The BRCA1/BARD1 Heterodimer Assembles Polyubiquitin Chains through an Unconventional Linkage Involving Lysine Residue K6 of Ubiquitin*

Received for publication, June 6, 2003, and in revised form, July 28, 2003 Published, JBC Papers in Press, July 30, 2003, DOI 10.1074/jbc.C300249200

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The BRCA1 tumor suppressor forms a heterodimer with the BARD1 protein, and the resulting complex functions as an E3 ubiquitin ligase that catalyzes the synthesis of polyubiquitin chains. In theory, polyubiquitination can occur by isopeptide bond formation at any of the seven lysine residues of ubiquitin. The isopeptide linkage of a polyubiquitin chain is a particularly important determinant of its cellular function, such that K48-linked chains commonly target proteins for proteasomal degradation, while K63 chains serve non-proteolytic roles in various signaling pathways. To determine the isopeptide linkage formed by BRCA1/BARD1-dependent polyubiquitination, we purified a full-length heterodimeric complex and compared its linkage specificity with that of E6-AP, an E3 ligase known to induce proteolysis of its cellular substrates. Using a comprehensive mutation analysis, we found that E6-AP catalyzes the synthesis of K48-linked polyubiquitin chains. In contrast, however, the BRCA1/BARD1 heterodimer directs polymerization of ubiquitin primarily through an unconventional linkage involving lysine residue K6. Although heterologous substrates of BRCA1/BARD1 are not known, BRCA1 autoubiquitination occurs principally by conjugation with K6-linked polymers. The ability of BRCA1/BARD1 to form K6-linked polyubiquitin chains suggests that it may impart unique cellular properties to its natural enzymatic substrates.

The BRCA1 tumor suppressor gene has been implicated in various cellular processes that include DNA repair, transcriptional regulation, and cell cycle checkpoint control (1). Its protein product contains an NH2-terminal RING domain and two COOH-terminal BRCT repeats. In vivo, BRCA1 exists as a heterodimer with BARD1, a related protein that displays a similar configuration of RING and BRCT motifs (2). The BRCA1/BARD1 heterodimer is stabilized by a 4-helix bundle formed by α-helices that immediately flank the RING domains of both polypeptides (3). Since most cellular BRCA1 polypeptides are found in association with BARD1 (4, 5), the BRCA1/BARD1 heterodimer is likely to be an essential mediator of BRCA1 function (6). This conclusion is strongly supported by analysis of Bard1-null mice, which display a characteristic phenotype that is essentially indistinguishable from that of Brca1-null animals (7).

Recent studies have uncovered an enzymatic role for the BRCA1/BARD1 heterodimer in protein ubiquitination (8–15). Ubiquitination occurs through a sequential process involving ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (16, 17). In the final step of this process, a ubiquitin monomer is covalently attached to a lysine residue on the ultimate substrate. The monoubiquitinated substrate can also serve as a nucleus for assembly of a polyubiquitin chain that, in many cases, will target the substrate for proteasome-mediated degradation. A common feature of many E3 ligases is the presence of a RING motif, and recent studies have established that sequences encompassing the RING domain of BRCA1 can catalyze the formation of polyubiquitin chains (8, 9). Moreover, the E3 ligase activity of BRCA1 is dramatically augmented by BARD1, and this enhanced activity is ablated by the C61G missense mutation, a germ line BRCA1 lesion associated with hereditary breast cancer (10–14). Therefore, many of the cellular functions attributed to BRCA1, including its ability to suppress tumor development, may be mediated through the enzymatic activity of the BRCA1/BARD1 heterodimer.

A better understanding of BRCA1-dependent tumor suppression should emerge once the natural enzymatic substrates of BRCA1/BARD1 have been identified and the effects of ubiquitination on the functions of these substrates are established. In vitro, BRCA1/BARD1 behaves as a processive enzyme that generates high molecular mass polyubiquitin chains (10–14). Ubiquitin has seven lysine residues, any one of which can potentially serve as a site of attachment during chain assembly (16, 17). Most commonly, proteins are conjugated to K48-linked chains in which each consecutive ubiquitin monomer forms an isopeptide bond with lysine residue 48 on the previously attached monomer (18). These proteins are usually targeted for degradation, presumably because 26 S proteasomes specifically recognize the K48 isopeptide linkage (19). In addition, chains assembled through either K29 or K63 have also been observed in vivo, and chains linked at these and other sites can be generated in vitro (20–22). While K48- and K29-linked chains target proteins for proteasomal destruction, K63-linked chains confer non-proteolytic signals that control various pathways, including post-replicative DNA repair and NF-κB activation (23). Here we have used mutation analysis to establish the linkage specificity of BRCA1/BARD1-dependent polyubiquitination. Surprisingly, BRCA1/BARD1 catalyzes ubiquitin polymerization primarily through lysine residue 6 to form an unconventional type of polyubiquitin chain.

* This work was supported by Grant CA97403 (Project 4) from the NCI. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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EXPERIMENTAL PROCEDURES

**Plasmids**—Baculoviral transfer vectors for expression of wild type (Fl-BRCA1–6H-wt/pVL1392) and mutant (Fl-BRCA1–6H-mt/pVL1392) BRCA1 were constructed by inserting cDNA sequences encoding full-length human BRCA1 with an NH2-terminal FLAG tag (NH2–MADYKDDDDK) and a COOH-terminal hexahistidine (His6) tag (HHHHHHHHHCOOH) into the pVL1392 plasmid (Pharmingen). The baculoviral vector for expression of full-length (GST-BARD1 fusion protein (GST-BARD1/pVL1392) was prepared by inserting full-length DNA sequences encoding glutathione S-transferase and full-length human BARD1 into pVL1392. A vector (6H-E6-AP/pET14b) for expression of E6-AP with an NH2-terminal His6 tag (NH2–MAHHHHHH) was generated by inserting cDNA sequences encoding full-length human UbcH5c (Image clone ID number 3447673) into 6H-pET14b. To generate a plasmid (Ub/psiT14*) encoding native wild type ubiquitin, the cDNA sequence for a single ubiquitin monomer was PCR amplified from MT123, a plasmid kindly provided by Dr. Dirk Bohmann (24), and inserted into pET14b, a derivative of pET14b from which sequences encoding the His6 tag had been excised. To obtain expression vectors encoding the Ub-R series of ubiquitin mutants, each of the seven lysine codons of the ubiquitin was individually changed to an arginine codon (CCG) by site-directed mutagenesis. Multiple rounds of mutagenesis were then performed to obtain expression vectors for “lysineless” (Ub-K0) ubiquitin and for the Ub-K series of ubiquitin mutants.

**Isolation of Full-length BRCA1/BARD1 Complexes**—To generate recombinant baculoviruses encoding the FLAG-BRCA1–6H polyepitide, SF9 cells were transfected with the Fl-BRCA1–6H-wt/pVL1392 vector using the BacuGold transfection kit (Pharmingen). Recombinant viruses encoding GST-BARD1 were obtained by transfecting SF9 cells with the GST-BARD1/pVL1392 vector. For expression of the BRCA1/BARD1 heterodimer, 500 ml of SF9 spinner cells were co-infected with BRCA1 and BARD1 recombinant baculoviruses at a multiplicity of infection of ~2 plaque-forming units/cell for each. Cells were harvested 72 h after infection, and the cell pellet was resuspended in lysis buffer (50 mM Hepes, pH 7.9, 250 mM NaCl, 0.1% Nonidet P-40, 10 mM 2-mercaptoethanol, 10% glycerol) with Complete EDTA-free protease inhibitor mixture (Roche Diagnostics). After 15 min on ice, the resuspended cells were sonicated, the cell debris removed by centrifugation at 30 K for 45 min at 4 °C, and the supernatant filtered through a 0.45-μm polyethersulfone filter (Nalgene). To purify the BRCA1/BARD1 complex, containing the ubiquitin monomers, was passed through a Ni-NTA agarose resin (Qiagen) and purified from cell lysates by Ni-NTA affinity chromatography. The supernatant was incubated with Ni-NTA agarose resin for 3 h at 4 °C, washed twice with lysis buffer (with protease inhibitors), twice with “Ni-NTA buffer” (25 mM Tris-HCl pH 7.9, 50 mM NaCl, 10 mM 2-mercaptoethanol, 10% glycerol, protease inhibitors) containing 10 mM imidazole, pooled into a column, and eluted sequentially with Ni wash buffer containing 30 mM and then 250 mM imidazole. The 250 mM imidazole eluate was pooled, adjusted to 250 mM imidazole, and the supernatant adjusted to pH 4.5–5.0 with concentrated acetic acid. Acid-precipitable proteins were removed by centrifugation, and the supernatant was adjusted to pH 5.1 with 6 N NaOH. This supernatant, containing the ubiquitin monomers, was passed through a 0.45-μm polyethersulfone filter and loaded onto a SP-Sepharose (Amersham Biosciences) column equilibrated with low salt buffer A at pH 5.1. The column was then washed with five column volumes of the same buffer, and the ubiquitin monomers were eluted with high-salt (100 mM NaCl) buffer A at pH 5.1. After pooling the peak fractions, the eluted ubiquitin monomers were desalted and neutralized on a PD10 column (Amersham Biosciences) equilibrated with Ni wash buffer and stored in aliquots at –80 °C. Ubiquitin preparations were analyzed by SDS-PAGE, and the yield of each was determined by comparing Coomassie staining with that of known quantities of bovine serum albumin and ubiquitin (Affinity Research).

In Vitro Ubiquitination—Ubiquitination reactions were conducted at 37 °C for 40 min in a 30-μl volume containing 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 2 mM NaF, 10 mM okadaic acid, 2 mM ATP, 0.6 mM ATP-regenerating system (Roche Diagnostics), and 20 ng of the appropriate purified GST-BARD1 fusion protein. Reactions were performed with 1 μg of ubiquitin monomer, 0.25 μg of 6H-UbcH5c, and a defined quantity of the purified BRCA1/BARD1 heterodimer (i.e. an amount containing 20 ng of the BRCA1 subunit) (Figs. 1B and 2A). However, experiments with the Ub-K ubiquitin series (Figs. 1C and 3A) and the BRCA1 auto ubiquitination assays (Fig. 4) were conducted using 0.125 μg 6H-UbcH5c and 0.25 μg ubiquitin. The E6-AP-dependent reactions were conducted with 240 ng of 6H-E6-AP, 0.6 μg of 6H-UbcH5c, and 1 μg of ubiquitin (Fig. 2B) or 300 ng of 6H-E6-AP, 1.25 μg of 6H-UbcH5c, and 1 μg of ubiquitin (Fig. 3B). After terminating the reactions with 6 μl of 5× SDS loading buffer (0.3 M Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 25% β-mercaptoethanol, 0.05% bromphenol blue), the reaction products were fractionated by SDS-PAGE and detected by immunoblotting. The ubiquit-in specific (FL-76 polyclonal and P4D1 monoclonal) and BRCA1-specific (C20 polyclonal) antibodies were purchased from Santa Cruz Biotechnology.

**RESULTS**

To express full-length BRCA1/BARD1 heterodimers, SF9 insect cells were co-infected with baculoviruses encoding FLAG-BRCA1–6H, a full-length BRCA1 polypeptide with an amino-terminal FLAG epitope and a COOH-terminal hexahistidine tag, and GST-BARD1, a fusion polypeptide containing GST linked to full-length BARD1. The BRCA1/BARD1 heterodimer was purified from SF9 lysates by sequential affinity chromatography on a Ni-NTA resin and glutathione-agarose beads (Fig. 1A, lane 2). In addition, a heterodimer containing the tumor-associated C61G mutation of BRCA1 was also expressed and purified (Fig. 1A, lane 3). Both were then tested for their ability to catalyze ubiquitin polymerization in the presence of UbcH5c, one of three closely related E2 enzymes (UbcH5a, UbcH5b, and UbcH5c) known to support BRCA1-dependent ubiquitination (8–14). The ubiquitination reactions were fractionated by SDS-PAGE, and the formation of polyubiquitin was assessed by immunoblotting with ubiquitin-specific antibodies. As expected, high molecular mass ubiquitinated products were generated in an ATP-dependent manner by the wild type (Fig. 1B, lane 3), but not the mutant (lane 4), BRCA1/BARD1 heterodimer. Ubiquitination reactions were also conducted using Ub-K0, a lysineless derivative of ubiquitin in which all seven lysines have been substituted with arginine. As such, Ub-K0 retains the potential for monoubiquitination but lacks the conjugation sites necessary for polyubiquitination. As shown in Fig. 1C, reactions with Ub-K0 only generate ubiquitinated products that migrate close to the molecular mass of FLAG-BRCA1–6H (lane 7) and presumably represent monoubiquitination of FLAG-BRCA1–6H at one or a few sites. This result is consistent with previous reports of BRCA1 auto ubiquitination (11–13) and indicates that the more slowly migrating products generated with wild type ubiquitin (Fig. 1C, lane 6) contain true polyubiquitin chains.

To evaluate the chain specificity of BRCA1/BARD1-dependent polyubiquitination, we used a panel of mutant ubiquitin molecules in which arginine was substituted for one of the seven lysine residues: Ub-R6, Ub-R11, Ub-R27, Ub-R29, Ub-R33, Ub-R48, and Ub-R63. For example, in Ub-R6, only lysine 6 has been changed to arginine. As shown in Fig. 2A polyubiquitin chains were generated with wild type ubiquitin (lane 2). However, with Ub-K0, a lysineless derivative of ubiquitin in which all seven lysines have been substituted with arginine, the ubiquitination products were generated in an ATP-dependent manner by the wild type (Fig. 1B, lane 3), but not the mutant (lane 4), BRCA1/BARD1 heterodimer. Ubiquitination reactions were also conducted using Ub-K0, a lysineless derivative of ubiquitin in which all seven lysines have been substituted with arginine. As such, Ub-K0 retains the potential for monoubiquitination but lacks the conjugation sites necessary for polyubiquitination. As shown in Fig. 1C, reactions with Ub-K0 only generate ubiquitinated products that migrate close to the molecular mass of FLAG-BRCA1–6H (lane 7) and presumably represent monoubiquitination of FLAG-BRCA1–6H at one or a few sites. This result is consistent with previous reports of BRCA1 auto ubiquitination (11–13) and indicates that the more slowly migrating products generated with wild type ubiquitin (Fig. 1C, lane 6) contain true polyubiquitin chains.
FLAG-BRCA1 missense mutation of BRCA1. Silver-stained bands representing the mutant heterodimer (lane 2 and asterisk lane 2 markers, PAGE, and visualized by silver staining. were purified from baculovirus-infected Sf9 cells, fractionated by SDS-PAGE, and immunoblotted with ubiquitin-specific antibodies. For B, reactions were conducted in the absence (lane 1) or presence of either wild type (lanes 2 and 3) or mutant (lane 4) BRCA1/BARD1. Reactions were also conducted in the absence (lane 2) or presence (lanes 1, 3, and 4) of ATP. For C, reactions were conducted using either the wild type (wt; lanes 5 and 6) or lysineless (K0; lane 7) form of ubiquitin, in the absence (lane 5) or presence (lanes 6 and 7) of wild type BRCA1/BARD1. Polymerized (Ub–) and free (Ub) forms of ubiquitin are indicated to the right of C, and the mobilities of the molecular mass markers are indicated in kilodaltons.

FIG. 1. In vitro polyubiquitination by the full-length BRCA1/BARD1 heterodimer. A, full-length BRCA1/BARD1 heterodimers were purified from baculovirus-infected Sf9 cells, fractionated by SDS-PAGE, and visualized by silver staining. Lane 1 shows molecular mass markers, lane 2 the wild type heterodimer (B/B-ut), and lane 3 a mutant heterodimer (B/B-ent) containing the tumor-associated C61G missense mutation of BRCA1. Silver-stained bands representing the full-length BRCA1/BARD1 heterodimer as an E3 ligase and UbcH5c as an E2 conjugating enzyme. B, reactions were conducted with E6-AP as an E3 ligase and UbcH5c as an E2 conjugating enzyme.

FIG. 2. The linkage specificities of BRCA1/BARD1 and E6-AP analyzed by in vitro ubiquitination with the Ub-R panel of ubiquitin mutants. In vitro ubiquitination reactions were performed with wild type ubiquitin (lanes 1) and with each of the seven Ub-R ubiquitin mutants (lanes 2–8). A, reactions were catalyzed using the BRCA1/BARD1 heterodimer as an E3 ligase and UbcH5c as an E2 conjugating enzyme. B, reactions were conducted with E6-AP as an E3 ligase and UbcH5c as an E2 conjugating enzyme. The mobilities of the molecular mass markers are indicated in kilodaltons.

FIG. 3. The linkage specificities of BRCA1/BARD1 and E6-AP determined by in vitro ubiquitination with the Ub-K panel of ubiquitin mutants. In vitro ubiquitination reactions were performed with lysineless ubiquitin Ub-K0 (lanes 1) and with each of the seven Ub-K ubiquitin mutants (lanes 2–8). A, reactions were catalyzed using the BRCA1/BARD1 heterodimer as an E3 ligase and UbcH5c as an E2 conjugating enzyme. B, reactions were conducted with E6-AP as an E3 ligase and UbcH5c as an E2 conjugating enzyme.

FIG. 4. Autoubiquitinated BRCA1 contains primarily K6-linked chains. BRCA1/BARD1-dependent ubiquitination reactions were fractionated by SDS-PAGE, and the BRCA1 polypeptides were detected by immunoblotting with a BRCA1-specific polyclonal antiserum. Reactions were performed in the absence of ubiquitin (lanes 1 and 4) or in the presence of either wild type ubiquitin (lane 2), Ub-R6 mutant ubiquitin (lane 3), lysineless Ub-K0 mutant ubiquitin (lane 5), or Ub-K6 mutant ubiquitin (lane 6). The mobilities of polyubiquitinated (BRCA1-Ub n) and free (BRCA1) forms of BRCA1 are indicated on the left.

biquitination was abolished in reactions conducted with Ub-R6, suggesting that BRCA1/BARD1 catalyzes the formation primarily of K6-linked ubiquitin polymers. Consistent with a previous study (12), the levels of polyubiquitination were reduced in Ub-R48 and Ub-R63 reactions, indicating that BRCA1/BARD1 may also induce, to a lesser extent, formation of K48- and K63-linked chains. As a control, we examined the linkage specificity of E6-AP, a ubiquitin ligase that can induce ubiquitination and degradation of the p53 tumor suppressor (25) and, like BRCA1, can function in collaboration with the UbcH5 family of E2 enzymes (26). As shown in Fig. 2B, E6-AP polyubiquitination was completely ablated in the Ub-K48 reaction, implying that E6-AP directs the synthesis of K48-linked chains.

To confirm the linkage specificities of BRCA1/BARD1 and E6-AP, in vitro reactions were also conducted using a panel of ubiquitin mutants in which all but one of the seven lysine residues are substituted with arginine: Ub-K6, Ub-K11, Ub-K27, Ub-K29, Ub-K33, Ub-K48, and Ub-K63. For example, in Ub-K6, all the lysines are changed to arginine with the exception of residue K6. As shown in Fig. 3A, robust polyubiquitination by BRCA1/BARD1 was only observed in reactions containing Ub-K6. On long exposures of the immunoblots, significantly lower levels of polyubiquitination were also detected in reactions conducted with Ub-K27, Ub-K48, and Ub-K63 (data not shown). These results confirm that while BRCA1/BARD1 preferentially catalyzes the formation of K6-linked polymers, it also has some capacity to induce synthesis of chains with other linkages. As shown in Fig. 3B, only Ub-K48 supported polymer formation by E6-AP, confirming that K48-linked chains are the major products of E6-AP-dependent ubiquitination.

Although heterologous substrates of BRCA1/BARD1-mediated polyubiquitination have not yet been identified, several groups have shown that the BRCA1 subunit of the heterodimer undergoes autoubiquitination in vitro (11–13). To determine whether K6-linked chains are conjugated to BRCA1 during autoubiquitination, BRCA1/BARD1-dependent reactions were conducted and the resulting ubiquitin-conjugates of BRCA1 were visualized by immunoblotting with BRCA1-specific antibodies (Fig. 4). As expected, only the unmodified FLAG-BRCA1–6H polypeptide was detected in reactions conducted in the absence of ubiquitin (lanes 1 and 4). In the presence of wildtype ubiquitin, high molecular mass species of polyubiquitinated BRCA1 were readily generated (lane 2), consistent with previous reports of BRCA1 autoubiquitination (11–13). In
contrast, however, polyubiquitinated BRCA1 was not observed in reactions conducted with Ub-R6 (lane 3), indicating that autoubiquitination primarily produces BRCA1 conjugates with K6-linked chains. The ability of BRCA1/BARD1 to induce K6-linked polyubiquitinated BRCA1 was confirmed by the observation that BRCA1 polyubiquitination occurs in reactions conducted with Ub-K6 (lane 6), the ubiquitin mutant that harbors a single lysine at residue 6, but not with the lysineless ubiquitin Ub-K0 (lane 5).

**DISCUSSION**

The identification of K6, an unconventional site for ubiquitin polymerization, as the principal linkage generated by BRCA1/BARD1 was unexpected. Nevertheless, several precautions were taken to ensure the proper fidelity of linkage formation during *in vitro* polyubiquitination. First, the reactions were conducted with a full-length heterodimer that contains the complete amino acid sequences of both BRCA1 and BARD1. Second, the ubiquitin monomers and their mutant derivatives were synthesized and purified without the use of epitope tags that could potentially bias linkage specificity. Third, to avoid misincorporation of lysine for arginine in the ubiquitin mutants, a known hazard of ubiquitin synthesis in bacteria (27), all arginine substitutions were generated by site-directed mutagenesis, and all ubiquitin monomers were expressed in a bacterial strain supplemented with exogenous tRNAs that recognize the problematic arginine codons (AGG and AGU). Finally, the fact that E6-AP-dependent ubiquitination produced primarily K48-linked polymers indicates that polyubiquitination through K48, the most common site for polymerization, can occur in our assays provided that the appropriate E3 is present. Although the linkage specificity of E6-AP had not been defined previously, the synthesis of K48 linked chains is consistent with the known ability of E6-AP to induce proteasomal degradation of substrates such as p53 (26).

While K29- and K48-linked chains can target their conjugated substrates for proteasomal degradation, K63 chains are believed to serve diverse non-proteolytic functions (23). In contrast, however, the cellular functions of K6 polyubiquitin chains are not known. To date, the production of K6 chains has only been observed in E3-independent reactions catalyzed by Rad6, the yeast ortholog of UbcH2 (22). Interestingly, although Rad6 forms exclusively K6 linkages in E3-independent reactions, it generates K48 chains in the presence of an appropriate E3 (22). This suggests that the chain linkage of ubiquitination can be influenced by the combined specificities of the collaborating E2 and E3 enzymes. A similar phenomenon is observed here, in that UbcH5c generates primarily K48 chains in the presence of E6-AP and K6 chains in the presence of BRCA1/BARD1.

A recent study using a selected panel of ubiquitin mutants (Ub-A48, Ub-A63, and Ub-A48/63) indicated that BRCA1/BARD1 can potentially generate K48- and K63-linked polymers (12). While our data are consistent with these observations, the use of a comprehensive panel of ubiquitin mutants reveals that K6 is the preferred linkage specificity of BRCA1/BARD1. Nonetheless, if BRCA1/BARD1 does catalyze, even to a modest extent, the formation of K48 or K63 linkages, then branched polymers could potentially be generated during BRCA1/BARD1-dependent ubiquitination. At present, the physiological functions of K6-linked chains, whether branched or not, have not been defined.

Important insights into the molecular functions of BRCA1 should emerge once the natural substrates of BRCA1/BARD1-dependent ubiquitination are identified. The only potential polyubiquitination substrate reported to date is BRCA1 itself, which undergoes autoubiquitination *in vitro* (11–13). Here we have shown that autoubiquitination results in conjugation of mostly K6 link chains to BRCA1. In light of this, it may be significant that the BRCA1 and BARD1 polypeptides appear to stabilize each other upon co-expression *in vivo* (7, 10, 28). Since BRCA1 autoubiquitination is catalyzed much more efficiently by the BRCA1/BARD1 heterodimer than by BRCA1 alone (11–13), it seems unlikely that autoubiquitination, if indeed it occurs *in vivo*, serves to target BRCA1 for proteasomal degradation. This in turn suggests that K6-linked chains may not promote proteasome-dependent proteolysis. Clearly, further studies will be necessary to determine the physiological functions of K6-linked polyubiquitin and its role in BRCA1-mediated tumor suppression.

**Acknowledgments**—We thank Drs. Wei Gu and Thomas Ludwig for their advice and critical comments on the manuscript. We are also very grateful to Dr. Dirk Bohmann for kindly providing ubiquitin cDNA plasmids.

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