 (1–304), compared with YFP alone. The relative percentage of cells displaying nuclear (and nuclear foci) staining of endogenous BRCA1 is shown below the confocal cell images.
keeping BRCA1-BARD1 complexes in the nucleus, where the BRCA1-BARD1 dimer is perhaps functionally most important. In this regard, the BRCA1-BARD1 complex was recently shown to display specific nuclear activities including the inhibition of mRNA polyadenylation through direct binding of BARD1 to the polyadenylation factor CstF-50 (44), and proposed involvement in DNA repair, as suggested by biochemical fractionation identifying BARD1 in four distinct BRCA1 nuclear-protein complexes (45).

The BRCA1-BARD1 dimer was also found to exhibit ubiquitin ligase activity (21–23). In the BRCA1-BARD1 structure, the zinc-containing RING domains loop out (31), possibly to trap protein substrates targeted by the strong ubiquitin E3 ligase activity of this complex (21–23). Given that BARD1 targets BRCA1 to the nucleus, it is quite possible that most ubiquitination/degradation substrates of this complex will be nuclear proteins, including targets such as RNA polymerase II (46). Since BRCA1 expression is cell-cycle regulated, BRCA1 itself may become ubiquitinated following dimerization with BARD1, so that following dissociation from BARD1 it is exported to the cytoplasm and degraded. This idea is consistent with the recent finding that BRCA1 and BARD1 stability is increased when the two proteins are co-expressed (47). It will be interesting to determine the signals that trigger dissociation of the BRCA1-BARD1 complex, to more directly address the cellular consequences and timing of BRCA1 nuclear export.

Our findings suggest that the rate of BRCA1 nuclear export will be highest in cells that poorly express BARD1, although
the cytoplasmic accumulation of BRCA1 may in turn be controlled by protein degradation. At this point we cannot exclude that other RING-binding proteins, namely the ubiquitin hydrolase BAP1 (18), also disrupt BRCA1 nuclear export. The masking of nuclear export signals was previously shown to regulate transport of the transcription factors, NF-AT (48) and PBX1/EXD (49), whose export signals become inaccessible to CRM1 following association with calcineurin and PREP1/HTH, respectively. Therefore, NES masking may prove a more general mechanism for regulating nuclear shuttling of proteins, and it will prove interesting to test for similar control of other tumor suppressors known to be regulated by nuclear export, such as p53 (50) and APC (32).

**BARD1 Promotes BRCA1 Nuclear Foci Formation after DNA Damage**—BARD1 localizes to nuclear dots during cellular DNA replication (S-phase) (6, 20), and after DNA damage nuclear BRCA1 redistributes into many more larger foci that co-stain with BARD1 and proteins associated with DNA repair and replication such as Rad51 (7), Rad50/hMre11/p95 (51), and proliferating cell nuclear antigen (6). In transient assays with YFP-tagged BRCA1, we observed similar BRCA1-BARD1 staining patterns as were previously observed for cellular BRCA1 (6, 20), both before and after DNA damage by 1-h methyl methanesulfonate treatment (Fig. 7). In particular, the cytoplasmic BRCA1 Δ306−1312 protein (no NLS) was shifted to the nucleus by BARD1, where it then formed DNA damage-inducible foci almost identical to that reported for endogenous BRCA1 exon 11 splice variant in mouse embryo fibroblasts (30). These important findings directly implicate BARD1 in the nuclear import of BRCA1 splice variants, and in the formation of DNA damage-inducible BRCA1 nuclear foci. Thus, BARD1 appears to target BRCA1 (wild-type or splice variant) to the nucleus for DNA repair.

We conclude that BARD1 is a critical regulator of BRCA1 subcellular localization. This activity was not negated by known breast cancer mutations within the BRCA1 RING domain, consistent with the structure of the BRCA1-BARD1 heterodimer, in which the zinc-binding sites of the RING finger loop out (31). In contrast, the ubiquitin ligase activity of BRCA1-BARD1 is abolished by BRCA1 RING mutations (21–23). Therefore, breast cancer mutations in BRCA1 do not block association with or nuclear targeting by BARD1, but can prevent other functions of the BRCA1-BARD1 complex. The potential impact of BARD1 cancer mutations (52) on BRCA1-BARD1 localization and function remains to be examined.

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BARD1 Induces BRCA1 Intranuclear Foci Formation by Increasing RING-dependent BRCA1 Nuclear Import and Inhibiting BRCA1 Nuclear Export

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