BARD1 Translocation to Mitochondria Correlates with Bax Oligomerization, Loss of Mitochondrial Membrane Potential, and Apoptosis

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The breast cancer regulatory protein-1 (BRCA1)-associated RING domain 1 (BARD1) gene is mutated in a subset of breast/ovarian cancers. BARD1 functions as a heterodimer with BRCA1 in nuclear DNA repair. BARD1 also has a BRCA1-independent apoptotic activity. Here we investigated the link between cytoplasmic localization and apoptotic function of BARD1. We used immunofluorescence microscopy and deconvolution analysis to resolve BARD1 cytoplasmic staining patterns and detected endogenous BARD1 at mitochondria. BARD1 was also detected in mitochondrial cell fractions by immunoblotting. The targeting of BARD1 to mitochondria was modestly stimulated by DNA damage and did not require BRCA1 as indicated by RNA interference and peptide-competition experiments. Transiently expressed yellow fluorescence protein-BARD1 localized to mitochondria, and the targeting sequences were mapped to both the N and C terminus of BARD1. Ectopic yellow fluorescence protein-BARD1 induced apoptosis and loss of mitochondrial membrane potential in MCF-7 breast tumor cells. BARD1 apoptotic function was associated with stimulation of Bax oligomerization at mitochondria. This distinguishes it from BRCA1, which is pro-apoptotic but did not induce Bax oligomerization. The cancer-associated BARD1 splice-variant ΔRIN (lacks the BRCA1 binding domain and ankyrin repeats) was recruited to mitochondria but did not stimulate apoptosis or alter membrane permeability. We propose that BARD1 has two main sites of action in its cellular response to DNA damage, the nucleus, where it promotes cell survival through DNA repair, and the mitochondria, where BARD1 regulates apoptosis.

BRCA1²-associated RING domain 1 (BARD1) is the primary binding partner of the breast and ovarian cancer susceptibility protein, BRCA1 (1). BARD1 is a putative tumor suppressor, and its gene is mutated in a subset of breast and ovarian cancer patients (2–4). The interaction between BRCA1 and BARD1 is important for regulation of their stability (5), localization (6), and function (7), and several lines of evidence indicate that the BARD1-BRCA1 heterodimer is the physiologically relevant form of BRCA1. The two proteins are coordinately expressed in different tissues and cell types in both Xenopus laevis and human cells, and they stabilize one another (5, 8, 9). The BRCA1-BARD1 heterodimer is important for cell viability, and loss of either or both proteins in mice results in accumulation of chromosomal abnormalities and early embryonic death due to severe cell proliferation defects (9, 10). BARD1 and BRCA1 are predominantly found in the cell nucleus where they co-localize in dots during S-phase of the cell cycle (11). Consistent with their role in the DNA damage checkpoint, genotoxic stress induces BARD1 and BRCA1 to redistribute in the nucleus into discrete foci that co-stain with DNA repair factors such as BRCA2 and Rad51 (12, 13). BRCA1 possesses E3 ubiquitin ligase activity, which is impaired by tumor-derived point mutations within theRING motif (14, 15) but stimulated by complex formation with BARD1 (8, 16–18).

Human BARD1 (777 amino acids) is smaller than BRCA1 (1843 amino acids), but both proteins share a similar domain structure, comprising an N-terminal RING finger and two C-terminal BRCT domains (1, 19). BARD1 differs from BRCA1 in that it also contains an ankyrin repeat domain known to mediate protein-protein interactions; the role of this domain in BARD1 is still under investigation. BRCA1 and BARD1 interact via their N-terminal RING domains (20). We previously discovered nuclear export sequences within the dimerization domain of both proteins and showed that heterodimerization caused inactivation of their nuclear export resulting in a strong nuclear accumulation of the dimer (21, 22). The ubiquitin ligase activity of BRCA1-BARD1 may contribute to its recently defined roles in DNA repair (9, 23) or mitotic regulation (24). The ubiquitination of H2AX links BRCA1-BARD1 ubiquitin ligase activity with chromosome rearrangement and formation of DNA damage-induced nuclear repair sites, as suggested also by the BRCA1-BARD1-dependent appearance of conjugated ubiquitin at damage-induced foci (25). The ubiquitination of γ-tubulin (26) indicates a role of

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2 The abbreviations used are: BRCA1, breast cancer regulatory protein-1; BARD1, BRCA1-associated RING domain 1; CMX-Ros, chloromethyl-X-rosamine; IR, ionizing radiation; NES, nuclear export sequence; NLS, nuclear localization sequence; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; YFP, yellow fluorescence protein; Ab, antibody; wt, wild type; IR, ionizing radiation.


**BARD1 Localizes to Mitochondria**

BARD1-BARD1 in centrosome duplication and chromosome segregation consistent with involvement in genomic integrity and mitosis. Other functional roles may depend on the subcellular localization patterns of BRCAl-BARD1.

The pro-apoptotic activity of BARD1 is the only known function that does not require its interaction with BRCAl (27). We previously identified BARD1 nuclear export (22) and nuclear import (28) sequences and used RNA interference to demonstrate that endogenous BRCAl can regulate accumulation of BARD1 in the nucleus (28). The apoptotic function of BARD1 is at least partly dependent on p53 (27) and is stimulated by the export of BARD1 from nucleus to cytoplasm (22). Jefford et al. (29) also observed a correlation between cytoplasmic staining of BARD1 and apoptosis. The co-expression of BRCAl inhibited BARD1 nuclear export and apoptotic activity (22, 30), consistent with the premise that BARD1 alone translocates to a specific cytoplasmic location to elicit an apoptotic response. In preliminary cell staining experiments we observed a granular cytoplasmic distribution of BARD1 reminiscent of mitochondrial staining. In this study we demonstrate that BARD1 does indeed locate at mitochondria and that this can occur independent of BRCAl. The mitochondrial targeting of BARD1 correlates with induction of apoptosis and loss of mitochondrial outer membrane potential. Further analysis revealed that BARD1 induces oligomerization of the pro-apoptotic factor Bax at mitochondria.

**MATERIALS AND METHODS**

**Cell Culture and Transfections**—The following human cell lines were grown under standard conditions in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum: U2OS osteosarcoma cells, HEK293T epithelial cells, HCT 116 and SW480 colon cancer cells, and MCF-7 breast cancer cells. HCC1937 breast cancer cells were grown in RPMI 1640 medium with 10% fetal calf serum. The immortalized but non-tumorigenic human breast epithelial cell line, MCF-10A, was cultured in Dulbecco’s modified Eagle’s medium/F12K containing 5% horse serum, insulin, epidermal growth factor, cholera toxin, and hydrocortisone. All cell lines were mycoplasma-negative.

Transient transfections were performed using Lipo-fectamine2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, 24 h after seeding, the cells were transfected at 50% confluence with 2 μg of DNA (per well in a 6-well plate) or 4 μg of DNA (T25 flask for Western blots or flow cytometry). At 6 h post-transfection, the transfection mix was removed and replaced with the appropriate medium containing 10% fetal calf serum. Cells were fixed and processed 24 h post-transfection for fluorescence microscopy and at 40 h post-transfection for flow cytometry analyses. For DNA damage experiments, at 45 h post-transfection cells were either left untreated or were treated with 15 gray ionizing radiation from a cesium-137 source with a 3-h recovery as described (13).

**Plasmid Construction**—The YFP-BARD1 wild-type (wt) and nuclear export signal (NES) mutant expression plasmids were described previously (22). The YFP-tagged BARD1 NLS mutant was described in Schüchner et al. (28). The plasmids pFLAG-BARD1 (1–188; Ref. 22) and pFLAG-BARD1 (171–461, 171–375; Ref. 28) were previously described as indicated, and YFP fusions of these constructs were obtained by amplifying the YFP gene from the pEYFP-C1 vector (Invitrogen) by PCR and inserting into the NotI site of the respective pFLAG-BARD1 plasmids. YFP-BARD1 (1–141 and 551–777) was kindly supplied by Wendy Au (Sydney). A YFP-tagged form of pBARD1(ΔRIN) was constructed by PCR-amplifying the BARD1(ΔRIN) cDNA (31) and inserting it into the Xhol/Xmal sites of pEYFP-C1. The forward primer (with Xhol site) was 5′-ATAATACGAGAAATGCGGATATTGC-3′, and the reverse primer (with Xmal site) was 5′-GACATCCGGGTCAGCTGAAAGGAGG-3′.

**Immunofluorescence Microscopy and Primary Antibodies**—Cells were grown on coverslips at 60% density and fixed in 3.7% formalin (tissue culture grade (Sigma), phosphate-buffered saline for 20 min followed by permeabilization with 0.2% Triton X-100, phosphate-buffered saline for 10 min at 24 h post-transfection and incubated with various antibodies as previously described (22). Live cells were incubated with 100 mM Mitotracker CMX-Ros (Molecular Probes) in culture medium for 30 min at 37 °C, then washed and fixed in ice-cold acetone: methanol (1:1) for 3 min at room temperature. Cells were then blocked using 3% bovine serum albumin in PBS for 1 h followed by the addition of primary antibodies as follows: rabbit polyclonal anti-BARD1 59L antibody (1:500, a kind gift from Prof. Richard Baer), Ethyl (Exon 4) rabbit polyclonal Ab against BARD1 (BL518) Exxon4 (1:500, Ethyl Laboratories); Novus rabbit polyclonal antibody against BARD1 Exxon 4 (NB100-319) (1:500, Novus Biologicals), mouse monoclonal anti-BRCA1 antibodies Ab-1, Ab-4 (1:200, Oncogene Research); mouse monoclonal anti-cytochrome c (1:200, BD Biosciences); mouse monoclonal anti-prohibitin (1:200, Abcam, Cambridge, UK); mouse anti-p53 (DO-1) monoclonal antibody (1:500, Santa Cruz Biotechnology); rabbit polyclonal antibody to apoptosis-inducing factor (1:200, BD Biosciences); anti-Bax polyclonal antibody 2772 against the N terminus (1:200, Cell Signaling Technology).

Bound antibodies were detected with either AlexaFluor 488-conjugated (1:500; Molecular Probes, Inc., Eugene, OR) or biotin-conjugated (1:500; DAKO) secondary anti-mouse or anti-rabbit antibodies. Biotinylated secondary antibodies were subsequently incubated with avidin D-Texas Red (Vector Laboratories, 1:1000 dilution) or AlexaFluor 594 diluted 1:1000 tertiary stains. Mitochondria were also detected by staining with the dye Nonylacridine orange (200 nM; Molecular Probes). Nuclei were counterstained with the DNA dye Hoechst 33285 (Sigma). Coverslips were mounted with Vectorshield aqueous mountant (Vector Laboratories) and observed and photographed using an Olympus BX51 fluorescence microscope at ×400 magnification. A SPOT32 camera was used for general image capture. Cell images for deconvolution were taken using the Axiosview 200M Cell Observer System (Zeiss) at 63× magnification, and z-stacks of ~21 incremental slices (0.3-μm step size) were captured for each cell. The AxioVision 4.5 software was used for image deconvolution and three-dimensional view reconstruction.

**Isolation of Mitochondrial Extracts**—Cells were lysed by mechanical homogenization using a small pestle, and mitochondrial extraction was performed using different mitochondria.
drial extraction kits (Alexis Biochemicals and Qiagen) according to the manufacturer’s instructions. The Qiagen kit benefits from an additional density gradient purification step. In brief, using the Qproteome mitochondria isolation kit (Qiagen), cells were suspended in lysis buffer to disrupt the plasma membrane without solubilizing it and to aid in the isolation of cytosolic proteins. Plasma membranes and compartmentalized organelles, such as nuclei, mitochondria, and the endoplasmic reticulum, remained intact and were pelleted by centrifugation at 1000 × g for 10 min. The resulting pellet was resuspended in disruption buffer, repeatedly passed through a narrow-gauge needle (26 or 21 gauge), and re-centrifuged to pellet nuclei, cell debris, and unbroken cells at 1000 × g for 10 min. The supernatant, which contains mitochondria, was re-centrifuged to pellet mitochondria at 6000 × g for 10 min. After removal of the supernatant, mitochondria were snap-frozen in liquid nitrogen, thawed on ice, and analyzed by SDS-PAGE as outlined below.

Immunoblotting—The mitochondrial or cytosolic fractions or total extracts were denatured in sample buffer (100 mM Tris- HCl, pH 6.8, 20% glycerol, 0.01% bromphenol blue, 10% β-mercaptoethanol, 5% SDS) and analyzed by SDS-PAGE and Western blot. 60–80 μg of protein extracts were loaded per lane and resolved by polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Millipore Corp.). The membranes were blocked using 5% skim milk powder in PBS containing 0.2% Tween 20 for 1 h at room temperature followed by primary antibody incubation for 2 h at room temperature using antibodies against BARD1 (1:1000 from Bethyl and Novus), PCNA (1:5000), β-tubulin (1:3000, Sigma), prohibitin (1:200, Abcam), cytochrome c (1:300, BD Biosciences), heat shock protein 70 (1:300, Affinity Bioreagents), and p53 (1:500, Santa Cruz). Incubation with secondary horseradish peroxidase-conjugated antibodies (1:10,000; Sigma) for 1 h was followed by detection by enhanced chemiluminescence (ECL; Amersham Biosciences). For detection of ectopic YFP-BARD1 proteins, T150 flasks (5 × 10^7 cells) were transfected with YFP-BARD1 constructs (12 μg of DNA and 12 μg of Lipofectamine), and cells were lysed and processed for mitochondrial extraction 24 h post-transfection.

Analysis of Apoptosis by Flow Cytometry, Release of CMX-Ros Dye, and Bax Oligomerization—At 40 h post-transfection, cells were detached with trypsin and resuspended in complete medium. Cells were pelleted by centrifugation at 1500 rpm for 5 min and washed in PBS and then centrifuged again at 1500 rpm for 5 min. Cells were resuspended in 400 μl of PBS and then fixed in 4.5 ml of ice-cold 85% ethanol at 4 °C for at least 1 h. Next, the cells were pelleted by centrifugation at 1500 rpm for 5 min and resuspended in 600 μl of PBS containing RNase A (1 mg/ml) and propidium iodide (2 mg/ml). YFP-expressing cells were gated, and the cell cycle/apoptosis profiles were determined using a BD Biosciences FACScalibur flow cytometer. The percentage of apoptosis in each sample was determined by quantifying the sub-G1 DNA content using CellQuest software. Cellular loss of mitochondria membrane potential was quantified using a single cell assay by scoring for loss of mitochondrial staining of the CMX-Ros MitoTracker dye in transfected cells, as described in Ref. 32. For detection of Bax oligomerization at mitochondria, MCF-7 cells were fixed in methanol-acetone at 4 °C and permeabilized, and cells were analyzed by flow cytometry using a BD Biosciences FACScalibur flow cytometer.

RESULTS

BARD1 Apoptotic Activity Requires Cytoplasmic Localization but Is Not Dependent on Nuclear-Cytoplasmic Shuttling—We previously described the identification of a NES at the N terminus of BARD1 and showed that mutagenesis of this sequence blocked BARD1 nuclear export and inhibited its apoptotic function by 50% (22). To determine whether the translocation of BARD1 from nucleus to cytoplasm contributed to its apoptotic activity, we compared the impact of both NES and NLS mutations. As shown in Fig. 1, transfection of MCF-7 breast tumor cells with wild-type YFP-BARD1 induced apoptosis as measured by flow cytometry, and this activity was reduced by the NES mutation L107A. We compared the effect of mutations in the primary NLS (NLS3) of human BARD1, which we previously showed reduced nuclear import (28). When expressed in MCF-7 cells, the NLS mutation had no effect on apoptosis (Fig. 1). These data indicate that the accumulation of BARD1 in the cytoplasm rather than its ability to shuttle from nucleus to cytoplasm that is important for its apoptotic activity.

![BARD1 Localizes to Mitochondria](image-url)
Detection of BARD1 at Mitochondria

We next examined in detail the cytoplasmic staining patterns of BARD1 using fluorescence microscopy and observed a granular/tubular staining pattern reminiscent of mitochondria. To test whether BARD1 localizes to mitochondria, we stained U2OS cells with different anti-BARD1 antibodies and antibody to the mitochondrial marker cytochrome c (Cyto C; stained red with AlexaFluor 594). Cells were then analyzed by fluorescence microscopy, and representative images revealing mitochondrial localization of endogenous BARD1 are shown. Each of three different BARD1 polyclonal anti-sera showed the same result by immunostaining, and only low background staining was visible in the absence of primary antibody (data not shown). To confirm that the actual overlapping staining patterns reflected true co-localization at mitochondria, we acquired a z-stack series of images using a Zeiss Axiovert microscope and used deconvolution software to resolve trans-sections of the cell. The deconvolved images revealed very clear localization of endogenous BARD1 at mitochondria (see Fig. 2B).

Detection of BARD1 at Mitochondria by Fluorescence Microscopy—We next examined in detail the cytoplasmic staining patterns of BARD1 using fluorescence microscopy and observed a granular/tubular staining pattern reminiscent of mitochondria. To test whether BARD1 localizes to mitochondria, we stained U2OS cells with different anti-BARD1 antibodies and antibody to the mitochondrial marker protein, cytochrome c, then fixed cells with acetone-methanol and analyzed them by fluorescence microscopy. The endogenous BARD1 pattern overlapped with the cytochrome c-positive-stained mitochondria in the cytoplasm (see Fig. 2A). Each of three different BARD1 polyclonal anti-sera showed the same result by immunostaining, and only low background staining was visible in the absence of primary antibody (data not shown). To confirm that the actual overlapping staining patterns reflected true co-localization at mitochondria, we acquired a z-stack series of images using a Zeiss Axiovert microscope and used deconvolution software to resolve trans-sections of the cell. The deconvolved images revealed very clear localization of endogenous BARD1 at mitochondria (see Fig. 2B).

Further control experiments were then performed, and these showed that endogenous BARD1 co-stained with the two mitochondrial-specific dyes Nonylacridine orange and the MitoTracker dye CMX-Ros (Molecular Probes) and also with the mitochondrial protein, prohibitin (see Ref. 34) as illustrated in supplemental Fig. S1. A deconvolution analysis of U2OS cells showed co-localization of BARD1 with prohibitin at mitochondria (supplemental Fig. S2). Moreover, we compared the mitochondrial localization pattern using a range of cell lines and
BARD1 Localizes to Mitochondria

BARD1 was originally discovered as a binding partner of BRCA1, and the two proteins are often detected in cell nuclei as a heterodimer (reviewed in Ref. 6). A recent report by Coene et al. (37) claimed that BRCA1 itself displays a mitochondrial staining pattern, although it was suggested that this was restricted to the hyperphosphorylated form of BRCA1, which is usually detected only in S-phase cells or after DNA damage. We, therefore, asked whether BARD1 staining at the mitochondria is regulated by, or dependent on the expression of BRCA1.

Mitochondrial Targeting of BARD1 Is Not Dependent on BRCA1—To address this question, we first compared the level of mitochondrial BARD1 in breast cancer cells that express wild-type BRCA1 (MCF10A) or truncated mutant BRCA1 (HCC1937). In Western blots, mitochondrial staining of full-length BARD1 was comparable in MCF10A and HCC1937 cells (Fig. 3A, B and C). Interestingly, BARD1 located more strongly at mitochondria (relative to cytoplasm) in the colon cancer cell lines SW480 and HCT116 than in the cell lines MCF-7 (breast cancer), MCF-10A (immortalized breast), and HEK293T (immortalized kidney epithelia). We note that a smaller BARD1 peptide of ~70 kDa was frequently observed in mitochondrial preparations, corresponding in size to a proteolytic cleavage product previously detected in colon cancer cells (36).

BARD1 Is Detected in Mitochondrial Cell Fractions—To confirm our microscopic analysis using a biochemical approach, we examined the localization of BARD1 cellular fractions highly enriched in mitochondria. Endogenous full-length BARD1 was strongly detected as a 97-kDa band in both mitochondrial and cytosolic fractions, as shown for U2OS osteosarcoma cells in Fig. 3A. Two different antibodies each detected wild-type BARD1 in mitochondria in addition to a higher molecular weight band which is yet to be fully characterized but which did not disappear after rigorous phosphatase treatments (data not shown). Integrity of the mitochondrial fractionation was validated by staining with antibody to α-tubulin, a cytoskeletal protein that does not locate at mitochondria, and this antibody detected tubulin in the cytosolic but not mitochondrial fractions as expected (Fig. 3A). Moreover, staining with antibody to the nuclear DNA replication protein PCNA showed no obvious nuclear contamination of the mitochondrial fractions (Fig. 3C and data not shown).

Staining for the mitochondria-localized proteins prohibitin and cytochrome c was positive for the mitochondrial fraction, as was p53, which was previously detected in mitochondria (35). BARD1 was also detected in mitochondrial fractions isolated from other cell lines (Fig. 3, B and C). Interestingly, BARD1 located more strongly at mitochondria in ~90–95% of YFP-transfected cells, and the same result was observed for the YFP-BARD1 (1–188) plasmid. Thus, disruption of the BRCA1-BARD1 interaction does not block mitochondrial staining of BARD1. C, BRCA1 was silenced by siRNA as described previously (22), and cells were stained for both BRCA1 (Ab1) and BARD1 (Bethyl Ab) and then fixed with formalin (similar results seen with methanol-acetone). BARD1 was found to co-stain with BRCA1 in nuclear foci but showed an additional mitochondrial staining in the cytoplasm that did not change after knockdown of BRCA1. The cell images shown are typical of three experiments.

FIGURE 4. BARD1 mitochondrial localization is not dependent on BRCA1. A, MCF-10A (wild-type BRCA1) and HCC1937 (express mutant BRCA1) cells were lysed, and mitochondrial (M) fractions were isolated (see “Materials and Methods”). Cell extracts were separated on 8% SDS-polyacrylamide gels, transferred to nitrocellulose, and analyzed by Western blotting with antibodies to BARD1 (Bethyl), α-tubulin, and prohibitin. There was no significant difference in BARD1 expression at mitochondria. C, cytosol. B, MCF-7 cells were transfected with YFP or YFP-tagged BARD1 (1–188) (contains the BRCA1 binding domain). At 48 h post-transfection, cells were stained for endogenous BARD1 (Bethyl Ab and AlexaFluor 594 red secondary) and for mitochondria with YFP or YFP-tagged BARD1 (1–188) (contains the BRCA1 binding domain). At 48 h post-transfection, cells were stained for both BRCA1 (Ab1) and BARD1 (Bethyl Ab) and then fixed with formalin (similar results seen with methanol-acetone). BARD1 was found to co-stain with BRCA1 in nuclear foci but showed an additional mitochondrial staining in the cytoplasm that did not change after knockdown of BRCA1. The cell images shown are typical of three experiments.
BARD1 Localizes to Mitochondria

**A**

| Sequence       | Relative mitochondrial localization |
|----------------|-------------------------------------|
| 1-141          | ++                                  |
| 1-188          | ++                                  |
| 171-375        | -                                   |
| 171-461        | -                                   |
| 551-777        | ++                                  |
| delta-RIN      | ++                                  |

**B**

| YFP-BARD1 | delta-RIN | 171-461 | 1-188 |
|------------|-----------|---------|-------|
| M          | M         | M       | M     |
| C          | C         | C       | C     |

**Figure 5.** BARD1 is targeted to mitochondria by N- and C-terminal sequences. A, mapping BARD1 mitochondrial targeting sequences. A series of YFP-BARD1 constructs (described in Ref. 28) spanning the length of BARD1 were transiently expressed in U2OS cells, and at 48 h post-transfection, cells were fractionated for mitochondrial enrichment. B, the mitochondrial (M) and cytosolic (C) extracts (−100 μg) were analyzed by Western blotting for BARD1 localization. Ectopic BARD1 wild-type, N-terminal (1–141 and 1–188), and C-terminal (551–777 and ΔRIN) sequences displayed staining in mitochondrial fractions (using anti-YFP or BARD1 antibodies), whereas central sequences (171–375 and 171–461) located poorly in mitochondria. Antibodies against mitochondrial (prohibitin, cytochrome c (cyto C), heat shock protein 70), cytosolic (α-tubulin), or nuclear/cytoplasmic (PCNA) markers were employed as shown. The data shown are typical of two different experiments and reveal that more than one sequence contributes to BARD1 recruitment to mitochondria.

(Fig. 4B and see supplemental Fig. S4 for full color images). Furthermore, we used a BRCA1-specific siRNA and control siRNA to silence endogenous BRCA1 expression as previously described (22). The knockdown of BRCA1 caused a diminution of BARD1 in nuclear foci but did not abolish staining of BARD1 at mitochondria (Fig. 4C). Furthermore, as will be described in the next section, a BARD1 sequence that lacks the BRCA1 binding domain retained the ability to localize at mitochondria (Fig. 5). Collectively, these experiments suggest that BRCA1 is not essential for targeting of BARD1 to mitochondria, although we do not exclude the possibility that the BRCA1-BARD1 heterodimer can assemble at mitochondria.

Identification of Distinct N-terminal and C-terminal Sequences That Contribute to BARD1 Mitochondrial Localization—Computer-assisted analysis of BARD1 did not identify a specific canonical mitochondrial import sequence. We, therefore, used a transient transfection system to map those sequence elements that target BARD1 to mitochondria. A series of fragments that span the length of BARD1 were tested (see Fig. 5A). We found that full-length YFP-BARD1 was consistently enriched in mitochondrial fractions (Fig. 5B). Unexpectedly, sequences that comprised either the N terminus (1–188) or C terminus (551–777) also displayed strong mitochondrial staining. The specificity of this staining is underscored by the lack of mitochondrial staining observed for sequences that locate in the center of BARD1 (i.e. 171–375, 171–461). The N-terminal sequence 1–141 showed a weaker mitochondrial staining than did 1–188, suggesting that the region 141–188 is important. The localization activity of the C terminus explains why the BARD1 splice variant, ΔRIN, also was detected in mitochondrial fractions. This cancer-associated isoform lacks exons 2–6 that contain the RING, BRCA1 binding, and ankyrin repeat domains. The deletion mapping indicates that more than one sequence contributes to BARD1 mitochondrial localization, perhaps reflecting involvement of multiple specific protein interactions.

**Wild-type BARD1, but Not the ΔRIN Splice Variant, Induces Apoptosis and Loss of Mitochondrial Membrane Potential**—We next compared the apoptotic activity of YFP-tagged wild-type BARD1 and the ΔRIN splice variant (deletes exons 2–6 or amino acids 54–523; Ref. 31). When transiently expressed in MCF-7 cells, wild-type BARD1 displayed apoptotic activity measured by flow cytometry and a release of the MitoTracker dye CMX-Ros in live cells correlating with the pre-apoptotic dissipation of mitochondrial membrane potential (32) (Fig. 6). In contrast, the ΔRIN splice variant was targeted to mitochondria (Fig. 5) but did not induce apoptosis or a loss of mitochondrial membrane potential in MCF-7 cells (Fig. 6). This result is consistent with data reported by Tsuzuki (31) and indicate that distinct sequences mediate BARD1 targeting to mitochondria and its apoptotic activity.

**Transient Expression of BARD1, but Not BRCA1, Induces Mitochondrial Bax Oligomerization in Apoptotic Cells**—Several apoptotic pathways converge on Bcl-2 family members at mitochondria, and the induction of Bax oligomerization and subsequent pore formation in the mitochondrial membrane is frequently linked to the apoptotic process (33). We tested the effect of YFP-BARD1 on endogenous Bax staining patterns in MCF-7 cells and found that cells transfected with YFP-BARD1 exhibited a >100% increase in Bax oligomerization compared with YFP-transfected cells (see Fig. 7, A and B, supplemental Fig. S5). To determine the specificity of this regulation, we transfected YFP-BARD1-ΔRIN into MCF-7 cells and found that it did not induce Bax oligomerization above background (i.e. YFP) levels (Fig. 7B). Moreover, YFP-BRCA1, which is pro-apoptotic in MCF-7 cells (30), also had no positive effect on Bax oligomerization. A closer examination of the Bax oligomerization was made in pFLAG-BARD1 transfected cells, and the intense Bax clusters were found to co-localize with the mitochondrial marker protein, prohibitin (Fig. 7C). Interestingly,
visual inspection of the Bax oligomerization-positive cells revealed that ~95% of those cells were apoptotic as indicated by Hoechst-stained abnormal nuclei (e.g. Fig. 7C; supplemental Fig. S5). These data lead us to speculate that the movement of BARD1 to mitochondria has two cellular consequences, loss of mitochondrial membrane potential and apoptosis, and that this cytological damage at least partly involves the induced oligomerization of Bax at mitochondria.

**FIGURE 6.** BARD1, but not BARD1-DΔRIN, induces apoptosis and loss of mitochondrial membrane potential in MCF-7 cells. A, YFP-tagged BARD1 (wt and ΔRIN) were transiently expressed in MCF-7 cells and compared by Western blot analysis of total cell extracts to demonstrate equivalent expression. The transfected cells were assayed in parallel for apoptotic activity by flow cytometry (as in Fig. 1) with graphs showing the mean ± S.E. from at least two independent experiments. The data are expressed relative to cells transfected with YFP plasmid alone. B, YFP and the YFP-tagged forms of BARD1 (wt and ΔRIN) were compared for loss of mitochondrial membrane potential, measured by loss of CMX-Ros staining. Cell images show BARD1-wt and ΔRIN plasmids transiently expressed in MCF-7 cells. The cells were stained before fixation with the Mitotracker dye CMX-Ros (Molecular Probes). Transfected cells were scored for loss of CMX-Ros mitochondrial staining, which is directly indicative of loss of membrane potential (32). C, quantitative comparison of reduced membrane potential. Graphs show the mean ± S.E. from at least two independent experiments. A minimum of 100 transfected cells were counted for each CMX-Ros slide.

**FIGURE 7.** YFP-tagged BARD1, but not BARD1-DΔRIN or BRCA1, induces Bax oligomerization. A, MCF-7 cells transfected with YFP or YFP-BARD1 were stained with Bax antibody (against the N terminus of Bax, Cell Signaling Technology), and transfected cells were analyzed by fluorescence microscopy. A fraction of transfected cells revealed a dramatic increase in staining intensity and clustering of Bax at mitochondria (see the arrows) compared with untransfected cells (representative images shown). B, quantification of cells transfected with different plasmids revealed that YFP-BARD1 induced >100% increase in the proportion of cells with Bax oligomerization compared with YFP alone. YFP-tagged BRCA1 and BARD1-DΔRIN were similar to the YFP control. Data shown are the mean ± S.E. from two experiments, scoring 100 cells per slide. C, MCF-7 cells were transfected with pFlag-BARD1 and stained for Bax and the mitochondrial marker, prohibitin. As shown, only one of two adjoining cells (arrow) displayed Bax oligomerization, and this co-localized with mitochondria. The same cell was apoptotic as revealed by a condensed and abnormal nucleus.

**BARD1 Increases at Mitochondria in Response to DNA Damage**—BARD1 is a known DNA damage response factor, and its expression was previously shown to increase after DNA damage in rodent cells (27). We treated MCF-7 cells with 15 gray of ionizing radiation, a dose that was found to stimulate apoptosis within 24 h (Fig. 8B). Within 3 h of ionizing radiation (IR) treatment, endogenous BARD1 was assayed by fractionation/Western blot and found to modestly increase in localization at mitochondria. An increase was also observed in the presence of a slightly slower migrating band likely to be the phospho-form of BARD1 induced by DNA damage (Fig. 8A). The same result was seen in two separate experiments. Furthermore, a small but consistent IR-dependent increase in mitochondrial BARD1 was detected using fluorescence microscopy (Fig. 8B). Our findings are summarized in a general diagram (Fig. 8C) that speculates on a link between movement of BARD1 to mitochondria and the induction of apoptosis as a part of the cellular response to DNA damage.
DISCUSSION

BARD1 is a putative tumor suppressor and is mutated in a subset of breast and ovarian cancers (2–4). BARD1 was first discovered as a binding partner for BRCA1, and the two proteins are coordinately expressed (5), forming a heterodimer that localizes predominantly in the nucleus (21, 22) and functions in DNA repair. In this study we used a combination of cell imaging and cellular fractionation/Western blotting to demonstrate a new localization site for BARD1; that is, at the mitochondria. BARD1 targeting to mitochondria was not dependent on BRCA1 in that it was not reduced by siRNA-mediated silencing of BRCA1, by peptide-mediated disruption of the BRCA1-BARD1 interaction, or by removal of the BRCA1 binding domain of BARD1. The translocation of BARD1 to mitochondria was increased by DNA damage and correlated with its apoptotic activity, providing an explanation for previous reports that link cytoplasmic localization of BARD1 to apoptosis (22, 29). The BARD1 apoptotic pathway involved stimulation of Bax oligomerization at mitochondria, an activity that was not demonstrated by pro-apoptotic BRCA1. We propose that BARD1 locates at two main cellular sites, the nucleus and mitochondria, and that its movement between these locations may contribute to regulation of cell survival and cell death, respectively (summarized in Fig. 8).

This study was prompted by our previous finding that BARD1 apoptotic activity correlated with its transport from nucleus to cytoplasm (22). Two mechanisms were proposed to explain how nuclear export of BARD1 stimulated apoptosis; (a) BARD1 removed an apoptosis inhibitor from the nucleus, or (b) BARD1 moves to a specific cytoplasmic location to trigger apoptosis. The data presented here favor the second supposition. Microscopic imaging of BARD1 disclosed staining at cytoplasmic tubular structures, which we confirmed as mitochondria (Fig. 2 and supplemental Figs S1–S3). There is very little known of BARD1 function outside of its role in the BRCA1-BARD1 complex, whereby it contributes to several diverse functions including ubiquitin ligase activity targeting substrates such as B23/nucleophosmin (38) and RNA polymerase II (39), DNA repair as part of the DNA damage response (9, 23), and the regulation of mitosis (24). We found that an NLS-mutated form of BARD1 that locates in the cytoplasm and is reduced in its nuclear entry retained a high level of apoptotic activity when transiently expressed in cells (Fig. 1). Conversely, an NES mutant of BARD1 that can enter but not exit the nucleus had reduced apoptotic activity. These findings indicate that BARD1 apoptotic function is correlated more with cytoplasmic positioning than with nuclear-cytoplasmic shuttling.

One likely explanation for the above results is that transiently expressed BARD1 accumulated to a limited extent at mitochondria in MCF-7 cells, where it triggered apoptosis and induced a striking pre-apoptotic loss of mitochondrial membrane potential as evidenced by loss of staining of the Mitotracker dye CMX-Ros (Fig. 6). The mitochondrial targeting of other tumor suppressor proteins including p53 (35) and a truncated form of p19-ARF (32) have also been linked to a loss of membrane potential and cell death. It is intriguing that both BARD1 and p53 display nuclear export activity when transiently expressed in cells (Fig. 1). Conversely, an NES mutant of BARD1 that can enter but not exit the nucleus had reduced apoptotic activity. These findings indicate that BARD1 apoptotic function is correlated more with cytoplasmic positioning than with nuclear-cytoplasmic shuttling.

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Mitochondria are present in almost all eukaryotic species and possess two membrane systems, an outer membrane in close communication with the cytosol and a folded inner membrane in addition to an aqueous matrix compartment (42). Mitochondria provide the cell with energy in the form of ATP produced by oxidative phosphorylation and play key roles in the metabolism of amino acids, iron, and lipids and in apoptosis. Most pro-apoptotic signaling pathways converge at mitochondria (42) and usually involve permeabilization of the outer membrane (43, 44). In this study we showed that transient expression of BARD1, but not BRCA1, increased the oligomerization of Bax at mitochondria and that >90% of those cells were apoptotic. Bax, a cytosolic Bcl-2 family protein, can translocate to mitochondria wherein it oligomerizes and inserts into the outer mitochondrial membrane, causing permeabilization and release of apoptogenic factors from the intermembrane space (44). This response is often induced by DNA damage (45).

In our preliminary experiments we observed a modest increase in BARD1 mitochondrial staining after DNA damage (Fig. 8), similar to but lower than that reported for p53, which is strongly targeted to mitochondria after genotoxic stress (35, 46). It is, therefore, possible that the BARD1 apoptotic effect is regulated by its interaction with other apoptotic regulators at mitochondria, such as p53, with which BARD1 was previously shown to associate (7, 27).

The BARD1 gene is mutated in a small frequency of breast/ovarian tumors (2–4), and one of the frequently reported mutations, Q564H, has been linked to defects in BARD1 apoptotic activity (27). We tested a YFP-tagged fusion of the BARD1 Q564H mutant; however, we observed no reduction in its ability to induce apoptosis (data not shown). We tested another naturally occurring splice variant of BARD1, ARIN (31), which has deleted the internal exons 2–6 including the RING domain and Ankyrin repeats, and found that this sequence exhibited very low levels of apoptosis. This is consistent with low apoptotic activity previously described for this fragment (31) and may explain why it is frequently detected in ovarian cancers (7).

We searched for a specific mitochondrial targeting signal in BARD1; however, like several other proteins (e.g. p53) that locate to the mitochondria, BARD1 does not possess a classical targeting element. Instead, our mapping data revealed the involvement of at least two different sequences located at opposing ends of BARD1. This suggests that the interaction with two or more proteins, as yet undefined, stimulates BARD1 mitochondrial recruitment.

The phosphorylated form of BRCA1 was previously reported to locate at mitochondria, and Coene et al. (37) used electron microscopy to provide evidence supporting a pool of BRCA1 co-locating with mitochondrial DNA in the matrix region. This led to the postulate that BRCA1 might contribute not only to maintenance of the nuclear genome but also the mitochondrial genome. A similar proposal was made for p53, which co-fractionated with certain mitochondrial DNA base excision repair enzymes in mitochondria (47). The propensity of BARD1 to heterodimerize with BRCA1 suggests that the heterodimer could assemble in both the nucleus and the mitochondria. We note that the heterodimeric form of BARD1 is less apoptotic than the monomeric form in MCF-7 cells (22, 30), raising the possibility that the BARD1-BRCA1 complex may contribute more to the repair of mitochondrial DNA damage. It is possible that the action of BARD1 in apoptosis or cell survival will be determined by distinct stimuli and response pathways and by its association with different binding partners. Future experiments that address these issues in the context of regulation of BARD1 at mitochondria should provide new insights into its multifunctional nature and potentially its regulated shuttling and action at different organelles.

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*BARD1 Localizes to Mitochondria*