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The UBXN1 Protein Associates with Autoubiquitinated Forms of the BRCA1 Tumor Suppressor and Inhibits Its Enzymatic Function[▽]

Foon Wu-Baer, Thomas Ludwig, and Richard Baer*

Institute for Cancer Genetics, Department of Pathology and Cell Biology, and Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, New York, New York 10032

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Although the BRCA1 tumor suppressor has been implicated in many cellular processes, the biochemical mechanisms by which it influences these diverse pathways are poorly understood. The only known enzymatic function of BRCA1 is the E3 ubiquitin ligase activity mediated by its highly conserved RING domain. *In vivo*, BRCA1 associates with the BARD1 polypeptide to form a heterodimeric BRCA1/BARD1 complex that catalyzes autoubiquitination of BRCA1 and *trans* ubiquitination of other protein substrates. In most cases, BRCA1-dependent ubiquitination generates polyubiquitin chains bearing an unconventional K6 linkage that does not appear to target proteins for proteasomal degradation. Since ubiquitin-dependent processes are usually mediated by cellular receptors with ubiquitin-binding motifs, we screened for proteins that specifically bind autoubiquitinated BRCA1. Here we report that the UBXN1 polypeptide, which contains a ubiquitin-associated (UBA) motif, recognizes autoubiquitinated BRCA1. This occurs through a bipartite interaction in which the UBA domain of UBXN1 binds K6-linked polyubiquitin chains conjugated to BRCA1 while the C-terminal sequences of UBXN1 bind the BRCA1/BARD1 heterodimer in a ubiquitin-independent fashion. Significantly, the E3 ligase activity of BRCA1/BARD1 is dramatically reduced in the presence of UBXN1, suggesting that UBXN1 regulates the enzymatic function of BRCA1 in a manner that is dependent on its ubiquitination status.

Germ line mutations of the BRCA1 tumor suppressor are responsible for many cases of hereditary breast and ovarian cancer (49). However, the mechanisms by which these mutations promote tumorigenesis have been difficult to ascertain, in part because BRCA1 has been implicated in a broad spectrum of cellular processes that includes—but is not limited to—cell cycle checkpoint control, DNA repair, mitotic spindle assembly, centrosome function, and transcriptional regulation (33, 34, 38, 51). The major isoform of BRCA1 is a large protein that harbors an N-terminal RING domain and two tandem C-terminal BRCT motifs (32). *In vivo*, BRCA1 associates with the structurally similar BARD1 protein to form a heterodimer that has E3 ubiquitin ligase activity (16, 50). Mice bearing null mutations of either *Brca1* or *Bard1* undergo embryonic lethality in indistinguishable manners, suggesting that the early developmental functions of both proteins are determined by the Brca1/Bard1 heterodimer (30). In addition, conditional inactivation of either gene in mouse mammary epithelial cells induces breast carcinomas that are phenotypically interchangeable and closely resemble the human breast tumors of BRCA1 mutation carriers (44). Thus, the BRCA1/BARD1 heterodimer is likely to mediate many aspects of BRCA1 function, including its critical role as a tumor suppressor in breast and ovarian tissues (1, 44).

The E3 ubiquitin ligase activity of BRCA1/BARD1 is the only known enzymatic function of BRCA1 (4, 16, 29, 54). E3 ligases catalyze the formation of an isopeptide linkage between the C-terminal carboxyl group of ubiquitin and the ε amino group in a lysine side chain of the protein substrate (37). Once ubiquitin is conjugated to the substrate, it can serve as a nucleus for assembly of a polyubiquitin chain in which each additional ubiquitin monomer forms an isopeptide bond with a lysine of the previously attached ubiquitin. The ubiquitin molecule has seven lysine residues that can potentially act as sites of attachment during chain assembly, and polymerization within a given chain is often restricted to certain lysine residues (e.g., K48 or K63) (37). In addition, "linear" ubiquitin chains can be assembled through canonical peptide bonds involving the C-terminal carboxyl group of one ubiquitin monomer and the N-terminal α amino group of the adjacent monomer (24).

In eukaryotes, proteins are often conjugated to K48-linked chains in which each consecutive ubiquitin monomer forms an isopeptide bond with lysine residue 48 of the previously attached monomer (3). These proteins are usually targeted for degradation, presumably because 19S proteasomal particles specifically recognize K48-linked polyubiquitin (12). However, polyubiquitin chains assembled through the other lysines of ubiquitin are also observed *in vivo*, and in some cases these function independently of proteasomal degradation (5, 20). For example, K63-linked chains confer nonproteolytic signals that control certain signaling pathways, including postreplication DNA repair in *Saccharomyces cerevisiae* and NF-κB activation in mammalian cells.

Structural studies suggest that the linkage specificity of polyubiquitination is determined in part by the orientation of the donor and accepter ubiquitin molecules within the catalytic complex formed by the E3 ligase and its cognate ubiquitin-charged E2 conjugating enzyme (11, 48). As such, linkage specificity should be an intrinsic feature of a given E3/E2 complex. In association with E2 enzymes of the UbcH5 family,

* Corresponding author. Mailing address: Institute for Cancer Genetics, Columbia University Medical Center, Irving Cancer Research Center, Rm. 503A, 1130 St. Nicholas Avenue, New York, NY 10032. Phone: (212) 851-5275. Fax: (212) 851-5220. E-mail: rb670@columbia.edu.

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BRCA1/BARD1 catalyzes *in vitro* polyubiquitination through an unconventional linkage involving lysine residue K6 of ubiquitin (35, 53). Indeed, although BRCA1/BARD1 is reported to ubiquitinate a number of proteins *in vitro* (4, 10, 25, 29, 35, 43, 45, 52, 53, 55), to date only four of these potential substrates (NPM, CtIP, RPB8, and BRCA1 itself) have been shown to be ubiquitinated *in vivo* in a BRCA1-dependent manner, and each was found to be conjugated to K6-linked chains (35, 43, 52, 55). Interestingly, the steady-state levels of these substrates are not reduced by BRCA1/BARD1-dependent ubiquitination, suggesting that K6-linked chains do not promote proteasomal degradation in a manner analogous to that for K48-linked polyubiquitin (35, 43, 52, 53, 55). As such, the functional consequences of BRCA1-mediated ubiquitination with K6-linked chains remain unclear.

Recent studies have uncovered a number of amino acid motifs that bind polyubiquitin and mediate downstream events initiated by polyubiquitination, such as proteasomal degradation and other proteasome-independent processes (15, 17, 18). This growing list of ubiquitin-binding motifs includes the ubiquitin-interacting motif (UIM), the ubiquitin-associated (UBA) domain, the Npl4 zinc finger (NZF), and the NEMO ubiquitin-binding domain (NUB/CoZi/UBAN). Several of these motifs bind with greater affinity to either monoubiquitin or polyubiquitin, and a subset of the latter can also discriminate between structurally distinct polyubiquitin chains in a manner that reflects their cellular function. For example, proteins conjugated to K48-linked polyubiquitin are targeted for degradation by cellular receptors that comprise either constitutive (e.g., Rpn10/S5a and Rpn13) or transient (Rad23 and Dsk2) subunits of the 19S proteasome particle (12, 19). Of note, these receptors harbor ubiquitin-binding domains that recognize K48-linked polymers in preference to monoubiquitin or other forms (e.g., K63 linked) of polyubiquitin. As an example of a proteasome-independent process, the RIP1 polypeptide is conjugated to at least two distinct forms of polyubiquitin, K63-linked chains and linear chains, during NF-κB signaling (5, 47). Polyubiquitinated RIP1 can then bind the regulatory subunits (TAB2/3 and NEMO, respectively) of the TAK1 and IKK kinase complexes, leading to enzymatic activation of both kinases and consequent induction of NF-κB signaling (5, 41). As such, TAB2/3 and NEMO serve as cellular receptors that recognize and execute the signaling functions of polyubiquitinated RIP. Significantly, the TAB2/3 and NEMO receptors each contain ubiquitin-binding domains (NZF and NUB/CoZi/UBAN, respectively) that specifically recognize either K63-linked or linear forms of polyubiquitin (9, 23, 26, 41). In a similar fashion, the downstream functions of BRCA1/BARD1 substrates may also be mediated by cellular receptors bearing an appropriate ubiquitin-binding domain.

Here we report that the UBXN1 protein serves as a cellular receptor for autoubiquitinated BRCA1. UBXN1 recognizes autoubiquitinated BRCA1 in a bipartite manner such that its UBA domain binds the K6-linked polyubiquitin chains conjugated to BRCA1 while its C-terminal sequences interact with the BRCA1/BARD1 heterodimer in a ubiquitin-independent fashion. As such, UBXN1 has the properties expected of a cellular receptor for K6-linked chains conjugated to BRCA1. Moreover, UBXN1 markedly inhibits the E3 ligase activity of BRCA1/BARD1. This inhibition requires the ubiquitin-binding activity of UBXN1, suggesting that UBXN1 suppression of BRCA1/BARD1 enzymatic functions is dependent on the ubiquitination state of BRCA1.

MATERIALS AND METHODS

Expression plasmids and two-hybrid screening. The following bacterial expression plasmids were constructed. The 6H-HA-BR304/pET14 plasmid, derived from the pET14b bacterial expression vector (Novagen), encodes BRCA1 residues 1 to 304, with an N-terminal tag of 47 amino acids that includes the hexahistidine sequence (6H) and three tandem hemagglutinin (HA) epitopes (3×HA). The BARD1*/pET28a plasmid, derived from the pET28a bacterial expression vector (Novagen), encodes untagged full-length BARD1. The GST-UBXN1/pGEX-KG plasmid, derived from the pGEX-KG bacterial expression vector, encodes a glutathione *S*-transferase (GST) fusion polypeptide containing residues 10 to 297 of UBXN1. The UBXN1/pMAL-c2 plasmid, derived from the pMAL-c2 bacterial expression vector, encodes a maltose-binding protein (MBP) fusion polypeptide containing full-length (residues 1 to 297) UBXN1. Plasmids encoding MBP fusion polypeptides containing various BRCA1 segments (see Fig. 3A), full-length UBXN1 (wild type and mutants) (see Fig. 10), and various UBXN1 segments (see Fig. 4) were constructed by inserting PCR-generated DNA fragments into the pMAL-c2 vector. Mammalian expression plasmids encoding ΔBRCA1 have been described previously (6). The 6H-Ub_2/pCMV mammalian expression plasmid was constructed to encode a polyprotein comprised of two tandem copies of His_6-tagged ubiquitin (CMV is cytomegalovirus). As with natural ubiquitin polyproteins, this product is processed posttranslationally into His_6-Ub monomers. Mammalian expression plasmids encoding full-length wild-type UBXN1 and its mutant derivatives were obtained by inserting PCR-generated cDNA fragments into the pCMV4 vector. To generate a bait for two-hybrid screening, the myc-(BR304-wt)-BARD/pGBKT7 yeast expression plasmid was constructed to encode a fusion polypeptide containing the DNA binding domain of yeast GAL4 (residues 1 to 147), a myc epitope tag, the N-terminal 304 amino acids of human BRCA1, and full-length human BARD1. A human lymphocyte cDNA library (HL4006AE) was then screened using a MatchMaker GAL4 two-hybrid system (BD Biosciences Clontech) by Leu-Trp-His triple selection according to the manufacturer's instructions.

Recombinant proteins and UBXN1-specific antibodies. The expression and purification of ubiquitin monomers, the 6H-UbcH5c conjugating enzyme, and full-length wild-type and I26A mutant BRCA1/BARD1 heterodimers have been described previously (53). For bacterial expression of glutathione *S*-transferase (GST) and maltose-binding protein (MBP) fusion polypeptides, Rosetta(DE3)/pLysS cells (Novagen) transformed with the appropriate expression plasmid were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and harvested 3 h later. The cell pellets were resuspended in a binding buffer (50 mM HEPES, pH 7.9, 0.4 mM NaCl, 0.1% NP-40, 10% glycerol) containing complete EDTA-free protease inhibitors (Roche) and 1 mM dithiothreitol (DTT), and cell suspensions were stored at −80°C. After thawing, the cell suspensions were lysed by sonication and centrifuged at 12,000 rpm for 15 min, and the supernatants were collected and bound in batch at 4°C for 1 h to 2.5 ml of the appropriate affinity resin (glutathione agarose or amylose agarose) equilibrated in binding buffer. The bound resins were then washed with buffer A (25 mM Tris-Cl, pH 7.9, 50 mM NaCl, 0.1% NP-40, 10% glycerol) containing 1 mM DTT. The GST fusion proteins bound to glutathione agarose resin were eluted with buffer A containing DTT and 10 mM glutathione, while the MBP fusion proteins bound to amylose resin were eluted with buffer A containing 1 mM DTT and 10 mM maltose. The eluted GST and MBP fusion proteins were desalted on PD10 columns (GE Healthcare) in buffer A with 1 mM DTT and stored at −80°C. Rabbit polyclonal antisera 960 and 961 were raised against a GST fusion protein containing the C-terminal 289 amino acids of human UBXN1 (residues 10 to 298).

GST and MBP pulldown assays. For each GST pulldown assay, 10 ng of GST or GST fusion protein was incubated with 100 ng of MBP fusion protein in 50 μl of "binding buffer" (50 mM HEPES-NaOH, pH 7.9, 250 mM NaCl, 0.1% NP-40, 10% glycerol, and 1 mM DTT) for 30 min at 4°C. After 10 μl of preequilibrated glutathione resin was added (1:1 with binding buffer), the mixture was rotated for 1 h at 4°C, washed three times with binding buffer, and boiled for 3 min in 30 μl of 2× sodium dodecyl sulfate (SDS) loading buffer (53). The supernatant was then fractionated by SDS-PAGE and immunoblotted with UBXN1 polyclonal (antiserum 961), BRCA1 monoclonal (Ab1; Calbiochem), ubiquitin monoclonal (P4D1; Santa Cruz), MBP polyclonal (New England BioLabs), GST polyclonal (Z-5; Santa Cruz), or HA monoclonal (12CA5) antibody. For each MBP pulldown assay, 10 ng of MBP or MBP fusion protein was incubated in 50 μl of

binding buffer for 30 min at 4°C with 75 ng of 6H-HA-BR304 polypeptide or 6H-HA-BR304/BARD1 heterodimer (see Fig. 2), 150 ng of baculovirus full-length BRCA1/BARD1 heterodimer (see Fig. 4B), or ~45 ng of polyubiquitinated BRCA1 (nickel-bound fraction) or free polyubiquitin (nickel-unbound fraction) (see Fig. 5). After 10 μl of preequilibrated amylose agarose resin was added (1:1 with binding buffer), the binding reaction was conducted and results were analyzed by immunoblotting as described for the GST pulldown assays. Western blots were developed using either SuperSignal West Pico chemiluminescent or Dura extended-duration substrate (Thermo Scientific).

Production of autoubiquitinated BRCA1 conjugates and free polyubiquitin chains. Preparative *in vitro* ubiquitination reactions were performed at 37°C for 3 h in 500 μl of PBDM buffer (25 mM Tris-Cl, pH 7.6, 5 mM $MgCl_2$, 10 mM creatine phosphate, 0.6 U/ml inorganic pyrophosphatase, and 0.6 U/ml creatine phosphokinase) (40) containing 0.75 mM DTT and 0.5 μg recombinant His_6-tagged human ubiquitin-activating enzyme E1 (Biomol). Purified full-length BRCA1/BARD1 heterodimers (0.75 μg), 6H-UbcH5c conjugating enzyme (0.26 μg), 2 mM ATP, and either wild-type or mutant (Ub-R6) ubiquitin were included. Some ubiquitination reaction mixtures were fractionated by being mixed for 1 h at 4°C with nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (100 μl) equilibrated in 25 mM Tris-Cl, pH 7.9, 250 mM NaCl, 0.1% NP-40, 10% glycerol, and 10 mM beta-mercaptoethanol. After the nickel-unbound fraction (containing free K6-linked polyubiquitin) was collected, the resin was washed three times with PBDM buffer containing 0.75 mM DTT and 50 mM NaCl. The nickel-bound fraction, containing BRCA1 and its ubiquitinated derivatives, was then eluted in 250 μl of binding buffer containing 125 mM imidazole. The nickel-bound and nickel-unbound fractions were stored at −80°C.

***In vitro* ubiquitination assays.** *In vitro* ubiquitination assays were conducted essentially as described previously (53), using 30 ng of purified BRCA1/BARD1 heterodimer in 30-μl reaction mixtures. For each reaction, a 0.125-μg aliquot of the purified 6H-UbcH5c conjugating enzyme was premixed for 10 min on ice with 0.5 μg of maltose-binding protein (MBP) or an MBP fusion polypeptide containing full-length UBXN1 (wild type or mutant), RAD23A, or Nemo. The 6H-UbcH5c premixes were then added to the ubiquitination reaction mixtures. After incubation for 40 min at 37°C, the reactions were terminated by adding 6 μl of 5× SDS loading buffer protein dye (53), and the reaction products were boiled for 3 min, fractionated by SDS-PAGE, and immunoblotted with ubiquitin-specific monoclonal (P4D1; Santa Cruz), BRCA1-specific monoclonal (Ab-1; Calbiochem), or MBP-specific polyclonal (New England BioLabs) antibody.

***In vivo* assays of BRCA1 autoubiquitination.** To detect BRCA1 autoubiquitination, 10^6 human 293 cells were seeded onto a 100-mm plate. After 24 h, the cells were transfected in 40 μl of FuGene reagent (Roche) with the Fl4-BR771/pCMV (5 μg), BARD1/pCMV (5 μg), 6H-Ub_2/pCMV (4 μg), and (wild-type or mutant) UBXN1/pCMV (2 μg) mammalian expression plasmids. The cells were harvested 40 h after transfection (some cultures were exposed to 12 Gy ionizing radiation 1 h prior to harvest), washed in PBS containing 5 mM *N*-ethylmaleimide (NEM), and centrifuged at 1,200 rpm for 2 min. Each cell pellet was lysed on ice for 15 min in 300 μl of NETN buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing 25 mM NaF, 5 mM NEM, complete EDTA-free protease inhibitors (Roche), and 1 mM DTT. The lysate was then spun at maximal speed in a microcentrifuge for 5 min at 4°C. After the "soluble fraction" was removed, the nuclear pellet was washed three times in digestion buffer (25 mM Tris-Cl, pH 7.9, 25 mM NaF, 10% glycerol, and 1 mM DTT) containing 100 mM NaCl. A chromatin extract was then prepared by treating the pellet with 75 μl of 1 mg/ml RNase-free DNase I (Roche) for 30 min at 37°C in digestion buffer containing 100 mM NaCl, 5 mM $MgCl_2$, and complete EDTA-free protease inhibitors. During this incubation, the pellet was further disrupted by pipetting. An additional 100 μl of digestion buffer containing 100 mM NaCl and protease inhibitors was then added, and the mixture was incubated at 37°C for 15 min longer. The mixture was spun at maximal speed in a microcentrifuge for 5 min at 4°C, and the supernatant (i.e., the "chromatin fraction") was collected. Aliquots (5 μg) of the chromatin extract were fractionated by SDS-PAGE and immunoblotted with BRCA1 monoclonal (Ab1; Calbiochem), UBXN1 polyclonal (antiserum 961), or phosphospecific γ-H2AX polyclonal (Upstate) antibody.

***In vivo* assays of double-strand-break repair by homologous recombination.** Assays were conducted with TOSA4 cells, a subclone of 293T cells that contains a single integrated copy of the DR-GFP recombination substrate (8). TOSA4 cells were cotransfected with expression vectors encoding the ISceI restriction endonuclease, along with either a wild-type or a mutant form of UBXN1, and the levels of UBXN1 expression were monitored 48 h later by immunoblotting. To evaluate homology-directed repair (HDR) of double-strand DNA breaks generated in the DR-GFP reporter by the ISceI restriction endonuclease, the number of GFP-positive cells was quantitated 48 h posttransfection by flow cytometry, and the average values and standard deviations of triplicate samples were calculated.

RESULTS

Identification of a UBA domain protein that interacts with BRCA1/BARD1 heterodimers. To identify proteins that recognize K6-linked polyubiquitin, we exploited the fact that K6-linked chains are generated upon BRCA1 autoubiquitination (35, 53). A yeast two-hybrid screen in which an N-terminal segment of BRCA1 (residues 1 to 304) and full-length BARD1 were expressed as a single fusion protein, together with the DNA-binding domain of yeast GAL4, was designed. Based on the "tethered catalysis" strategy used to screen for phosphodependent protein interactions (13), we reasoned that this GAL4-BRCA1-BARD1 fusion protein would be enzymatically active and would autoubiquitinate its BRCA1 moiety *in vivo* so as to create a two-hybrid bait bearing K6-linked polyubiquitin chains. This bait was then used in a yeast two-hybrid screen of a human lymphocyte cDNA library. In all, 37 positive isolates were obtained upon screening ~5 million library transformants. Nucleotide sequencing revealed that the 37 positive clones represent 21 independent cDNAs that encode 14 distinct proteins. We anticipated that these proteins might fall into three classes: (i) proteins that interact with either BRCA1 or BARD1, (ii) proteins that bind a surface of BRCA1/BARD1 that forms only upon heterodimerization, and (iii) proteins that interact with the K6-linked polyubiquitin chains of the autoubiquitinated BRCA1-BARD1 bait. Thus, each positive clone was tested in a yeast two-hybrid mating assay for its ability to interact with the original bait (GAL4-BRCA1-BARD1), a bait lacking BARD1 sequences (GAL4-BRCA1), and a bait lacking BRCA1 sequences (GAL4-BARD1). Of the 21 independent cDNAs obtained in the two-hybrid screen, 17 were able to interact with either GAL4-BRCA1 or GAL4-BARD1 alone (as well as with GAL4-BRCA1-BARD1). In contrast, the other 4 cDNAs retained the ability to bind GAL4-BRCA1-BARD1 but failed to interact with either GAL4-BRCA1 or GAL4-BARD1. One of the four cDNAs encodes a segment of the Rho GDP dissociation inhibitor 2. At present, we do not know whether the interaction represented by this single isolate is relevant to BRCA1/BARD1 function. However, the other three independent clones encode a single protein, and remarkably, it contains a ubiquitin-binding domain of the UBA class (Fig. 1A). This protein is designated UBXN1 by the HUGO Nomenclature Committee but has also been described by other names, including LOC51035, Y33K, SAKS1, and 2B28. In addition to the N-terminal UBA domain, UBXN1 harbors a C-terminal UBX motif that adopts a three-dimensional fold similar to that of ubiquitin. Although UBXN1 serves as a substrate for stress-activated protein kinases (31) and its overexpression confers stability to certain proteins (21), the cellular functions of UBXN1 remain poorly understood.

To ascertain whether UBXN1 also interacts with BRCA1/BARD1 *in vivo*, 293 cells were transfected with plasmids encoding proteins with an N-terminal Flag epitope (Fig. 1B). Cell lysates were then immunoprecipitated on M2 beads and an agarose resin conjugated with Flag-specific antibodies and fractionated by PAGE. Each immunoprecipitate was then evaluated for the presence of endogenous BRCA1 or BARD1

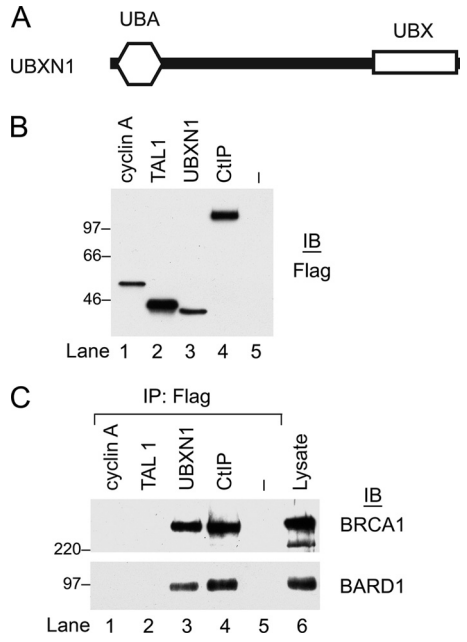

FIG. 1. UBXN1 associates with BRCA1/BARD1 *in vivo*. (A) Map of human UBXN1, displaying the UBA and UBX domains. (B) 293 cells were transfected with vectors encoding Flag-tagged derivatives of cyclin A (lane 1), TAL1 (lane 2), UBXN1 (lane 3), and CtIP (lane 4) or with the empty vector (lane 5). Protein expression was confirmed by immunoblotting (IB) with Flag-specific antibodies. (C) Cell lysates were immunoprecipitated (IP) on Flag-specific agarose beads and fractionated by SDS-PAGE (lanes 1 to 5), along with an aliquot of untreated cell lysate (lane 6). Coimmunoprecipitation of endogenous BRCA1 and BARD1 polypeptides was determined by immunoblotting with the respective antisera (50). The electrophoretic mobilities of molecular size markers are indicated in kilodaltons.

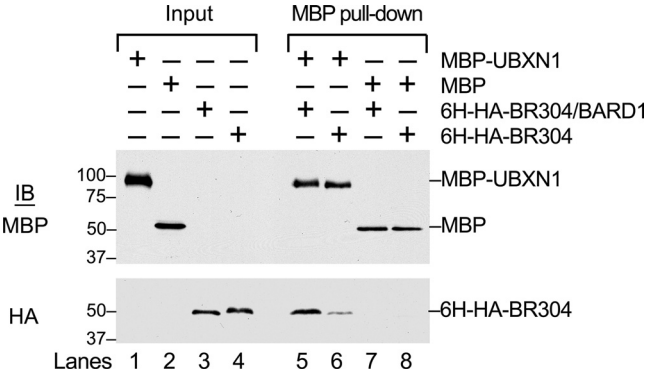

FIG. 2. UBXN can bind both BRCA1 and the BRCA1/BARD1 heterodimer independently of ubiquitin. Maltose-binding protein (MBP) (lane 2) and a polypeptide containing MBP fused to full-length UBXN1 (MBP-UBXN1) (lane 1) were expressed in *E. coli*, purified by affinity chromatography on amylose agarose, fractionated by SDS-PAGE, and detected by immunoblotting with an MBP-specific antibody (upper panel). In addition, 6H-HA-BR304, a polypeptide containing a hexahistidine tag, three tandem HA epitopes, and the N-terminal 304 residues of BRCA1, was expressed in *E. coli* either alone or together with full-length untagged BARD1. The 6H-HA-BR304 polypeptide (lane 4) or the 6H-HA-BR304/BARD1 heterodimer (lane 3), respectively, was then purified from *E. coli* lysates by nickel chromatography, fractionated by SDS-PAGE, and detected by immunoblotting with HA-specific antibodies (lower panel). To evaluate *in vitro* interactions between UBXN1 and BRCA1, the 6H-HA-BR304 polypeptide (lanes 6 and 8) and the 6H-HA-BR304/BARD1 heterodimer (lanes 5 and 7) were incubated with either MBP (lanes 7 and 8) or MBP-UBXN1 (lanes 5 and 6). Each reaction mixture was then loaded onto amylose-agarose beads, and the bound material was examined by immunoblotting with MBP-specific (upper panel) and HA-specific (lower panel) antibodies.

by immunoblotting (Fig. 1C). As expected, proteins serving as negative controls (cyclin A and TAL1) did not coimmunoprecipitate BRCA1 or BARD1 (Fig. 1C, lanes 1 and 2). Significantly, however, Flag-tagged UBXN1 readily coimmunoprecipitated both BRCA1 and BARD1 (Fig. 1C, lane 3) and did so at levels comparable to those for Flag-tagged CtIP (Fig. 1C, lane 4), a known BRCA1-associated protein (56).

UBXN1 can interact directly with BRCA1 independently of ubiquitin conjugation. Since UBXN1 harbors a ubiquitin-binding motif, its *in vivo* association with BRCA1/BARD1 could reflect direct interactions between the UBXN1 and the BRCA1/BARD1 polypeptides and/or UBA-mediated recognition of the K6-linked chain of autoubiquitinated BRCA1. In Fig. 1C, the endogenous BRCA1 species that coimmunoprecipitates with UBXN1 displays the electrophoretic mobility expected for unmodified (i.e., not ubiquitinated) BRCA1, suggesting that UBXN1 can interact with BRCA1 independently of ubiquitin. Therefore, we tested whether it binds BRCA1 polypeptides and BRCA1/BARD1 heterodimers expressed and purified from *Escherichia coli*. As prokaryotes lack the enzymatic machinery for ubiquitin conjugation, recombinant proteins expressed in *E. coli* are "naked" in the sense that they are devoid of ubiquitin modifications. Therefore, bacterial cells were (i) transformed with an expression vector encoding 6H-HA-BR304, a polypeptide containing a hexahistidine tag, three tandem HA epitopes, and the N-terminal 304 BRCA1 residues, including the sequences necessary for BARD1 association, or (ii) cotransformed with vectors encoding 6H-HA-BR304 and full-length untagged BARD1. The 6H-HA-BR304 polypeptide or the 6H-HA-BR304/BARD1 heterodimer, respectively, was then purified from bacterial lysates by affinity chromatography on Ni-NTA agarose beads. In addition, maltose-binding protein (MBP) and a polypeptide containing MBP fused to full-length UBXN1 (MBP-UBXN1) were expressed in *E. coli*, purified by amylose chromatography, and incubated with either the 6H-HA-BR304 polypeptide or the 6H-HA-BR304/BARD1 heterodimer. The incubation reaction mixture was then loaded onto amylose agarose beads, and after being washed, the bound material was fractionated by PAGE. As shown in Fig. 2, the 6H-HA-BR304/BARD1 heterodimer was specifically retained on the beads in the presence of MBP-UBXN1 (lower panel, lane 5) but not MBP alone (lane 7). This indicates that UBXN1 can indeed interact with "naked" BRCA1/BARD1 heterodimers independently of ubiquitin. Curiously, although yeast two-hybrid assays did not detect an *in vivo* interaction between UBXN1 and BRCA1 in the absence of BARD1, the isolated 6H-HA-BR304 polypeptide also bound amylose beads in the presence of MBP-UBXN1 (Fig. 2, lower panel, lane 6) though to a reproducibly weaker extent than the 6H-HA-BR304/BARD1 heterodimer (lane 5). Together, these results suggest that UBXN1 interacts with N-terminal sequences of BRCA1 (residues 1 to 304) and that the interaction is enhanced upon incorporation of these sequences into the BRCA1/BARD1 heterodimer.

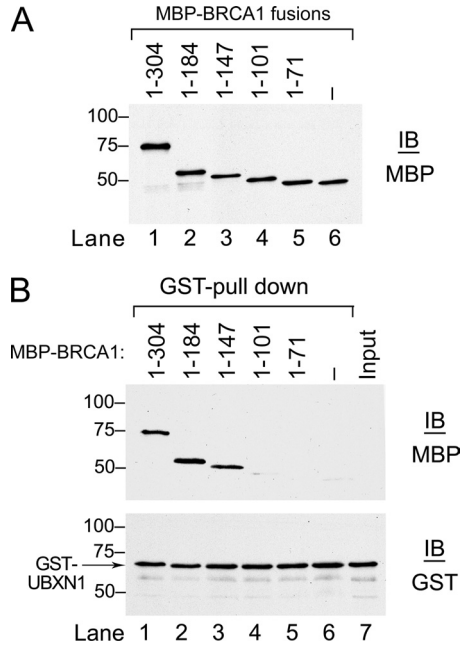

FIG. 3. Ubiquitin-independent interaction with UBXN1 requires an N-terminal region of BRCA1 that includes the RING domain and its BARD1-binding sequences. (A) A panel of MBP-BRCA1 fusion proteins containing the indicated N-terminal BRCA1 residues was expressed in *E. coli*, purified by amylose chromatography, and analyzed by immunoblotting with an MBP-specific antibody. (B) To identify BRCA1 sequences required for interaction with UBXN1, each of the MBP-BRCA1 polypeptides was incubated with GST-UBXN1, a GST fusion protein containing full-length UBXN1. Each reaction mixture was then loaded onto glutathione-agarose beads, and the bound material was examined by immunoblotting with MBP-specific (upper panel) and GST-specific (lower panel) antibodies.

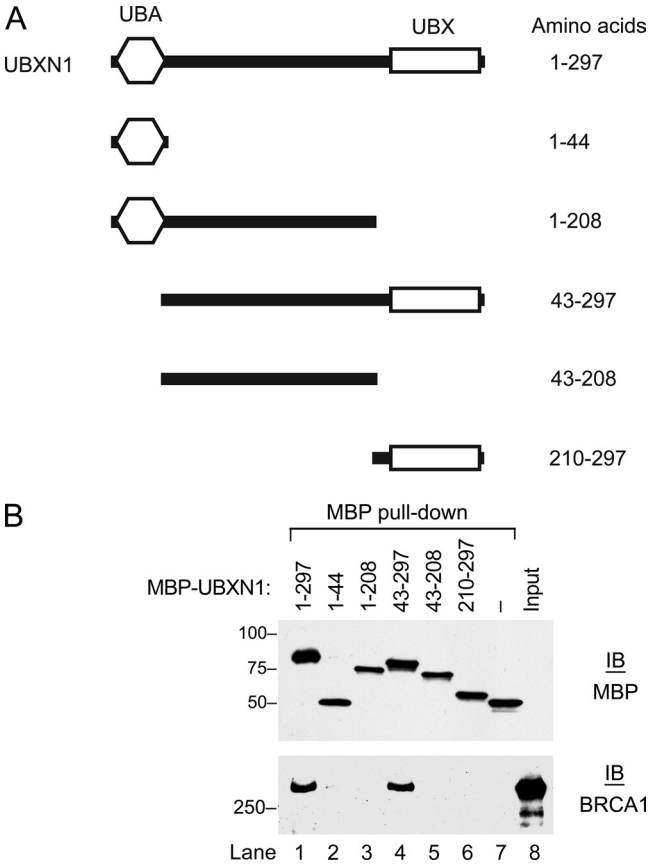

FIG. 4. The UBA domain is not required for ubiquitin-independent binding between UBXN1 and BRCA1. (A) A panel of MBP-UBXN1 fusion proteins containing the indicated segments of UBXN1 was expressed in *E. coli* and purified by amylose chromatography. (B) The MBP-UBXN1 fusion proteins were incubated with a full-length BRCA1/BARD1 heterodimer purified from baculovirus-infected Sf9 cells (53). Each reaction mixture was then loaded onto amylose-agarose beads, and the bound material was fractionated by SDS-PAGE (lanes 1 to 7), together with an aliquot of the input BRCA1/BARD1 heterodimer (lane 8). Immunoblotting was performed with MBP-specific (upper panel) and BRCA1-specific (lower panel) antibodies.

To locate the region of interaction within BRCA1, different N-terminal BRCA1 segments were expressed in bacteria as MBP fusion proteins and purified by amylose chromatography (Fig. 3A), while a glutathione *S*-transferase (GST) fusion polypeptide containing full-length UBXN1 was expressed in *E. coli* and purified by glutathione chromatography (Fig. 3B, lower panel). Aliquots of GST-UBXN1 were then incubated with each of the MBP-BRCA1 fusion proteins and loaded onto glutathione-agarose beads, and the bound material was fractionated by PAGE. As shown in Fig. 3B (upper panel), only MBP-BRCA1 polypeptides containing larger (e.g., residues 1 to 147), not smaller (e.g., residues 1 to 101), N-terminal segments of BRCA1 were retained on the beads in the presence of GST-UBXN1. This places the UBXN1-binding region of BRCA1 in the immediate vicinity of its RING domain and its BARD1-interacting sequences.

To identify the BRCA1-interacting sequences of UBXN1, different UBXN1 segments (Fig. 4A) were expressed in bacteria as MBP fusion proteins, purified by amylose chromatography, and incubated with the full-length BRCA1/BARD1 heterodimer purified from baculovirus-infected Sf9 cells (53). The incubation mixture was then loaded onto amylose beads, and the bound material was fractionated by PAGE. Immunoblotting with an MBP-specific antibody confirmed that comparable quantities of the different MBP-UBXN1 polypeptides were loaded on the beads (Fig. 4B, upper panel). Immunoblotting with a BRCA1-specific antibody (Fig. 4B, lower panel) revealed that BRCA1 was retained on beads in the presence of MPB fused to full-length UBXN1 (lane 1) and a large UBXN1 segment lacking the UBA domain (lane 4) but not the other UBXN1 segments tested (lanes 2, 3, 5, and 6). Thus, the BRCA1 interaction is mediated by a C-terminal portion of UBXN1 (residues 43 to 298) that excludes the UBA domain.

The UBA domain of UBXN1 binds K6-linked polyubiquitin chains. Given the presence of a UBA domain, we examined whether UBXN1 interacts with K6-linked polyubiquitin chains. To generate K6-linked chains, *in vitro* ubiquitination reactions were conducted using BRCA1/BARD1 heterodimers isolated from baculovirus-infected Sf9 cells and the E2 conjugating enzyme UbcH5c (53). The reaction products were then analyzed by immunoblotting with either ubiquitin- or BRCA1-specific antibodies (Fig. 5, lanes 1 to 3). High-molecular-weight polyubiquitin was readily generated when these reactions were

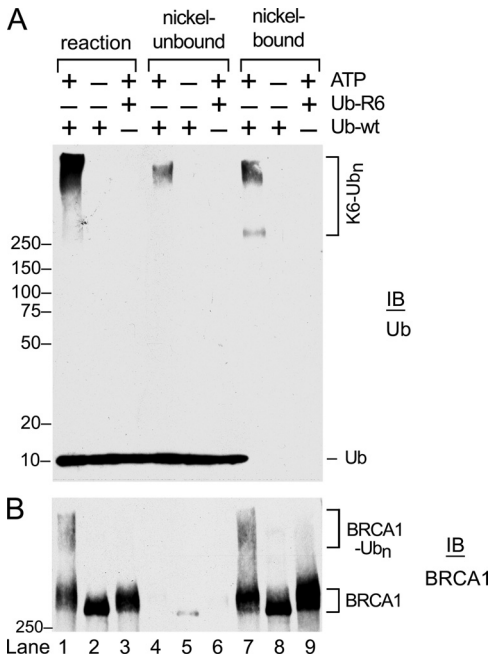

FIG. 5. Preparation of free K6-linked polyubiquitin chains and BRCA1 polypeptides conjugated with K6-linked polyubiquitin. Three parallel *in vitro* ubiquitination reactions were conducted using the purified full-length BRCA1/BARD1 heterodimer and the UbcH5c ubiquitin conjugating enzyme, as described previously (53). The reaction mixtures were supplied with either wild-type ubiquitin (Ub-wt) (lanes 1 and 2) or a mutant ubiquitin (Ub-R6) in which lysine residue 6 is substituted with arginine (lane 3). In addition, a control reaction was performed in the absence of ATP (lane 2). The reaction products were then analyzed by immunoblotting with a ubiquitin-specific (A) or a BRCA1-specific (B) antibody. The electrophoretic mobilities of free K6-linked polyubiquitin (K6-Ub_n), free ubiquitin monomers (Ub), K6-linked polyubiquitinated BRCA1 (BRCA1-Ub_n), and unmodified BRCA1 (BRCA1) are indicated. Aliquots of the three reaction mixtures (lanes 1 to 3) were also subjected to affinity chromatography on Ni-NTA agarose beads, and the resulting nickel-unbound (lanes 4 to 6) and nickel-bound (lanes 7 to 9) fractions were analyzed by immunoblotting. The nickel-unbound fraction of the reaction mixture supplied with the wild type (lane 4) was subsequently used as the source of free K6-linked polyubiquitin and free ubiquitin monomers for the experiment with results shown in Fig. 6, while the nickel-bound fraction (lane 7) was used as the source of both polyubiquitinated and unmodified BRCA1/BARD1 heterodimers for the experiment with results shown in Fig. 7.

conducted with wild-type ubiquitin (Fig. 5A, upper panel, lane 1) but not with a mutant ubiquitin (Ub-R6) in which residue K6 is substituted with arginine (lane 3), consistent with previous data showing that BRCA1/BARD1 heterodimers, in association with UbcH5 conjugating enzymes, primarily synthesize K6-linked polyubiquitin (35, 53). Immunoblotting with BRCA1 antibodies also revealed the presence of BRCA1 polypeptides conjugated to K6-linked chains among the reaction products (Fig. 5B, lower panel, lane 1).

Apart from ubiquitin, all protein components of these *in vitro* reaction mixtures, including BRCA1, possess hexahistidine tags. Thus, to separate the ubiquitin monomers and free polyubiquitin chains from these components, the reaction mixtures were passed through Ni-NTA agarose beads. Aliquots of both the nickel-bound and -unbound fractions were then analyzed by immunoblotting. As expected, BRCA1 polypeptides, including their ubiquitinated derivatives, bound the Ni-NTA agarose beads (Fig. 5B, lower panel, lane 7) and were absent from the unbound fraction (lane 4). This indicates that the high-molecular-weight chains detected with antiubiquitin antibodies in the unbound fraction correspond to free K6-linked chains (Fig. 5A, upper panel, lane 4) while those in the bound fraction represent K6-linked chains conjugated to BRCA1 (lane 7). Thus, the nickel-unbound fraction (lane 4) contains free K6-linked polyubiquitin chains as well as free ubiquitin monomers.

To determine whether UBXN1 interacts with free K6-linked polyubiquitin, the nickel-unbound fraction (Fig. 5, lane 4) was incubated with MBP fusion proteins containing either full-length UBXN1 or various segments of UBXN1 (Fig. 4A). The reaction mixture was then loaded onto amylose beads, and the bound material was fractionated by PAGE. Immunoblotting with MBP antibodies confirmed that comparable amounts of the MBP-UBXN1 fusion proteins were bound to the beads (Fig. 6, upper panels). Immunoblotting with ubiquitin-specific antibodies revealed that K6-linked polyubiquitin, but not ubiquitin monomers, bound avidly to MBP fusion proteins containing full-length UBXN1 (Fig. 6, lower panels, lanes 2 and 9). K6-linked polyubiquitin also bound an N-terminal segment (UBXN1 residues 1 to 203) that excludes the UBX domain (lane 4). Although a smaller N-terminal segment (residues 1 to 44) containing only UBA domain sequences was not sufficient to retain polyubiquitin (lane 3), this may reflect an inability of the shorter fragment to adopt the proper UBA fold. Alternatively, the UBA domain may be required but not sufficient for recognition of polyubiquitin, as was recently demonstrated for the UBA domain of XIAP (14).

Structural studies show that the UBA domain consists of three tightly packed α helices (15, 17, 18). Ubiquitin binding is mediated by a hydrophobic patch that includes the conserved "MGF loop" between helices 1 and 2 of the UBA fold (36, 46). To confirm that the UBA domain of UBXN1 is required for recognition of K6-linked polyubiquitin, we examined the binding properties of an MBP-UBXN1 fusion polypeptide containing an amino acid substitution (M13T) in the MGF loop. The M13T substitution is modeled after a pathogenic mutation (M404T) of the p62 protein observed in Paget's disease of bone that ablates ubiquitin binding by p62 (27, 28). As shown in Fig. 6, the M13T substitution abolishes the interaction of UBXN1 with K6-linked polyubiquitin (compare lanes 9 and 10). In contrast, UBXN1 recognition of K6-linked chains is unaffected by mutation (R219A) of the conserved arginine residue in the UBX fold (lane 11). Together, these data indicate that UBXN1 binds K6-linked polyubiquitin chains through its UBA domain.

UBXN1 binding to BRCA1/BARD1 heterodimers is enhanced by autoubiquitination. The data presented so far indicate that the C-terminal sequences of UBXN1 can bind BRCA1 in a ubiquitin-independent manner (Fig. 4) while the N-terminal UBA domain of UBXN1 can recognize K6-linked polyubiquitin (Fig. 6). Thus, unlike "naked" BRCA1/BARD1 heterodimers, autoubiquitinated BRCA1/BARD1 can interact with UBXN1 through two distinct modes. To ascertain whether autoubiquitination enhances the interaction between UBXN1 and BRCA1/BARD1, an *in vitro* ubiquitination reaction was conducted using

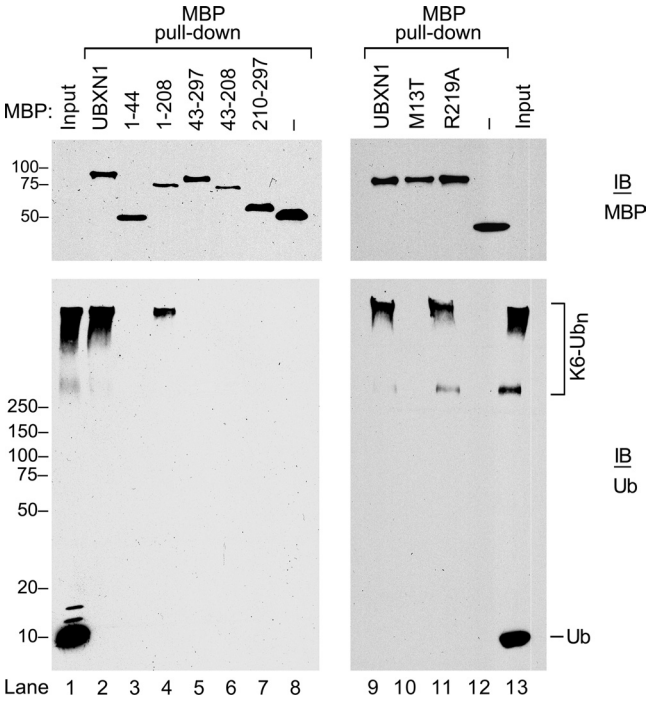

FIG. 6. The UBA domain of UBXN1 binds K6-linked polyubiquitin chains. The nickel-unbound fraction of a BRCA1-driven ubiquitination reaction mixture (Fig. 5, lane 4) was incubated with MBP (lanes 8 and 12) or the indicated MBP-UBXN1 fusion proteins (lanes 2 to 7 and 9 to 11). The MBP-UBXN1 fusion proteins contain full-length wild-type UBXN1 (residues 1 to 297) (lanes 2 and 9), the indicated segments of UBXN1 (lanes 3 to 7), or full-length mutant UBXN1 bearing the M13T (lane 10) or R219A (lane 11) amino acid substitution. Each reaction mixture was then loaded onto amylose-agarose beads, and the bound material was separated by SDS-PAGE (lanes 2 to 12), together with aliquots of the input nickel-unbound fraction (lanes 1 and 13). The presence of the MBP fusion proteins was confirmed by immunoblotting with MBP-specific antibodies (upper panel), while the associations with K6-linked polyubiquitin and free monoubiquitin were evaluated by immunoblotting with Ub-specific antibodies (lower panel).

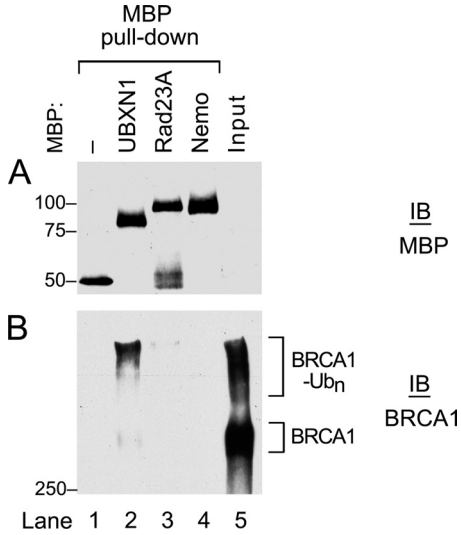

FIG. 7. UBXN1 preferentially binds autoubiquitinated BRCA1/BARD1 heterodimers. The nickel-bound fraction of a BRCA1-driven ubiquitination reaction mixture (Fig. 5, lane 7) was incubated with MBP (lane 1), the MBP-UBXN1 fusion protein (lane 2), an MPB fusion protein containing the known ubiquitin-binding protein RAD23A (lane 3), or NEMO (lane 4). Each reaction mixture was then loaded onto amylose-agarose beads, and the bound material was fractionated by SDS-PAGE, together with an aliquot of the input nickel-bound fraction (lane 5). The presence of the MBP fusion proteins was confirmed by immunoblotting with MBP-specific antibodies (A), while the association with polyubiquitinated and unmodified BRCA1/BARD1 heterodimers was evaluated by immunoblotting with BRCA1-specific antibodies (B).

a purified full-length BRCA1/BARD1 heterodimer in which the BRCA1 subunit is tagged with hexahistidine (53). The reaction products were then passed through Ni-NTA agarose beads, and the bound fraction was washed and eluted with imidazole. As expected, the eluted material includes both "naked" BRCA1 polypeptides that migrate at the expected molecular mass of ~210 kDa and higher-molecular-mass forms conjugated to K6-linked polyubiquitin (Fig. 7B, lane 5) (35, 53). This material was then incubated with comparable levels of the parental MBP polypeptide or the indicated MBP fusion polypeptide (Fig. 7A). As shown in Fig. 7B, autoubiquitinated forms of BRCA1 readily bound the MBP-UBXN1 polypeptide (lane 2) but not the MBP fusions containing RAD23A, which has two UBA domains that preferentially recognize K48-linked chains (39), or NEMO, which harbors a distinct type of ubiquitin-binding motif that binds linear chains (26, 41). A small quantity of naked BRCA1 also interacted with MBP-UBXN1 (Fig. 7A, lane 2); however, MBP-UBXN1 bound a significantly greater proportion of the input autoubiquitinated BRCA1 than the input naked BRCA1, indicating that the interaction with UBXN1 is greatly enhanced upon BRCA1 autoubiquitination.

UBXN1 inhibits the E3 ligase activity of BRCA1/BARD1. To determine whether UBXN1 influences the catalytic properties of BRCA1/BARD1, we examined its effect on BRCA1-mediated ubiquitination. For this purpose, biochemically pure, full-length BRCA1/BARD1 heterodimers were generated (53) with or without the I26A mutation, an isoleucine-to-alanine substitution in the RING domain of BRCA1. This mutation disrupts the interaction of BRCA1 with all E2 enzymes known to support BRCA1-dependent ubiquitination and thereby renders the BRCA1/BARD1 heterodimer enzymatically inert (2, 7). The purified heterodimers were then used for *in vitro* ubiquitination reactions with the E2 conjugating enzyme UbcH5c, and polyubiquitin formation was assessed by immunoblotting with ubiquitin-specific antibodies. As expected, polyubiquitin chains were generated by wild-type, but not I26A mutant, BRCA1/BARD1 heterodimers (Fig. 8A, lanes 2 and 5, respectively). To assess the effect of UBXN1 on BRCA1-mediated ubiquitination, maltose-binding protein (MBP) and a polypeptide containing MBP fused to full-length UBXN1 (MBP-UBXN1) were expressed in *E. coli* and purified from bacterial lysates by amylose chromatography. The BRCA1-dependent ubiquitination reactions were then conducted in the presence of equimolar quantities of either MBP-UBXN1 or MBP (Fig. 8C, lane 3 or 4, respectively). As shown in Fig. 8A, BRCA1-dependent ubiquitination was dramatically inhibited by the presence of MBP-UBXN1 (lane 3) but not the parental MBP polypeptide (lane 4). To assess whether UBXN1 also influ-

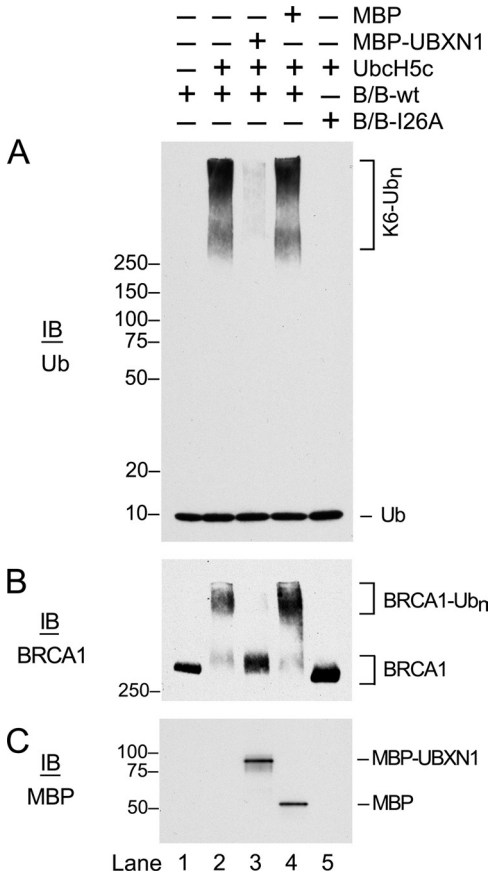

FIG. 8. UBXN1 inhibits the enzymatic activity of the BRCA1/BARD1 heterodimer. *In vitro* ubiquitination reactions were conducted in the presence (lanes 2 to 5) or absence (lane 1) of UbcH5c and either the full-length wild-type BRCA1/BARD1 heterodimer (B/B-wt) (lanes 1 to 4) or an enzymatically defective heterodimer containing the I26A mutation of BRCA1 (B/B-I26A) (lane 5). The indicated reaction mixtures were supplemented with either purified maltose-binding protein (MBP) (lane 4) or an MBP fusion protein containing full-length UBXN1 (MBP-UBXN1) (lane 3). Aliquots of each reaction mixture were then evaluated by immunoblotting with ubiquitin-specific (A), BRCA1-specific (B), and MBP-specific (C) antibodies. The electrophoretic mobilities of K6-linked polyubiquitin (K6-Ub_n), free ubiquitin monomers (Ub), K6-linked polyubiquitinated BRCA1 (BRCA1-Ub_n), and unmodified BRCA1 (BRCA1) are indicated.

ences BRCA1 autoubiquitination, the reaction products were examined by immunoblotting with BRCA1-specific antibodies. As seen in Fig. 8B, BRCA1 autoubiquitination was also suppressed by MBP-UBXN1 (lane 3) but not by MBP alone (lane 4). Significantly, the ability to inhibit BRCA1-mediated polyubiquitination (Fig. 8A) and BRCA1 autoubiquitination (Fig. 8B) is specific to UBXN1, since MBP fusion polypeptides containing other ubiquitin-binding proteins (i.e., RAD23A and NEMO) had no effect (data not shown).

To ascertain whether UBXN1 also modulates the enzymatic activity of BRCA1/BARD1 *in vivo*, we examined the effect of UBXN1 expression on BRCA1 autoubiquitination. Nishikawa et al. (35) have shown that autoubiquitinated conjugates of BRCA1 can be detected *in vivo* by overexpression of a truncated BRCA1 polypeptide (amino acid residues 1 to 772) that retains the N-terminal RING domain and forms heterodimers

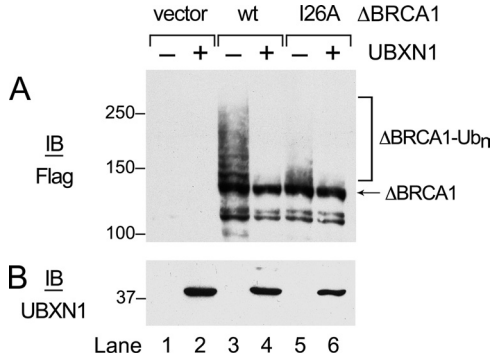

FIG. 9. UBXN1 inhibits BRCA1 autoubiquitination *in vivo*. 293 cells were transfected with a Flag-tagged segment of BRCA1 (ΔBRCA1, amino acids 1 to 771) with (lanes 5 and 6) or without (lanes 3 and 4) the I26A mutation. All cultures were also cotransfected with exogenous BARD1 and ubiquitin, and the indicated cultures were transfected with a vector encoding UBXN1 (lanes 2, 4, and 6) or the corresponding empty vector (lanes 1, 3, and 5). The cells were lysed 48 h posttransfection, and chromatin extracts were examined by immunoblotting with FLAG-specific antibodies (A) and UBXN1-specific antiserum (B). As shown, the autoubiquitinated ΔBRCA1 conjugates detected with Flag-specific antibodies (lane 3) were eliminated upon overexpression of UBXN1 (lane 4). wt, wild type.

with BARD1. Therefore, we transfected 293 cells with expression vectors (6) encoding Flag-tagged derivatives of a similar BRCA1 segment (ΔBRCA1, amino acids 1 to 771), with or without a mutation (I26A) that ablates its enzymatic activity (2). As expected, autoubiquitinated ΔBRCA1 conjugates were observed in the chromatin fraction of cells transfected with wild-type (Fig. 9A, lane 3), but not I26A mutant (lane 5), ΔBRCA1. Significantly, ΔBRCA1 autoubiquitination was suppressed by overexpression of exogenous UBXN1 (lane 4), indicating that the enzymatic activity of BRCA1/BARD1 can be inhibited by UBXN1 *in vivo*.

Inhibition of BRCA1/BARD1-mediated ubiquitination is dependent on the ubiquitin-binding activity of UBXN1. Since the interaction of UBXN1 with BRCA1 is greatly enhanced by autoubiquitination (Fig. 7), we asked whether the ubiquitin-binding properties of UBXN1 influence its ability to inhibit BRCA1-dependent ubiquitination. As shown in Fig. 10, mutation of the UBX domain (R219A) had no effect on UBXN1 inhibition of BRCA1-mediated *in vitro* ubiquitination (compare lanes 3 and 5). In contrast, a missense mutation (M13T) in the UBA domain of UBXN1 that ablates its recognition of K6-linked polyubiquitin (Fig. 6B) impairs the ability of UBXN1 to inhibit BRCA1-dependent ubiquitination (compare lanes 3 and 4). This suggests that the ubiquitin-binding activity of UBXN1 is required for its ability to inhibit the enzymatic activity of autoubiquitinated BRCA1. We also examined the effects of these mutations on UBXN1 inhibition of ΔBRCA1 autoubiquitination *in vivo*. As shown in Fig. 11, ΔBRCA1 autoubiquitination was suppressed by overexpression of either wild-type UBXN1 (lane 5) or R219A mutant UBXN1 (lane 9), and this effect was not influenced by genotoxic stress from ionizing radiation (lanes 6 and 10). In contrast, UBXN1 suppression of ΔBRCA1 autoubiquitination was ablated by mutation (M13T) of the UBA domain (lane 7). Indeed, ΔBRCA1 autoubiquitination was reproducibly en-

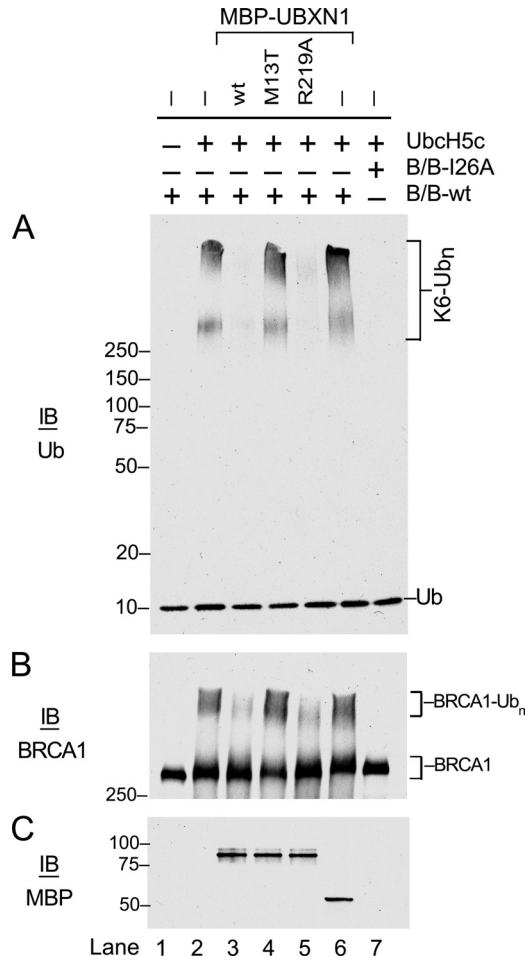

FIG. 10. The ubiquitin-binding activity of UBXN1 is required for inhibition of BRCA1 enzymatic function. *In vitro* ubiquitination reactions were conducted in the presence (lanes 2 to 7) or absence (lane 1) of UbcH5c and either the full-length wild-type BRCA1/BARD1 heterodimer (B/B-wt) (lanes 1 to 6) or an enzymatically defective heterodimer containing the I26A mutation of BRCA1 (B/B-I26A) (lane 7). The indicated reaction mixtures were also supplemented with either purified maltose-binding protein (MBP) (lane 6) or MBP fusion proteins containing full-length UBXN1 (MBP-UBXN1) (lanes 3 to 5). The MBP-UBXN1 fusions harbored the wild-type UBXN1 sequence (lane 3) or contained a missense mutation in either the UBA (M13T) (lane 4) or the UBX (R219A) (lane 5) domain. Aliquots of each reaction mixture were then immunoblotted with ubiquitin-specific (A), BRCA1-specific (B), and MBP-specific (C) antibodies. The electrophoretic mobilities of K6-linked polyubiquitin (K6-Ub_n), free ubiquitin monomers (Ub), K6-linked polyubiquitinated BRCA1 (BRCA1-Ub_n), and unmodified BRCA1 (BRCA1) are indicated.

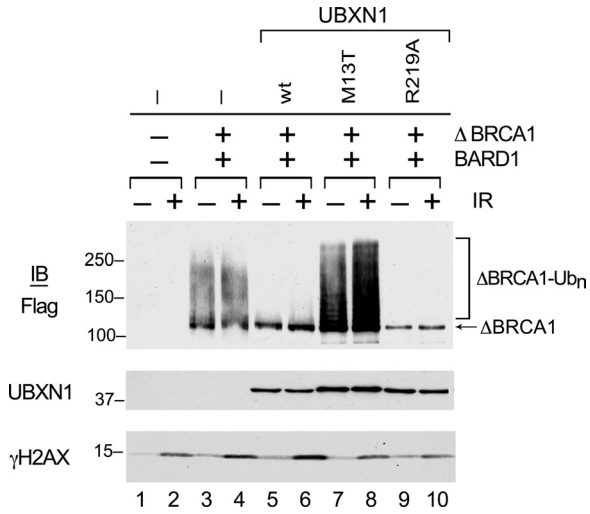

FIG. 11. The ubiquitin-binding activity of UBXN1 is required for *in vivo* inhibition of BRCA1 enzymatic function. The indicated cultures of 293 cells were cotransfected with BARD1 and a Flag-tagged derivative of ΔBRCA1 (lanes 3 to 10). All cultures were cotransfected with exogenous ubiquitin, and the indicated cultures were transfected with wild-type (lanes 5 and 6), M13T mutant (lanes 7 and 8), or R219A mutant (lanes 9 and 10) UBXN1. The indicated cultures were subjected to 12 Gy of ionizing radiation (IR) at 1 h prior to harvest. Cells were lysed 48 h posttransfection, and chromatin extracts were examined by immunoblotting with FLAG-specific antibodies (upper panel), UBXN1-specific antiserum (middle panel), and γH2AX phosphospecific antiserum (lower panel). As shown, the autoubiquitinated ΔBRCA1 conjugates detected with Flag-specific antibodies (lanes 3 and 4) were eliminated upon overexpression of wild-type UBXN1 (lane 5 and 6) and R219A mutant UBXN1 (lanes 9 and 10) but not M13T mutant UBXN1 (lanes 7 and 8).

hanced upon overexpression of M13T mutant UBXN1 (compare lanes 3 and 7), presumably reflecting a dominant negative effect of this mutant on the activity of endogenous UBXN1. In any event, these results indicate that the ubiquitin-binding properties of UBXN1 are required for inhibition of BRCA1-mediated ubiquitination both *in vitro* and *in vivo*.

Although UBXN1 overexpression suppresses BRCA1 autoubiquitination *in vivo* (Fig. 9 and 11), it appears to have no effect on homology-directed repair (HDR) of double-strand DNA breaks, a BRCA1-dependent process that promotes genome stability (33). As shown in Fig. 12, HDR levels are unchanged by overexpression of either wild-type or mutant UBXN1 (8). This observation is consistent with the notion that the E3 ligase activity of BRCA1/BARD1 is dispensable for BRCA1-dependent HDR (42).

DISCUSSION

As the only known enzymatic property of BRCA1, the E3 ligase activity is likely to mediate at least some of the many cellular functions now attributed to BRCA1. Although the BRCA1/BARD1 heterodimer can catalyze both BRCA1 autoubiquitination and *trans*-ubiquitination of other proposed substrates, the functional consequences of BRCA1-mediated ubiquitination remain unclear (1, 51). *In vitro*, BRCA1/BARD1 can generate different forms of polyubiquitin, depending on the identity of the collaborating E2 conjugating enzyme. For example, in the presence of the UbcH5 conjugases (UbcH5a, UbcH5b, and UbcH5c), BRCA1/BARD1 primarily synthesizes chains bearing the unconventional K6 linkage (35, 53). However, with certain combinations of other E2 enzymes, BRCA1/BARD1 can catalyze formation of either K48- or K63-linked polyubiquitin (7). To date, the only substrates shown to be ubiquitinated in a BRCA1-dependent manner *in vivo* are those conjugated to K6-linked chains, including NPM/B23, CtIP, RPB8, and BRCA1 itself (35, 43, 52, 53, 55). Interestingly, multiple lines of evidence suggest that proteins bearing K6-linked chains are not targeted for proteasomal

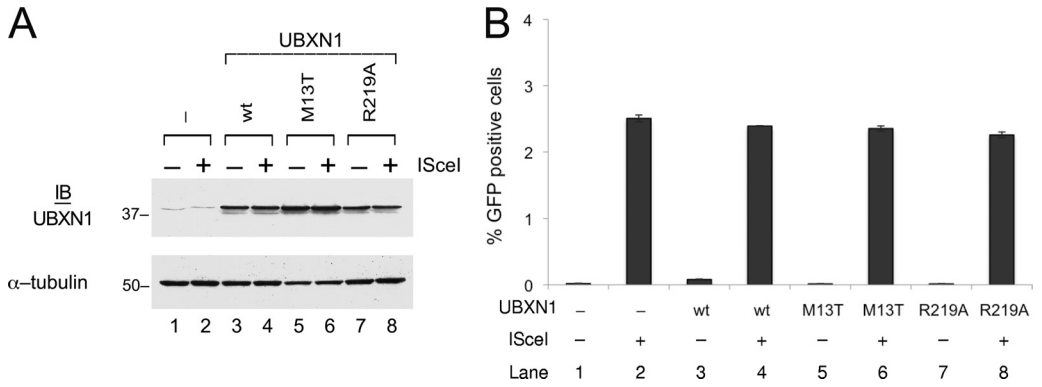

FIG. 12. UBXN1 overexpression does not affect homology-directed repair of double-strand DNA breaks. The effects of UBXN1 overexpression were evaluated with TOSA4 cells, a subclone of 293T cells that contains a single integrated copy of the DR-GFP recombination substrate (8). (A) TOSA4 cells were cotransfected with the ISceI endonuclease (lanes 2, 4, 6, and 8) and wild-type (lanes 3 and 4), M13T mutant (lanes 5 and 6), or R219A mutant (lanes 7 and 8) UBXN1. At 48 h posttransfection, the levels of endogenous (lanes 1 and 2) and overexpressed (lanes 3 to 8) UBXN1 were assessed by immunoblotting. (B) To evaluate homology-directed repair of ISceI-induced double-strand breaks in DR-GFP, the number of GFP-positive TOSA4 cells was quantitated at 48 h posttransfection by flow cytometry. The error bars indicate standard deviations. As expected, ISceI expression induced homology-directed repair of DR-GFP (compare lanes 1 and 2). However, repair levels were not altered upon overexpression of wild-type (lane 4) or mutant (lanes 6 and 8) UBXN1.

degradation. First, although control polypeptides conjugated with K48-linked polyubiquitin are readily degraded upon *in vitro* incubation with purified 26S proteasomes, this treatment results in deubiquitination, but not degradation, of BRCA1 polypeptides autoubiquitinated with K6-linked chains (35). Second, while BRCA1/BARD1 readily catalyzes autoubiquitination of the BRCA1 subunit (4), leading to its conjugation with K6-linked chains both *in vivo* and *in vitro* (35, 53), autoubiquitination is unlikely to increase BRCA1 turnover since BRCA1 and BARD1 are known to stabilize each other upon coexpression in mammalian cells (16, 22, 30). Third, the stabilities of the four proteins known to be conjugated to K6-linked polyubiquitin *in vivo* in a BRCA1-dependent manner (NPM, CtIP, RPB8, and BRCA1) are not reduced upon coexpression of BRCA1/BARD1, further suggesting that K6-linked chains do not promote proteasomal degradation (35, 43, 52, 53, 55). As such, the functional consequences of BRCA1-mediated ubiquitination with K6-linked chains remain unclear.

Ubiquitin-dependent processes are ultimately mediated by cellular receptors that harbor ubiquitin-binding domains (15, 17, 18). Although a variety of different ubiquitin-binding motifs have been identified to date, the UBA motif is among the most common. At least 37 distinct human proteins harbor a UBA domain, including the RAD23A and RAD23B polypeptides that promote proteasomal degradation of K48-polyubiquitinated proteins. Our data indicate that UBXN1 associates with autoubiquitinated BRCA1/BARD1 heterodimers in a bipartite manner such that its UBA domain binds the conjugated K6-linked chains of BRCA1 while its C-terminal sequences interact with BRCA1/BARD1 in a ubiquitin-independent fashion. Interestingly, cellular receptors for polyubiquitinated proteins can achieve specificity of recognition by forming bipartite interactions with their ligands. For example, NEMO has two modes of interaction with polyubiquitinated RIP1, one involving direct binding between NEMO and RIP1 and another involving recognition of RIP-conjugated polyubiquitin by the ubiquitin-binding domain (NUB/UBD/UBAN) of NEMO (9). Although either mode can be detected in isolation by *in vitro* and *in vivo* assays of protein interaction, both modes are required for physiological signaling in the NF-κB pathway (9). If a similar bipartite mechanism governs the interaction between UBXN1 and BRCA1, then UBXN1 may be able to discriminate autoubiquitinated BRCA1/BARD1 heterodimers from unmodified heterodimers and from other polypeptides conjugated with K6-linked polyubiquitin, as well as from free K6-linked chains. In this manner, UBXN1 would serve as a cellular receptor that specifically recognizes autoubiquitinated BRCA1/BARD1.

Recent work suggests that BRCA1-dependent ubiquitination, whether leading to autoubiquitination or to *trans* ubiquitination of heterologous substrates, is not required for all BRCA1 activities (42). This is exemplified by the fact that embryonic stem (ES) cells expressing enzymatically inert Brca1 are viable, whereas Brca1-null ES cells are not. In addition, cells lacking Brca1 ligase activity are competent for homology-directed repair (HDR) of double-strand DNA breaks, a pathway known to be dependent on Brca1. Consistent with these results, UBXN1 overexpression does not impair the HDR pathway (Fig. 12) despite the fact that it inhibits BRCA1 autoubiquitination *in vivo* (Fig. 9 and 11). Thus, the E3 ligase activity appears to be required for some, but not all, BRCA1 functions (42). Nonetheless, this activity is likely to be important given the stringent phylogenetic conservation of the BRCA1 RING domain and the occurrence of tumorigenic RING mutations in some families with hereditary breast cancer.

Since the consequences of BRCA1 autoubiquitination remain poorly understood, a precise role for UBXN1 in the BRCA1 pathway is difficult to ascertain. Nevertheless, our data show that UBXN1 dramatically inhibits the E3 ligase activity of BRCA1/BARD1 and that suppression of BRCA1 enzymatic function is dependent on the ubiquitin-binding activity of UBXN1. These findings suggest that UBXN1 serves as an inhibitor of the BRCA1/BARD1 complex that regulates its enzymatic activity in a manner that is dependent on the ubiquitination status of BRCA1. Of interest, this would allow for spatial and/or temporal control of BRCA1-mediated ubiquitin-

ation. Spatial regulation could be determined simply by the cellular distribution of UBXN1, such that high UBXN1 concentrations in a particular setting (e.g., the cytoplasm) would locally inhibit the E3 ligase activity of BRCA1/BARD1. In addition, the presence of UBXN1 may act to limit the duration of BRCA1 enzymatic function. In this scenario, physiological induction of BRCA1/BARD1 enzymatic activity, whether in response to DNA damage or other potential stimuli, would simultaneously promote *trans* ubiquitination of BRCA1 substrates and autoubiquitination of its BRCA1 subunit. While *trans* ubiquitination of its substrates would allow downstream effector functions of BRCA1 to ensue, BRCA1 autoubiquitination would enhance the affinity of UBXN1 for BRCA1/BARD1 and thereby promote UBXN1 inhibition of BRCA1/BARD1 enzymatic activity. This manner of feedback inhibition would ensure that the E3 ligase function of BRCA1/BARD1 is confined to a restricted time interval. Thus, although the mechanisms that govern the induction of BRCA1 enzymatic activity *in vivo* are not yet known, autoubiquitination and the consequent association with UBXN1 might provide an effective means to downregulate this activity where and when it is no longer required.

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