Hyperphosphorylation of the BARD1 Tumor Suppressor in Mitotic Cells*\[S\]

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Atish D. Choudhury, Hong Xu, Ami P. Modi, Wenzhu Zhang, Thomas Ludwig, and Richard Baer

From the Institute for Cancer Genetics and the Departments of Pathology and Anatomy and Cell Biology, Columbia University Medical Center, New York, New York 10032

Although the BRCA1 tumor suppressor has been implicated in a number of cellular processes, it plays an especially important role in the DNA damage response as a regulator of cell cycle checkpoints and DNA repair pathways. In vivo, BRCA1 exists as a heterodimer with the BARD1 protein, and many of its biological functions are mediated by the BRCA1-BARD1 complex. Here, we show that BARD1 is phosphorylated in a cell cycle-dependent manner and that the hyperphosphorylated forms of BARD1 predominate during M phase. By mobility shift analysis and mass spectrometry, we have identified seven sites of mitotic phosphorylation within BARD1. All sites exist within either an SP or TP sequence, and two sites resemble the consensus motif recognized by cyclin-dependent kinases. To examine the functional consequences of BARD1 phosphorylation, we used a gene targeting knock-in approach to generate isogenic cell lines that express either wild-type or mutant forms of the BARD1 polypeptide. Analysis of these lines in clonogenic survival assays revealed that cells bearing phosphorylation site mutations are hypersensitive to mitomycin C, a genotoxic agent that induces interstrand DNA cross-links. These results implicate BARD1 phosphorylation in the cellular response to DNA damage.

Although hereditary breast cancer can often be traced to germ line mutations in the BRCA1 tumor suppressor gene, it is still unclear how BRCA1 lesions promote mammary carcinogenesis (1). The major isoform of BRCA1 is a large polypeptide that contains a RING domain at its N terminus and two tandem copies of the BRCT domain at the C terminus (2). In vivo, BRCA1 exists primarily as a heterodimer with BARD1, a distinct but related protein that also harbors an N-terminal RING motif and two C-terminal BRCT domains (3). Both proteins colocalize within the same nuclear foci of S phase cells (4, 5), and their interaction is mediated by sequences encompassing their respective RING motifs (3). Co-immunoprecipitation analysis indicates that most of the cellular pool of endogenous BRCA1 exists in the form of a heterodimer with BARD1 (6, 7), and structural studies have shown that dimerization occurs through a four-helix bundle formed by α-helices flanking the RING motifs of both proteins (8). Because the phenotypes of mice null for either Brca1 or Bard1 are essentially indistinguishable (9), the major functions of both proteins are likely to be mediated by the BRCA1/BARD1 heterodimer. This notion is supported by the fact that BRCA1 and BARD1 together form a potent enzymatic complex that can catalyze ubiquitin polymerization in vitro (10–17). Recent studies have also shown that BARD1 is required for nuclear retention of BRCA1 (18) and that, together with BRCA1, it modulates mRNA processing during the DNA damage response (19, 20) and promotes homology-directed repair of chromosomal breaks (21).

It is now apparent that phosphorylation exerts important controls over BRCA1 function. Early studies established that BRCA1 is hyperphosphorylated in several cellular settings. During normal cell cycle progression, hyperphosphorylation of BRCA1 occurs at the G1/S transition, and various phosphorylated forms of BRCA1 are apparent throughout the subsequent S, G2, and M phases (5, 22–25). In addition, some BRCA1 phosphorylation events are induced in response to growth factor signaling (26, 27). The phosphorylation status of BRCA1 also changes dramatically when cells are subjected to genotoxic stress (5, 24). For example, in cells exposed to ionizing radiation (IR), the ATM kinase phosphorylates BRCA1 at a series of sites that includes Ser1387, Ser1423, and Ser1524 (28, 29), whereas Chk2 phosphorylates Ser988 (30). Indeed, several of these modifications have now been implicated in certain downstream functions of BRCA1. Thus, BRCA1 phosphorylation at Ser1387 is required for the IR-induced intra-S (but not G2/M) checkpoint (31), whereas phosphorylation at Ser1423 is necessary for the IR-induced G2/M (but not intra-S) checkpoint (32, 33). In addition, phosphorylation at Ser988 (but not Ser1387 or Ser1423) is required for BRCA1 modulation of homologous and non-homologous DNA recombination (34). These results indicate that the downstream functions of BRCA1 can be influenced by specific phosphorylation events.

By most measures, the biological functions of BRCA1 appear to be mediated through the heterodimeric BRCA1/BARD1 complex (35). Here, we provide evidence that BARD1 is also subject to post-translational phosphorylation and that hyperphosphorylated forms of BARD1 arise primarily during mitosis. Each of the seven identified BARD1 phosphorylation sites resides...
within an SP or TP sequence motif, indicating the involvement of proline-directed kinases. Significantly, cells that express mutant BARD1 polypeptides that lack these phosphorylation sites are hypersensitive to genotoxic stress, suggesting a role for BARD1 phosphorylation in the cellular response to DNA damage.

EXPERIMENTAL PROCEDURES

Cell Culture—The HCT116, HeLa, and H1299 cell lines were obtained from American Type Culture Collection. HCT116 cells were cultured in McCoy's 5A medium, and HeLa and H1299 cells were cultured in Dulbecco's modified Eagle's medium; both media were supplemented with 100 μg/ml penicillin/streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. For cell cycle analysis, HeLa cells were synchronized in prometaphase by a thymidine/nocodazole block and subjected to fluorescence-activated cell sorting (FACS) and Western analysis as described previously (36).

Antibodies—The BARD1-specific polyclonal antiserum (3) and monoclonal antibody (36) have been described. Other antibodies included anti-α-tubulin (Ab-1, Oncogene Science Inc.); anti-BRCA1 C-20, anti-Cdc27 (H-300), and anti-hemagglutinin (HA) (Y-11, all from Santa Cruz Biotechnology, Inc.); and anti-FLAG (M2, Sigma). A phosopho-specific antiserum that recognizes phospho-Thr299 of human BARD1 was generated and affinity-purified at Zymed Laboratories Inc. (South San Francisco, CA) from rabbits immunized with the phosphothreonine-containing peptide CSPRNVEPTPEK conjugated to keyhole limpet hemocyanin. For detection of exogenous BARD1 polypeptides with the phospho-Thr299-specific antiserum (e.g. Fig. 5B), direct Western analysis was performed as described (36), except that 5% bovine serum albumin was used (instead of milk) in the TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) buffer for blocking and for primary antibody incubation. For detection of endogenous BARD1 polypeptides with the phospho-specific antiserum (see Fig. 5C), cell lysates in 50 μl of low salt Nonidet P-40 buffer (supplemented as described previously (36), except using 2× phosphate inhibitors: 10 mM NaF and 0.2 mM Na3VO4) were precleared with 50 μl of protein G-Sepharose beads (20% slurry) and then incubated overnight at 4 °C. The beads were washed once with low salt Nonidet P-40 buffer, twice with high salt (1M NaCl) Nonidet P-40 buffer, and once again with low salt Nonidet P-40 buffer. The bound proteins were eluted from the beads by denaturing at 100 °C in 2× sample buffer, and once again with low salt Nonidet P-40 buffer. The bound proteins were then incubated with 25 μl of anti-FLAG antibody M2 beads (20% slurry) overnight at 4 °C. The beads were washed three times with 2× Buffer A (20 mM HEPES, pH 7.9, 200 mM NaCl, 0.2% Nonidet P-40, and 20% glycerol) and eluted twice for 3 h each with 500 μg/ml of phospho-specific antiserum in 100 μl of BC200 buffer. The eluted protein was then concentrated and subjected to electrophoresis in a 10% polyacrylamide gel containing 2% sodium dodecyl sulfate. The gel was stained with Coomassie blue, dried, and exposed to x-ray film. For detection of tryptic peptides derived from the phospho-Thr299 residue, trichloroacetic acid-precipitated pellets were resuspended in 20 μl of 1× protein loading dye, denatured at 70 °C for 10 min, and alkylated by incubation with 2 μl of 125 mM iodoacetamide for 1 h in the dark (37). The sample was separated by SDS-PAGE on a 4–12% Tris-glycine gel, which was then fixed and stained with GelCode Blue stain reagent (Pierce) according to the manufacturer's protocol.

Phosphorylation of BARD1—To generate cell lines that stably express an exogenous BARD1 polypeptide, H1299 cells were transfected with a mammalian expression plasmid encoding human full-length BARD1 with a N-terminal FLAG epitope (NH2-MDYKD-DDKGGSGS) and a C-terminal HA epitope (GGYPYDVPDYA-COOH) in the pIRESpuro vector (Clontech). H1299 cell transformants were then selected in 2 μg/ml puromycin and maintained in 1 μg/ml puromycin. To generate FLAG-BARD1-HA polypeptides for mass spectrometry, H1299 cells expressing FLAG-BARD1-HA (subclone 7) were seeded on 16 cm-diameter plates; and at ~50% confluence, the cells were treated with 100 ng/ml nocodazole for 16 h. The cells were then collected by scraping to yield a cell pellet of ~1 ml. All lysis buffers were supplemented with 0.1 mM dithiothreitol, protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride and Roche Protease Inhibitor Cocktail), and phosphatase inhibitors (5 mM NaF and 0.1 mM Na3VO4). The cell pellet was resuspended in 25 ml of Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA) for 15 min, followed by vigorous shaking for 15 s upon addition of 0.5 μl of 10% Nonidet P-40. After centrifugation at 3000 rpm for 5 min, the pellet was lysed in 2.5 ml of Buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA) by vortexing for 15 min at 4 °C. After centrifugation at 3000 rpm for 5 min, the supernatant was then added to 2.5 ml of Buffer D (20 mM HEPES, pH 7.9, 1 mM EDTA) and 100 μl of 10% Nonidet P-40. The lysates were clarified by centrifugation at 25,000 × g for 30 min and filtration through a 0.45-μm filter. The lysates were then incubated with 250 μl of anti-FLAG antibody M2 beads (20% slurry) overnight at 4 °C. The beads were washed three times with BC200 buffer (20 mM Tris-HCl, pH 7.9, 200 mM NaCl, 0.2% Nonidet P-40, and 20% glycerol) and eluted twice for 3 h each with 500 μg/ml of phospho-specific antiserum in 100 μl of BC200 buffer. The eluted protein was then concentrated and subjected to electrophoresis in a 10% polyacrylamide gel containing 2% sodium dodecyl sulfate. After phosphorylation, the trichloroacetic acid-precipitated pellet was resuspended in 20 μl of 1× protein loading dye, denatured at 70 °C for 10 min, and alkylated by incubation with 2 μl of 125 mM iodoacetamide for 1 h in the dark (37). The sample was separated by SDS-PAGE on a 4–12% Tris-glycine gel, which was then fixed and stained with GelCode Blue stain reagent (Pierce) according to the manufacturer's protocol.

Analysis of BARD1 Phosphorylation Sites—BARD1 in-gel enzymatic digestion and extraction from gel bands were performed essentially as described (38, 39). Briefly, two Coomassie Blue-stained BARD1 protein bands containing a total protein amount of 1–2 μg were excised and divided into four portions. Each portion was digested with 0.2 μg of modified trypsin, endopeptidase Lys-C, endopeptidase Asp-N, or endopeptidase Glu-C (Roche Applied Science). After digestion, each portion was added to 10 μl of 1% trifluoroacetic acid containing 0.5 μl (dry volume) of a 1:1 mixture of POROS R2 and R3 beads (Applied Biosystems, Foster City, CA) directly into the solution containing the gel pieces. The peptides were extracted in the presence of the POROS beads for 3–12 h. The solution containing the POROS beads was aspirated into an Eppendorf Geloader tip pinched at its tip. The column formed at the pipette tip was washed with 0.1% trifluoroacetic acid in water, and the peptides were directly eluted onto the MALDI plate with 1 μl of matrix solution (2× dilution of a saturated solution of 2,5-dihydroxybenzoic acid in 60% methanol and 5% acetic acid). BARD1 phosphorylation sites were analyzed by MALDI-QqTOF mass spectrometry and tandem mass spectrometry on a QStar XL mass spectrometer with an oMALDI ion source. MALDI-phosphopeptide fragmentation was generated in a proteolytic digest by a nitrogen laser operating at 20 Hz. MALDI mass spectra were collected at m/z 700–7000 for 2 min. MALDI tandem mass spectra were acquired for a period of time ranging from 1 to 6 min with the binning factor set at 8 (maximum number available in Analyst QS SP7) for sensitive fragment ion detection.

BARD1 Knock-out Targeting Vector (see Fig. 6A)—The targeting constructs were generated using the pBlueScript II-SK plasmid (Stratagene) as a backbone. The homology arms were derived from bacteriophage λ clones containing human genomic BARD1 DNA (40). The 5′-homology arm spans a 3.6-kb region that includes the first 23 bp of exon 2 and sequences from the preceding intron. The 3′-homology arm spans a 3.6-kb region immediately downstream of exon 3. The arms flank a cassette that includes 1) the hygromycin resistance gene flanked by two loxP sites, 2) the herpes simplex virus thymidine kinase polyadenylation signal, and 3) the primer sequence GTGACAGAAGATT-GTCCTGTGGTGC to facilitate PCR screening of transformants. The diptheria toxin gene was inserted 5′ of the left homology arm for negative selection of random integration events.

BARD1 Knock-in Targeting Vector (see Fig. 6B)—The 5′-arm was derived from the targeting construct containing human genomic BARD1 DNA (40) and spans a 3.6-kb region that includes the first 23 bp of exon 2 and sequences from the preceding intron. The 3′-homology arm was generated by PCR amplification of genomic DNA from HCT116 cells with Platinum Taq High Fidelity polymerase (Invitrogen); it corresponds to a 3.5-kb region of genomic DNA including exon 3 and sequences from the preceding intron. The arms flank a cassette that includes 1) cDNA sequences encoding residues 61–777 of human BARD1 phosphorylation in the cellular response to DNA damage.
Phosphorylation of the BARD1 Tumor Suppressor

Fig. 1. BARD1 is hyperphosphorylated in mitotic cells. A, electrophoretic mobility shift analysis of BARD1 in nocodazole-arrested cells. HeLa cells were synchronized in prometaphase by a thymidine/nocodazole block. The mitotic cells were then collected by shake-off, and at time 0, the cells were replated at low density to induce cell cycle progression from prometaphase. Cells harvested at various times (1–5 h) after induction were subjected to FACScan analysis to determine the cell cycle distribution and to Western analysis to monitor expression of BARD1, BRCA1, Cdc27, and α-tubulin. Asynchronous cells (first lane) and nocodazole-arrested cells (N; second lane) were also examined. B, the electrophoretic mobility shift of mitotic BARD1 is attributable to phosphorylation. Lysates of asynchronous HeLa cells (lanes 1 and 2), nocodazole-arrested cells (lanes 3 and 4), and cells released from nocodazole block for 2 h (lanes 5 and 6) or 5 h (lanes 7 and 8) were incubated in the presence (+) or absence (−) of λ-phosphatase (λ-Pase). The digested lysates were then examined by immunoblotting with the BARD1-specific antisera, C, the hyperphosphorylated BARD1 species of mitotic cells interact with BRCA1. Lysates of asynchronous HeLa cells (lane 1), nocodazole-arrested cells (lane 2), and cells released from nocodazole block for 2 h (lane 3) or 5 h (lane 4) were immunoprecipitated (IP) with the anti-BARD1 (upper panel) or anti-BRCA1 (lower panel) monoclonal antibody. The immunoprecipitates were then examined by Western blotting (WB) with the anti-BARD1 polyclonal antiserum. D, localization of the mitotic BARD1 phosphorylation sites by electrophoretic mobility shift analysis. HeLa cells were transfected with expression plasmids encoding each of the indicated fragments of BARD1 fused to an N-terminal tag containing three tandem copies of theFLAG epitope. The transfected cells were allowed to cycle asynchronously (odd-numbered lanes) or were arrested in prometaphase by a thymidine/nocodazole block (even-numbered lanes). The electrophoretic mobilities of the transfected BARD1 fragments were examined by immunoblotting with the anti-FLAG antibody. As shown, mitotic mobility shifts were apparent with BARD1 fragments 129–228 (lane 1), 189–287 (lane 2), 249–349 (lane 3), 309–409 (lane 4), and 369–469 (lane 5).

RESULTS

BARD1 Is Hyperphosphorylated during Mitosis—Our previous study showed that BARD1 expression varies during cell cycle progression in a manner reminiscent of BRCA1 (36). Thus, the steady-state levels of BARD1 polypeptides are low in resting (G0) cells and G1 cycling cells, but increase markedly as cycling cells enter S phase and remain high throughout G2 phase and mitosis. In the course of this study, we also noticed that the electrophoretic mobility of BARD1 from nocodazole-treated cells is retarded on SDS-polyacrylamide gels relative to that of BARD1 from unsynchronized cells. To investigate this phenomenon further, HeLa cells were arrested in prometaphase by a thymidine/nocodazole block, released into nocodazole-free medium, and collected at hourly time points. The BARD1 polypeptides from these cells were then visualized by Western analysis. As shown in Fig. 1A, the electrophoretic mobilities of the mitotic BARD1 polypeptides were retarded relative to that of the major BARD1 isoform from asynchronous cells (compare first and second lanes). The mobility shifts of mitotic BARD1 are due to phosphorylation because λ-phosphatase treatment converted these isoforms to a hypermobile band

BARD1; 2) an internal ribosome entry site followed by the neomycin resistance gene, with both flanked by loxP sites; 3) the herpes simplex virus thymidine kinase polyadenylation signal; and 4) the primer sequence ACTTGTCCAAGTTACAAAGCTTAGTAGACAGC to facilitate PCR screening of transformants. The diphtheria toxin gene was inserted 3′ of the right homology arm for negative selection of random integration events.

Transfection and Selection of HCT116 Cells—The targeting vectors were linearized by restriction digestion with NotI (Promega) and purified by phenol/chloroform extraction and precipitation in 70% ethanol. Cells (6 × 10⁶) were seeded on each of three 100-mm plates; after 20 h, the cells on each plate (at ~70% confluence) were transfected with 10 µg of the linearized targeting construct and 50 µl of Lipofectamine in serum-free Opti-MEM I according to the protocol of Invitrogen. 24 h after transfection, the cells from each plate were trypsinized and seeded on twenty 96-well plates in medium containing 100 µg/ml hygromycin B (Roche Applied Science) for knock-out targeting or 300–325 µg/ml G418 (Invitrogen) for knock-in targeting. Starting after ~19 days, cells from wells containing a single colony were transferred to fresh 96-well plates in medium containing 50 µg/ml hygromycin B or 100 µg/ml G418. After screening, cells were cultured in medium without the drug used for selection.

Screening HCT116 Transformants—Cells from each well of a 96-well plate were trypsinized and collected, leaving ~20% to repopulate the well; and genomic DNA was purified using the QIamp DNA blood mini kit (Qiagen Inc.). Transformants were screened by PCR using Platinum Taq High Fidelity polymerase according to the protocol of Invitrogen with primers GTGACAAGGAATGTCCTGTGGTGCG and GTCCATGATTTGAATCTGTCACATGC for knock-in targeting and with primers AGCTTTGCAAGTTACAAAGCTTAGTAGACAGC and CCACCGGGTGTCACATGC for knock-out targeting. The genomic DNAs from six clones were linearized by restriction digestion with NotI (Promega) and purified using the QIAamp DNA blood mini kit, and 10-µg aliquots of BglII-digested DNA were subjected to Southern hybridization with the radiolabeled BARD1 5′-probe (a 1.3-kb segment of genomic BARD1 located between exon 1 and the region corresponding to the 5′-homology arm of the targeting constructs).

Western Analysis of HCT116 Transformants—Cells were arrested by a thymidine/nocodazole block as described above and collected by scrap-
Phosphorylation of the BARD1 Tumor Suppressor

FIG. 2. Identification of mitotic BARD1 phosphorylation sites by mutation analysis. A, electrophoretic mobility shift analysis of BARD1 fragment 280–388. HeLa cells were transfected with expression plasmids encoding FLAG-tagged BARD1 fragment 280–388 with the wild-type BARD1 sequence (lanes 1 and 2) or with the indicated amino acid substitutions (lanes 3–8). The transfected cells were allowed to cycle asynchronously (A) or were arrested in prometaphase by a thymidine/nocodazole block (N). The electrophoretic mobilities of the transfected BARD1 fragments were then examined by immunoblotting with the anti-FLAG antibody. B, analysis of BARD1 fragment 189–287. HeLa cells were transfected with expression plasmids encoding FLAG-tagged BARD1 fragment 189–287 with the wild-type BARD1 sequence (lane 1) or with the indicated amino acid substitutions (lanes 2–10). The transfected cells were allowed to cycle asynchronously (lanes 7 and 9) or were arrested in prometaphase by a thymidine/nocodazole block (lanes 1–6, 8, and 10), and the transfected BARD1 fragments were examined by immunoblotting with the anti-FLAG antibody. C, analysis of BARD1 fragment 146–240. HeLa cells were transfected with expression plasmids encoding FLAG-tagged BARD1 fragment 146–240 with the wild-type BARD1 sequence (lanes 9 and 10) or with the indicated amino acid substitutions (lanes 1–8 and 11–16). The BARD1 fragments from asynchronous (odd-numbered lanes) and nocodazole-arrested (even-numbered lanes) cells were examined by immunoblotting with the anti-FLAG antibody. D, analysis of BARD1 fragment 146–240. HeLa cells were transfected with expression plasmids encoding FLAG-tagged BARD1 fragment 146–240 with the wild-type BARD1 sequence (lanes 2 and 5) or with the indicated amino acid substitutions (lanes 1, 3, 4, and 6–9). The BARD1 fragments from asynchronous (lanes 1–3) and nocodazole-arrested (lanes 4–9) cells were examined by immunoblotting with the anti-FLAG antibody. E, analysis of BARD1 fragment 280–435. HeLa cells were transfected with expression plasmids encoding FLAG-tagged BARD1 fragment 280–435 with the wild-type BARD1 sequence (lane 1) or with the indicated amino acid substitutions (lanes 2–5). The BARD1 fragments from nocodazole-arrested cells were examined by immunoblotting with the anti-FLAG antibody.

(Fig. 1B, compare lanes 3 and 4). Hyperphosphorylated BARD1 was absent, however, in cells that had progressed into G2 phase after being released from the nocodazole block for >2 h (Fig. 1A). In this respect, the pattern of BARD1 phosphorylation parallels that of Cdc27, a component of the anaphase-promoting complex known to be specifically phosphorylated during mitosis (Fig. 1A). Thus, the hyperphosphorylated BARD1 polypeptides of mitotic cells are either degraded or dephosphorylated at the M/G1 transition.

To ascertain whether the hyperphosphorylated BARD1 species of mitotic cells retain the capacity to interact with BRCA1, synchronized HeLa cell lysates were immunoprecipitated with either the anti-BARD1 or anti-BRCA1 monoclonal antibody, and the immunoprecipitates were examined by immunoblotting with the BARD1-specific antibody. As shown in Fig. 1C, equivalent proportions of hypophosphorylated and hyperphosphorylated BARD1 were recovered in the BARD1 (upper panel) and BRCA1 (lower panel) immunoprecipitates. Thus, mitotic phosphorylation of BARD1 does not appreciably alter its in vivo association with BRCA1.

Mapping BARD1 Phosphorylation Sites by Mutation Analysis—To identify the mitotic phosphorylation sites of BARD1, we initially tested whether individual fragments of the BARD1 polypeptide display an electrophoretic mobility shift when expressed exogenously in nocodazole-arrested cells. Thus, HeLa cells were transfected with a series of expression vectors encoding different BARD1 fragments with a common N-terminal tag containing three tandem FLAG epitopes. Lysates were then prepared from 1) cells grown asynchronously and 2) cells arrested in prometaphase with nocodazole. Western analysis of the cell lysates with the anti-FLAG antibody revealed that fragments containing BARD1 amino acids 129–228, 189–287, and 280–388 were visibly shifted in mitotic cells compared with asynchronously cycling cells (Fig. 1D, lanes 4, 6, and 8). The remaining BARD1 fragments (residues 1–120, 385–488, 464–574, 563–681, and 661–777) did not display an obvious mobility shift in mitotic cells.

The consensus sequence for phosphorylation by the CDK1-cyclin B complex, the major cyclin-dependent kinase of mitotic cells, is (S/T)PX(S/R)K (41). BARD1 fragment 280–388 contains several sequences of this type, two of which are conserved in the mouse: Ser288 and Thr299. As shown in Fig. 2A, the mitotic mobility shift of this fragment was abolished by mutation of Thr299 to Ala (lane 6), but not mutation of Ser288 to Ala (lane 4). Thus, Thr299 is a potential mitotic phosphorylation site of BARD1.

Because there are no obvious phosphorylation motifs within BARD1 fragment 189–287, we used site-directed mutagenesis
sites. In addition, a careful comparison of both Ser184 and Ser186 (Cdues 146–240 (Fig. 2), mitotic BARD1 phosphorylation site.

As shown, phosphorylation of some (Ser251, Thr299, Ser391, and Thr394), but not all (Ser148, Ser184, and Ser186), of the phosphorylation sites yielded a visible mitotic shift in the context of full-length BARD1.

to mutate all serine and threonine residues within this interval to alanine. Simultaneous mutation of both Ser184 and Ser186 abolished the mitotic shift of this fragment (Fig. 2B, lane 4), as did mutation of Ser251 alone (lane 10), but not Ser394 alone (lane 8), indicating that Ser251 represents another possible mitotic BARD1 phosphorylation site.

A similar strategy of exhaustive mutagenesis within residues 146–240 (Fig. 2C) revealed that simultaneous mutation of both Ser184 and Ser186 (lanes 8 and 16), but not Ser184 alone (lane 12) or Ser186 alone (lane 14), substantially reduced the mitotic shift of BARD1 fragment 146–240. This indicates that both Ser184 and Ser186 are potential mitotic phosphorylation sites. In addition, a careful comparison of lanes 7 and 8 suggests that fragment 146–240 still retains a modest mitotic shift even when it harbors both the S184A and S186A mutations. This shift was not observed, however, in a fragment bearing simultaneous mutations of Ser148, Ser151, Ser184, and Ser186 (Fig. 2D, lanes 7–9). Thus, it is likely that Ser184 and/or Ser186 represents an additional site of BARD1 phosphorylation during mitosis. Phosphorylation at Ser186 (but not Ser184) was confirmed by mass spectrometric analysis of BARD1 polypeptides purified from mitotic cells (see below).

Although the T299A mutation abolished the mitotic shift of fragment 280–386 (Fig. 2A), the shift of BARD1 fragment 280–435 was only partly reduced by this mutation (Fig. 2E, lane 2). Thus, additional phosphorylation sites exist between residues 388 and 435 of BARD1. Because each of the five phosphorylation sites identified above resides within an SP or TP sequence, we evaluated the effect of mutating the three remaining SP/TP sequences (Ser251, Ser294, and Ser401) in fragment 280–435. Indeed, simultaneous alanine substitution of Thr299, Ser294, and Thr394 abolished the mitotic shift of this fragment (lane 5). Because smaller mitotic shifts were also apparent in fragments bearing either the T299A and S394A mutations (lane 4) or the T299A and S391A mutants (lane 3), Thr394 and Ser294 both represent potential mitotic BARD1 phosphorylation sites.

Thus, mutation analysis identified seven potential phosphorylation sites responsible for the visible mitotic shift of exogenous BARD1 fragments during nocodazole block (Fig. 2). All seven sites are present in SP/TP motifs, and two of these, Ser148 and Thr299, resemble the cyclin-dependent kinase consensus sequence. As shown in Fig. 3, a full-length BARD1 polypeptide that bears alanine substitutions at all seven sites did not demonstrate a mitotic shift in HeLa cells (lane 2). When each phosphorylation site was individually restored to this polypeptide, Ser251, Thr299, Ser391, and Thr394 (lanes 10, 12, 14, and 16), but not Ser148, Ser184, and Ser186 (lanes 4, 6, and 8), generated a detectable mitotic shift. Thus, phosphorylation of some (but not all) of the identified sites yields a visible mitotic shift in the context of full-length BARD1.

Mapping BARD1 Phosphorylation Sites by Mass Spectrometry—Our mutation analysis identified seven potential mitotic phosphorylation sites in BARD1. To confirm these results, we performed mass spectrometric analysis of BARD1 polypeptides purified from mitotic cells. To obtain a source of purified cellular BARD1 polypeptides, we stably transformed human lung carcinoma H1299 cells with a puromycin-resistant expression vector encoding full-length BARD1 with an N-terminal FLAG epitope and a C-terminal HA epitope (MDYKDDDDKGGS) and a C-terminal HA epitope (GGYPYDVPDYA). Cell extracts from puromycin-resistant H1299 subclones (subclones 2, 5, 7, and 8) that express endogenous BARD1-HA were subjected to affinity chromatography using anti-FLAG antibody beads. Bound proteins were eluted from the beads with FLAG peptide (Sigma), fractionated by SDS-PAGE, and visualized by staining with colloidal Coomassie Blue. 10- and 50-ng aliquots of bovine serum albumin (BSA) were fractionated in separate lanes. The mobilities of the molecular mass markers (in kilodaltons) are indicated on the left, and the FLAG-BARD1-HA band is marked with an arrow.

![Fig. 3. Individual phosphorylation sites confer electrophoretic mobility shifts on full-length BARD1 polypeptides.](Image)

![Fig. 4. Purification of BARD1 from mitotic cells.](Image)
BARD1-specific antiserum. As shown in Fig. 4A (upper panel), FLAG-BARD1-HA polypeptides were readily detected in immunoprecipitates from H1299 subclones 2, 5, 7, and 8. When the extracts were examined by direct immunoblotting with the BARD1-specific antiserum, each of these four subclones displayed a clearly migrating doublet that included both endogenous BARD1 and the exogenous FLAG-BARD1-HA polypeptide (lower panel). The expression levels of exogenous FLAG-BARD1-HA varied in the different subclones, ranging from ∼2- to 10-fold higher than that of endogenous BARD1.

For purification of FLAG-BARD1-HA, cultured cells of H1299 subclone 7 were arrested in mitosis with nocodazole. A lysate of these cells was immunoprecipitated with anti-FLAG antibody-conjugated agarose (Sigma), and the immunoprecipitated protein was eluted with the FLAG peptide. The eluate was denatured, alkylated with iodoacetamide, and separated by SDS-PAGE (Fig. 4B). The band corresponding to FLAG-BARD1-HA was then excised from the gel and digested with a protease encompassing this phosphothreonine residue. To confirm the phospho-specificity of the affinity-purified antisera, exogenous FLAG-BARD1-HA polypeptides were immunoprecipitated from cell lysates of H1299 subclone 7 (Fig. 4A) with the anti-FLAG monoclonal antibody; the immunoprecipitates were fractionated by SDS-PAGE, and phosphorylated BARD1 was detected by immunoblotting with the phospho-Thr299-specific antiserum. As shown in Fig. 5A, Thr299-phosphorylated FLAG-BARD1-HA was observed in untreated immunoprecipitates (first lane), but not in immunoprecipitates treated with λ-phosphatase (second lane). To evaluate Thr299 phosphorylation with respect to cell cycle progression, the FLAG-BARD1-HA-expressing H1299 cells were arrested in prometaphase with nocodazole, released into nocodazole-free medium, and collected at various time points. The BARD1 polypeptides from these cells were then visualized by Western analysis. As shown in Fig. 5B, the exogenous FLAG-BARD1-HA polypeptide was expressed at all time points after nocodazole release, consistent with the fact that its transcription is driven by the cytomegalovirus promoter of the expression plasmid. In contrast, Thr299-phosphorylated BARD1, although prominent in nocodazole-arrested cells, was absent in cells released from the nocodazole block for 9–15 h. However, it reappeared in cells released for 17 h, a time when the synchronized cell population began to enter the subsequent M phase. Thus, Thr299 phosphorylation occurs in a cell cycle-dependent manner. To ascertain whether Thr299 phosphorylation of endogenous BARD1 polypeptides is also regulated with respect to the cell cycle, HeLa cells were arrested in prometaphase with nocodazole, released into nocodazole-free medium, and collected at various time points. As shown in Fig. 5C, BARD1 polypeptides were observed at time points corresponding to late G1 phase (9 h) and S phase (13 and 16 h). However, Thr299-phosphorylated BARD1 did not reappear until later time points, when the synchronized cells had entered the subsequent M phase (20 and 23 h).

**Generating Stable Cell Lines That Express Only Mutant Forms of BARD1—** Functional analysis of BRCA1 phosphorylation has benefited from the use of HCC1937, a human cell line derived from a familial breast tumor that expresses truncated (but not full-length) BRCA1 (43). Indeed, specific functions have been ascribed to individual BRCA1 phosphorylation sites by comparing the phenotypes of HCC1937 derivatives reconstituted with either full-length wild-type BRCA1 or full-length mutant BRCA1 bearing defined phosphorylation site substitutions (31–34). However, because mutant BARD1 cell lines are not currently available, this approach is not applicable to the analysis of BARD1 phosphorylation. Instead, we sought to generate cells expressing mutant BARD1 by applying a combined knock-out/knock-in strategy in HCT116, a line of human colorectal carcinoma cells that has been used for somatic cell knock-out of numerous genes (44), including p53, p21, SMAD4, securnin, 14-3-3σ, and DNMT1 (45–50). For this purpose, we constructed a hygromycin-based promoterless targeting vector to knock-out the BARD1 gene by homologous recombination (Fig. 6A). The coding sequences of human BARD1 are derived from 11 exons that are distributed over ∼65 kb of genomic DNA (40). As illustrated in Fig. 6A, homologous recombination with the knock-out vector would replace a 4.9-kb fragment of genomic BARD1 DNA encompassing coding exon 3 and part of coding exon 2 with a hygromycin cassette that is in-frame with the remaining exon 2 coding sequence. As a result, the “knock-out” allele would encode a hybrid protein that includes the N-terminal 60 residues of BARD1 fused to the hygromycin gene product. Because most of the RING domain is encoded by the deleted sequences of exons 2 and 3, the knock-out allele cannot encode an enzymatically active BARD1 polypeptide. Upon transfection of HCT116 cells with this vector, we screened hygromycin-resistant clones for BARD1 targeting using a rapid PCR strategy (49), and properly targeted clones were confirmed by Southern analysis (data not shown). With this approach, we obtained five independent HCT116 clones that were successfully targeted on one of the two BARD1 alleles.

**Identification of BARD1 Phosphorylation Sites by Mass Spectrometry**

| Phosphorylation sites | MS/MS<sup>a</sup> analyzed phosphopeptide | Enzyme | m/z |
|-----------------------|------------------------------------------|-------|-----|
| Ser<sup>247</sup>     | 145-MWFSPSR<sup>150</sup>               | Trypsin | 903.3 |
| Ser<sup>250</sup>     | 172-DASQQDSYEFVpSP<sub>p</sub>SPPADYSER<sup>194</sup> | Trypsin | 2642.0 |
| Ser<sup>250</sup>     | 379-KNSMDFISLpSPGTPPTSLSSSYR<sup>265</sup> | Trypsin | 3049.3 |
| Thr<sup>299</sup>     | 165-MWFSPSR<sup>189</sup>               | Lys-C  | 1118.5 |
| Thr<sup>299</sup>     | 235-SNEVTPER<sup>302</sup>              | Lys-C  | 1228.6 |
| Ser<sup>251</sup>     | 178-DSYEFVpSP<sub>p</sub>SP<sub>p</sub>SP<sup>189</sup> | Asp-N  | 1455.5 |
| Ser<sup>251</sup>     | 235-SKQLVSFSQPSVSP<sub>p</sub>SQINGE<sup>257</sup> | Glu-C  | 2600.2 |

<sup>a</sup> MS/MS, tandem mass spectrometry.

<sup>b</sup> Mass spectrometry analysis alone narrowed down the phosphorylation site to Ser<sup>247</sup>, Ser<sup>250</sup>, and Ser<sup>251</sup>. Ser<sup>251</sup> is the likely site of phosphorylation based on mutation analysis (Fig. 2B).
Phosphorylation of the BARD1 Tumor Suppressor

Fig. 5. Phosphorylation of BARD1 Thr299 is regulated with respect to the cell cycle. A, specificity of the phospho-Thr299-specific antiserum. Cell lysates of nocodazole-arrested FLAG-BARD1-HA-expressing H1299 cells (subclone 7) were immunoprecipitated (IP) with the anti-FLAG monoclonal antibody. Both untreated and phospho-Thr299-specific antiserum were then examined by Western blotting (WB) with either the BARD1-specific antiserum (upper panel) or the phospho-Thr299-specific antiserum (α pT299; lower panel). B, mitotic phosphorylation at Thr299 on exogenous FLAG-BARD1-HA polypeptides. FLAG-BARD1-HA-expressing H1299 cells (subclone 7) were synchronized in prometaphase by a thymidine/nocodazole block (N). The mitotic cells were then collected by shake-off, and at time 0, the cells were replated at low density to induce cell cycle progression from prometaphase. Cells harvested at various times (0–23 h) after induction were subjected to FACs analyses to determine the cell cycle distribution, and cell lysates were analyzed by immunoblotting with the anti-HA1 antibody (upper panel), the phospho-Thr299-specific antiserum (middle panel), and the anti-α-tubulin antibody (lower panel). C, mitotic phosphorylation at Thr299 on endogenous BARD1 polyptides. HeLa cells were synchronized by a thymidine/nocodazole block. The mitotic cells were then collected and replated at low density to induce cell cycle progression from prometaphase. Cells harvested at various times (0–23 h) after induction were subjected to FACs analyses to determine the cell cycle distribution, and cell lysates were analyzed by immunoblotting with the anti-HA1 antibody (upper panel), the phospho-Thr299-specific antiserum (middle panel), and the anti-α-tubulin antibody (lower panel).

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g the remaining wild-type allele of BARD1+/− cells with a neomycin-based derivative of the knock-out vector. However, these experiments repeatedly generated neomycin-resistant subclones in which the inactive (but not the wild-type) BARD1 allele was disrupted, suggesting that BARD1 expression is essential for the survival of HCT116 cells in culture. This result is consistent with a previous study in which a conserved gene targeting failed to generate viable clones of Bard1-null mouse embryonic stem cells (9).

Having rendered one allele of BARD1 inactive, we used a “knock-in” strategy to replace the remaining germ line allele of BARD1+/− HCT116 cells with CDNA sequences encoding either wild-type or mutant BARD1. In this way, we sought to generate a series of isogenic HCT116 clones that express only either a wild-type or mutant form of BARD1. We initially replaced the functional BARD1 allele of BARD1+/− HCT116 cells with a CDNA sequence encoding wild-type BARD1. To do so, the knock-in targeting vector illustrated in Fig. 6B was constructed. Upon homologous recombination, this vector would replace a 1.3-kb fragment of genomic BARD1 DNA encompassing the 3′-region of coding exon 2 with a cassette that includes both the wild-type CDNA sequence for residues 61–777 of BARD1 and a neomycin coding sequence (Fig. 6B). The vector was designed such that the incorporated BARD1 cDNA sequence of this cassette is in-frame with the retained segment of BARD1 coding exon 2, thereby recreating a full-length BARD1 coding sequence whose expression is driven by the endogenous BARD1 promoter. Expression of the neomycin sequence is likewise driven by the BARD1 promoter, but requires an internal ribosome entry site for protein translation.

BARD1+/− HCT116 cells were then transfected with this vector, and >1000 neomycin-resistant clones were screened in pools of six for BARD1 targeting events by long-range PCR. Oligonucleotide primers were designed such that PCR amplification would yield DNA fragments of different sizes from the wild-type (4.85 kb) and knock-in (3.8 kb) alleles, but would not amplify a fragment from the knock-out allele (which lacks a priming site for one of the two primers). As shown in Fig. 7A, PCR amplification of genomic DNA identified rare pools of neomycin-resistant clones that harbored the PCR fragment expected of a properly targeted knock-in BARD1 allele. The relevant clones from these pools were then identified, and their genotypes were confirmed by both PCR amplification (Fig. 7B) and Southern analysis (Fig. 7C). As illustrated in Fig. 7C, this experiment yielded seven subclones of BARD1+/− HCT116 cells in which the germ line BARD1 allele was successfully replaced with the wild-type knock-in BARD1 cassette. The genotype of these subclones was designated BARD1wt− (Fig. 6C).

To evaluate the function of the BARD1 phosphorylation sites, two mutant derivatives of the knock-in targeting vector were constructed. One derivative, BARD1 mutant A (mutA), harbored alanine substitutions at the two phosphorylation sites that resemble the cyclin-dependent kinase consensus sequence: Ser184 and Thr299. The other derivative, BARD1 mutant B (mutB), carried alanine substitutions at the five remaining phosphorylation sites: Ser148, Ser186, Ser251, Ser391, and Thr394. With these targeting vectors, the same knock-in strategy was used to replace the remaining germ line allele of BARD1+/− HCT116 cells with CDNA sequences encoding mutant BARD1. In this manner, we generated five independent subclones of BARD1+/− HCT116 cells with CDNA sequences encoding mutant BARD1. In this manner, we generated five independent subclones of BARD1+/− HCT116 cells with CDNA sequences encoding mutant BARD1. In this manner, we generated five independent subclones of BARD1+/− HCT116 cells with CDNA sequences encoding mutant BARD1. In this manner, we generated five independent subclones of BARD1+/− HCT116 cells with CDNA sequences encoding mutant BARD1. In this manner, we generated five independent subclones of BARD1+/− HCT116 cells with CDNA sequences encoding mutant BARD1. This subclone was designated BARD1mutA− and BARD1mutB−, respectively (Fig. 6C).

Close scrutiny of the Southern blots revealed that some subclones displayed unequal ratios of the knock-out and knock-in alleles (i.e. BARD1mutA− subclone 2 and BARD1mutB− subclones 9, 11, and 13), suggesting amplification of either the knock-out or knock-in chromosome; these subclones were not used in subsequent studies.

To confirm proper expression of the knock-in BARD1 alleles, extracts were prepared from the parental BARD1+/− HCT116 cells and from multiple independent subclones representing each of the three knock-in genotypes (BARD1wt−, BARD1mutA−, and BARD1mutB−). The extracts were then im-

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H. Xu and R. Baer, unpublished data.
FIG. 6. Knock-out/knock-in strategy for gene targeting in the BARD1 locus of HCT116 cells. A, knocking out the BARD1 locus. The map of the germ line BARD1 locus illustrates the first three coding exons of BARD1; the AUG initiation codon is also indicated. The knock-out targeting vector is illustrated below the germ line BARD1 locus: the 5′-homology (“left”) arm has 3.6 kb of genomic BARD1 DNA that includes the 5′-segment of coding exon 2, and the 3′-homology (“right”) arm has 3.6 kb of genomic DNA. The two arms encompass a hygromycin cassette arranged such that the hygromycin coding sequence (hyg, denoted by a rectangle) is in-frame with the BARD1 coding sequences of exon 2. Another cassette containing the diphtheria toxin gene (DT, denoted by a triangle) driven by the human β-actin gene promoter was included in the vector to select for recovery of homologous recombination events. Upon homologous recombination, a 4.9-kb fragment of genomic BARD1 DNA encompassing coding exon 3 and the 3′-segment of coding exon 2 is replaced with the hygromycin cassette. On the resultant knock-out allele, transcription of the hygromycin gene is driven by the BARD1 promoter and employs the herpes simplex virus thymidine kinase polyadenylation signal at the 3′-end of the hygromycin cassette. The positions of the PCR primers used to screen for gene targeting events are indicated with arrows. The triangles flanking the hygromycin coding sequence represent lox recombination signals not relevant to the current experiment. B, knocking in the BARD1 cDNA sequence. The knock-in targeting vector is illustrated below the germ line BARD1 locus: the left arm has 3.6 kb of genomic BARD1 DNA that includes the 5′-segment of coding exon 2, and the right arm has 3.6 kb of genomic DNA. The two arms encompass a cassette containing BARD1 cDNA sequences and a neomycin gene (each denoted by a rectangle). The cDNA sequences, which encode BARD1 residues 61–777, are arranged in-frame with the 5′-segment of coding exon 2 so as to recreate the full-length BARD1 coding sequence. Another cassette containing the diphtheria toxin gene (denoted by a rectangle) driven by the human β-actin gene promoter was included in the vector to select for recovery of homologous recombination events. Upon proper recombination, a 1.3-kb fragment of genomic BARD1 DNA containing the 3′-segment of coding exon 2 is replaced with the BARD1-neomycin cassette. On the resultant knock-in allele, transcription of the BARD1-neomycin cassette is driven by the endogenous BARD1 promoter and employs the herpes simplex virus thymidine kinase polyadenylation signal. Translation of the neomycin promoter is facilitated by an internal ribosome entry site. The positions of the PCR primers used to screen for gene targeting events are indicated with arrows. The triangles flanking the internal ribosome entry site (IRES)-neomycin sequence are lox recombination signals not relevant to the current experiment. C, structures of the BARD1 alleles represented in the four HCT116 genotypes. All four genotypes contain one knock-out BARD1 allele. BARD1wt−/− cells also contain a germ line BARD1 allele. In BARD1mutB−/− cells, the remaining germ line BARD1 allele is replaced with a knock-in allele encoding wild-type BARD1. In BARD1mutA−/− and BARD1mutB−/− cells, the germ line BARD1 allele is replaced with a knock-in allele encoding BARD1 mutA (S148A and T299A) or BARD1 mutB (S184A, S186A, S251A, S391A, and T394A), respectively.
Phosphorylation of the BARD1 Tumor Suppressor

Phosphorylation of the BRCA1 tumor suppressor occurs with cell cycle progression and during the cellular response to DNA damage (5, 22–30), and some of these phosphorylation events have been shown to impact specific BRCA1 functions (31–34). Because the downstream effects of BRCA1 are mediated primarily through the BRCA1/BARD1 complex, post-translational modifications of the BARD1 polypeptide are also likely to influence BRCA1-mediated tumor suppression. Here, we have shown that BARD1 is phosphorylated in a cell cycle-dependent manner and that hyperphosphorylated BARD1 polypeptides accumulate primarily during mitosis. This differs from the major pattern observed with BRCA1, in which hyperphosphorylated BRCA1 species become prominent at the G2/S transition and remain so throughout the subsequent G2 and M phases. Nonetheless, a recent study has shown that at least one site within BRCA1 is phosphorylated at the G2/M transition (53). This event, which is mediated by the mitotic kinase Aurora A (53), should be contemporaneous with the cell cycle-dependent phosphorylation of BARD1 reported here. The fact that both BRCA1 and BARD1 are subjected to specific post-translational modifications during M phase is consistent with growing evidence that BRCA1 has important functions during mitosis (36, 53–60) and that disruption of these functions may be partly responsible for the genomic instability observed in Brca1-deficient cells (61, 62).

In this study, we identified seven residues of BARD1 that are phosphorylated specifically in cells treated with nocodazole, a microtubule poison that induces prometaphase arrest by activating the spindle assembly checkpoint. As such, in unstressed cells, these sites are likely to be phosphorylated prior to or during mitosis. It is possible, however, that some of these modifications do not occur in unperturbed cells, but instead represent specific responses to nocodazole treatment. Using a phospho-specific antiserum, we have shown that mitotic phosphorylation of BARD1 does indeed occur at Thr299 independently of nocodazole treatment. Formal proof that the other six sites are also modified in unperturbed cells will require production of the cognate phospho-specific antibody reagents. Interestingly, all seven BARD1 phosphorylation sites occur within either an SP or TP sequence, suggesting the involvement of proline-directed kinases. Two sites (Ser148 and Thr299) resemble the consensus sequence (S/T)P(X)R/K) recognized by cyclin-dependent kinases (41) and as such could be substrates for the mitotic CDK1-cyclin B1 complex (60). In contrast, none of the other sites bears a clear resemblance to current consensus motifs for the two other major mitotic kinases, PLK1 and Aurora A (63, 64).

In a recent study, Hayami et al. (60) also observed mitotic phosphorylation of BARD1 and showed that an N-terminal fragment of BARD1 (residues 1–320) is an efficient substrate for in vitro phosphorylation with either CDK2-cyclin E1 or CDK1-cyclin A1. They also demonstrated that this fragment (but not a corresponding fragment bearing simultaneous alanine substitutions at Ser148, Ser251, and Thr299) is hyperphosphorylated upon cotransfection with either CDK2-cyclin E1/A1 or CDK1-cyclin B1. Although we did not obtain evidence for Ser288 phosphorylation in our study, we did observe phosphorylation at Ser148, Ser251, and Thr299 by both mutation analysis and mass spectrometry. Remarkably, Hayami et al. (60) found that cotransfection with CDK2-cyclin E1/A1 (but not CDK1-cyclin B1) reduced the enzymatic activity of the BCC1/BARD1 complex by inducing nuclear export of BRCA1. However, this behavior could not be attributed to BARD1 phosphorylation because BARD1 polypeptides harboring substitutions at the four sites (Ser148, Ser251, Ser288, and...
Thr\textsuperscript{299}) were also down-regulated by cotransfection with CDK2-cyclin E\textsubscript{1}/A\textsubscript{1}.

To evaluate the functional consequences of BARD1 phosphorylation, we devised a knock-in strategy to generate isogenic cell lines that express only wild-type BARD1 or defined BARD1 mutants. This entailed knocking out one allele of \textit{BARD1} in HCT116 colorectal carcinoma cells and replacing the remaining allele with a knock-in \textit{BARD1} cDNA construct. In this manner, we generated knock-in HCT116 subclones that encode 1) wild-type BARD1, 2) BARD1 mutA (harboring the S148A and T299A mutations), or BARD1 mutB (harboring the S184A, S186A, S251A, S391A, and T394A mutations). In contrast, we were unable to derive \textit{BARD1}-null subclones of HCT116 by knocking out both \textit{BARD1} alleles in succession. This is consistent with a previous futile attempt to generate \textit{Bard1}\textsuperscript{-null} embryonic stem cells (9) and suggests that BARD1 function is essential for the viability of both embryonic stem cells and HCT116 cells. However, the fact that we successfully obtained subclones expressing the mutA and mutB forms of BARD1 indicates that these are hypomorphic mutations and that BARD1 phosphorylation, at least at the seven sites ablated in these mutants, is not required for cell viability.

Although the BRCA1 tumor suppressor is involved in a diverse spectrum of cellular processes, it plays an especially important role in the cellular response to genotoxic stress (65–67). In particular, BRCA1 is required for several of the cell cycle checkpoints activated by DNA damage and has also been implicated in certain repair pathways, such as homology-directed repair of double-stranded DNA breaks (66). As such, cells bearing hypomorphic lesions of BRCA1 show enhanced sensitivity to DNA-damaging agents, especially those that induce interstrand DNA cross-links, such as mitomycin C (51, 52). Recent studies have shown that inactivation of BARD1 also impairs homology-directed repair and promotes chromosome instability (9, 21). In this study, we observed that cells expressing BARD1 mutA (which lacks the Ser\textsuperscript{148} and Thr\textsuperscript{299} phosphorylation sites) or BARD1 mutB (which lacks the Ser\textsuperscript{184}, Ser\textsuperscript{186}, Ser\textsuperscript{251}, Ser\textsuperscript{391}, and Thr\textsuperscript{394} sites) are hypersensitive to the DNA-cross-linking reagent mitomycin C. Although the precise molecular mechanism by which these cells are rendered hypersensitive is not known, these data implicate BARD1 phosphorylation in the cellular response to genotoxic stress.

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\textbf{REFERENCES}
1. Wooster, R., and Weber, B. L. (2003) \textit{N. Engl. J. Med.} \textbf{348}, 2339–2347
2. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., Ding, W., Bell, R., Rosenbluh, J., Hussey, C., Tran, T., McClure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher, H., Yakumo, K., Ghodsi, M., Shaffer, D., Stone, S., Bauer, S., Wray, C., Bogden, R., Dayanand, P., Ward, J., Tonin, P., Narod, S., Bristow, P. R., Norris, P. H., Holzinger, L., Morris, P., Rosteck, P., Lai, M., Barrett, J. C., Lewis, C., Neuhausen, S., Cannon-Albright, L., Coldgar, D., Wiseman, R., Kamb, A., and Skolnick, M. H. (1994) \textit{Science} \textbf{266}, 66–71
3. Wu, L. C., Wang, Z. W., Tsai, J. T., Spellman, M. A., Phung, A., Xu, X. L., Yang, M.-C. W., Hwang, L.-Y., Bowcock, A. M., and Baer, B. (1996) \textit{Nat. Genet.} \textbf{14}, 430–440
4. Jin, Y., Xu, X. L., Yang, M.-C. W., Wei, F., Aiy, T.-C., Bowcock, A. M., and Baer,
Hyperphosphorylation of the BARD1 Tumor Suppressor in Mitotic Cells
Atish D. Choudhury, Hong Xu, Ami P. Modi, Wenzhu Zhang, Thomas Ludwig and Richard Baer

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