BRCA1-Associated Protein 1 Interferes with BRCA1/BARD1 RING Heterodimer Activity

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Abstract

The breast and ovarian tumor suppressor BRCA1 constitutes a RING heterodimer E3 ligase with BARD1. BRCA1-associated protein 1 (BAP1) is a ubiquitin COOH-terminal hydrolase that was initially identified as a protein that bound to the RING finger domain of BRCA1. However, how BAP1 contributes to the E3 activity of BRCA1/BARD1 is unclear. Here, we report that BAP1 interacts with BARD1 to inhibit the E3 ligase activity of BRCA1/BARD1. Domains comprised by residues 182-365 of BAP1 interact with the RING finger domain of BARD1, and surface plasmon resonance spectroscopy (BIAcore) analyses showed that BAP1 interferes with the BRCA1/BARD1 association. The perturbation resulted in inhibition of BRCA1 autoubiquitination and NPM1/B23 ubiquitination by BRCA1/BARD1. Although BAP1 was capable of deubiquitinating the polyubiquitin chains mediated by BRCA1/BARD1 *in vitro*, a catalytically inactive mutant of BAP1, C91S, still inhibited the ubiquitination *in vitro* and *in vivo*, implicating a second mechanism of action. Importantly, inhibition of BAP1 expression by short hairpin RNA resulted in hypersensitivity of the cells to ionizing irradiation and in retardation of S-phase progression. Together, these results suggest that BAP1 and BRCA1/BARD1 coordinately regulate ubiquitination during the DNA damage response and the cell cycle. [Cancer Res 2009;69(1):111–9]

Introduction

Germ-line mutations of *BRCA1* predispose women to early-onset familial breast and ovarian cancers (1). In addition, impairment of BRCA1 pathways is likely responsible for basal-like breast cancer, a subset of breast cancer categorized by a gene expression profile that is associated with poor prognosis and distinctive chemosensitivity, implying the importance of BRCA1 not only in familial breast cancers but also in sporadic cancers (2, 3). BRCA1 is a multifunctional protein that participates in various cellular supercomplexes to execute its tasks, including homologous recombination repair of DNA double-strand breaks, chromatin remodeling, cell cycle checkpoints, transcription, apoptosis, and regulation of centrosome duplication (4, 5). In many of these complexes, BRCA1 exists as a RING heterodimer with BARD1 (6) whose formation is required for ubiquitin E3 ligase activity (7).

Ubiquitination is a multistep process whereby ubiquitin is first activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent process, then transferred to a ubiquitin carrier protein (E2), and, finally, transferred to the ε-amino group of lysine in the substrate protein in a reaction catalyzed by a ubiquitin ligase (E3; ref. 8). Both BRCA1 and BARD1 contain an NH_2-terminal RING finger domain and COOH-terminal tandem BRCT domains. The cleft composed by the RING finger of BRCA1 interacts with E2 (9). The BARD1 RING finger domain is required to support this interaction by binding to the BRCA1 RING finger from a different direction (9).

BARD1 is also required for BRCA1 protein stability (7, 10), and therefore, deficiency of *BARD1* in mice causes similar phenotypes to that of *BRCA1* (11). These include basal-like breast cancers generated by mammary-specific inactivation of *BARD1* (12). Because frequency, latency, and histopathology of the cancer in the conditional *BARD1*-mutant mice were indistinguishable from those that developed in conditional *BRCA1*-mutant mice and double conditional *BARD1/BRCA1*-mutant mice, the tumor suppressor activities of both genes are likely mediated through the BRCA1/BARD1 heterodimer (12). However, how the dimer interaction and E3 ligase activity are regulated is poorly understood.

BRCA1-associated protein 1 (BAP1) is a nuclear-residing ubiquitin COOH-terminal hydrolase (UCH), a subfamily of deubiquitinating enzymes (DUB), which was originally identified by the yeast two-hybrid technique as a protein that interacted with the RING finger domain of BRCA1 and enhanced BRCA1-mediated cell growth suppression (13). DUBs are proteases that catalyze the hydrolysis of the isopeptide linkage that joins the COOH-terminal glycine of ubiquitin and a lysine side chain on the target polypeptide, resulting in removal of single ubiquitin moieties from ubiquitin chains or cleavage of the isopeptide bond between ubiquitin and the substrate protein (14). The DUB enzymes are classified into five categories: UCH, the ubiquitin-specific processing proteases (UBP or USP), Jab1/Pad1/MPN domain containing metalloproteinases (JAMM), Otu domain ubiquitin-aldehyde binding proteins (OTU), and Ataxin-3/Josephin domain containing proteins (Ataxin-3/Josephin). Whereas the USP family includes at least 58 members, the UCH family consists of only 4 members: UCH-L1, UCH-L3, UCH-L5/UCH37, and BAP1 (14). Whereas UCH enzymes are generally small, containing only a proteolytic core domain, BAP1 is a relatively large protein with an NH_2-terminal proteolytic core domain and a COOH-terminal domain that contains nuclear localization signals and interacts with the BRCA1 RING finger (13). However, the role of BAP1 in BRCA1 function is poorly understood; for example, whether BAP1 affects the E3 ligase activity of BRCA1/BARD1 remains to be determined. A previous report showed that BAP1 was incapable of deubiquitinating autoubiquitinated BRCA1, although it possessed ubiquitin hydrolase activity (15).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Here, we report that BAP1 inhibits the E3 ligase activity of BRCA1/BARD1 by binding to BARD1 and perturbing RING heterodimer formation in addition to deubiquitinating BRCA1/BARD1-mediated ubiquitination. Down-regulation of BAP1 in cells by short hairpin RNA (shRNA) resulted in S-phase retardation and ionizing irradiation (IR) hypersensitivity, a phenotype similar to BRCA1 deficiency, suggesting that BRCA1-mediated ubiquitination and BAP1-mediated ubiquitination-suppression may coordinately function in these cellular processes. The results show the first example that a DUB directly inhibits E3 ligase activity.

Materials and Methods

Plasmids. cDNAs for full-length human BAP1 and UCH-L3 were amplified by PCR from a HeLa cell cDNA library using Pfu polymerase (Stratagene) and subcloned into pcDNA3 or pET-3E vector in frame with the appropriate NH_2-terminal tag. Mammalian expression plasmids for BRCA1, BARD1, and quadruple repeated hemagglutinin (HA)-ubiquitin were previously described (7, 16). For simultaneous expression of BRCA1 and BARD1 in *Escherichia coli*, FLAG-BRCA1$^{1-304}$ and BARD1$^{1-320}$ were subcloned into pCold and pRSF-1b vectors, respectively, in frame with a hexa-histidine (His) tag. The fused pGEX-4T3/pET-15b construct for the simultaneous expression of glutathione *S*-transferase (GST)-HA-ROC1 and His-FLAG-CUL1$^{324-776}$ is a gift from Dr. Zhen-Qiang Pan (Mount Sinai School of Medicine, New York, NY). The missense mutant pET-His-BAP1 C91S was generated by site-directed mutagenesis (Stratagene). Truncated mutants pcDNA3-FLAG-BAP1 (1-490, 240-729, 1-188, 182-365, 358-537, and 529-729) and pET-His-BAP1 (1-683) were generated by introducing a stop codon and/or *Bam*H1 restriction site by site-directed mutagenesis followed by *Bam*H1 digestion to eliminate the appropriate fragment. For expression of BAP1 shRNA, annealed oligonucleotides GATCCCCcacaagtctcaagagtcaTTCAAGAGAtgactcttgagacttgtggTTTTTC and TCGAGAAAAAccacaagtctcaagagtcaTCTCTTGAAtgactcttgagacttgtggGGG were introduced into the *Bgl*2/*Xba*I-digested pSuper-Retro-Puro-shRNA plasmid. All plasmids used were verified by DNA sequencing.

Cell culture and transfection. HeLa cervical carcinoma cells and HEK-293T transformed human kidney cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic agent (Life Technologies, Inc.) in 5% CO_2 at 37°C. HEK-293T cells were transfected using the standard calcium phosphate precipitation method. For each transfection, total plasmid DNA was adjusted by adding the parental pcDNA3 vector. Cells were harvested 36 h after transfection and subjected to further analyses. To generate cell lines that stably expressed either shRNA specific for BAP1 or control shRNA, HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) with pSuper-Retro-Puro-shRNA plasmids. Four days after transfection, cells were diluted, seeded, and selected with 5 μg/mL puromycin. Colonies of the transformants were obtained after 5 d of culture and were further amplified and maintained in 2.5 μg/mL puromycin. Cell synchronization by a double thymidine block was previously described (17). Progression through the cell cycle was monitored by flow cytometry analysis of cells stained with propidium iodide using FACSCalibur (Becton Dickinson). To study the sensitivity of cells to IR, cells were irradiated at the indicated doses and cultured in fresh medium for 3 d. Cell viabilities were analyzed in triplicate by CellTiter-Blue (Promega Corp.) according to the manufacturer's instructions.

Immunologic techniques. Mouse monoclonal antibodies to BAP1 (C-4, Santa Cruz Biotechnology), HA (12CA5, Boehringer Mannheim), Myc (9E10, BabCo), FLAG (M2, Sigma), conjugated ubiquitin (FK2, Nippon Bio-Test), α-tubulin (DM1A, NeoMarkers), and NPM1/B23 (FC82291, Sigma) as well as rabbit polyclonal antibody to BARD1 (BL518, Bethyl) and BRCA1 (C20, Santa Cruz Biotechnology) were purchased commercially. Immunoprecipitation and immunoblotting methods, including the detection of *in vivo* autoubiquitinated BRCA1, were previously described (16, 18).

Purified proteins. His-FLAG-BRCA1$^{1-304}$, His-BAP1, His-BAP1 C91S, His-FLAG-BAP1, and His-UCH-L3 were purified from BL21/DE3 bacteria cells following isopropyl-L-thio-B-D-galactopyranoside (IPTG) induction as previously described (7, 16). Purification of His-UbcH5c, His-BRCA1$^{1-304}$, His-BARD1$^{14-189}$, GST-BRCA1$^{1-342}$, and His-FLAG-HA-NPM1 was previously described (7, 16, 18). To purify His-FLAG-BRCA1$^{1-304}$ and His-BARD1$^{1-320}$ as a RING heterodimer, BL21/DE3 bacteria cells were first transformed with pCold-His-FLAG-BRCA1$^{1-304}$ and selected with ampicillin. Cells were then transformed with pRSF-1b-His-BARD1$^{1-320}$ and selected with ampicillin and kanamycin. His-FLAG-BRCA1$^{1-304}$ was first expressed by IPTG induction at 15°C for 24 h followed by expression of His-BARD1$^{1-320}$ at 25°C for 16 h. Proteins were extracted under naturing conditions and purified with nickel beads as described (7). Fractions containing the heterodimer were further purified with AKTA purifier (HiLoad 16/60 Superdex 200 pg column, GE Healthcare) according to the manufacturer's instructions (Supplementary Fig. S1). GST-HA-ROC1/His-FLAG-CUL1$^{324-776}$ was purified as described elsewhere (19). Rabbit E1 (Affiniti), mammalian ubiquitin (Affiniti), and isopeptidase T (Boston Biochem) were purchased commercially.

Surface plasmon resonance (BIAcore) analysis. Analyses were performed using a BIAcore 3000 instrument (GE Healthcare). Purified His-FLAG-BAP1, His-BRCA1$^{1-304}$, or anti-FLAG antibody was immobilized (700–3,000 resonance units) on the surface of each CM5 sensor chip (GE Healthcare) using an amine coupling kit (GE Healthcare) according to the manufacturer's instructions. In some experiments, FLAG-tagged protein (complex) was first immobilized on the tip through the anti-FLAG antibody. Binding of proteins was measured at a flow rate of 10 μL/min in 145 mmol/L NaCl/10 mmol/L HEPES (pH 7.4) containing 100μg/mL or indicated concentration of proteins and 0.005% surfactant P20 (GE Healthcare). An equivalent volume of each protein sample was injected over a surface with no protein immobilized to serve as a blank sensorgram for subtraction of the bulk refractive index background. Regeneration of the surfaces was achieved by injection of 20 μL of 10 mmol/L glycine-HCl (pH 2.0). Data were analyzed using the manufacturer's software (GE Healthcare).

GST pull-down assays. Indicated amounts of GST-BRCA1$^{1-342}$ protein were mixed with His-BARD1$^{14-189}$, His-BAP1, or both and 10 μL of glutathione-agarose beads in 1 mL of buffer containing 50 mmol/L Tris-HCl (pH 7.5), 0.5% NP40, 150 mmol/L NaCl, 50 mmol/L NaF, and 1 mmol/L DTT. After rotation at 4°C for 2 h, proteins bound to glutathione-agarose beads were washed thrice, boiled in Laemmli SDS-loading buffer with 0.1 mol/L DTT, and resolved by SDS-PAGE followed by Sypro Ruby staining (GE Healthcare).

Ubiquitin ligation and deubiquitination assays. The procedure used for the *in vitro* ubiquitin ligation assay was performed as previously described (7, 16) with a reaction mixture (30 μL) that contained 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L $MgCl_2$, 2 mmol/L NaF, 10 nmol/L okadaic acid, 2 mmol/L ATP, 0.6 mmol/L DTT, 1 μg mammalian ubiquitin, 40 ng E1, 0.3 μg UbcH5c, 1 μg His-FLAG-BRCA1$^{1-304}$/His-BARD1$^{1-320}$, and 1 μg or indicated amount of either His-BAP1, His-UCH-L3, or isopeptidase T. In some experiments, E3 was substituted with GST-HA-ROC1/His-FLAG-CUL1$^{324-776}$. For ubiquitination of NPM1, 0.5 μg His-FLAG-NPM1 was added to the reaction. The ubiquitination products were detected by immunoblot with the indicated antibodies.

For the *in vitro* deubiquitination assay, the ubiquitin ligation reactions were carried out as described above with GST-BRCA1$^{1-342}$/His-BARD1$^{14-189}$ as the E3. The reactions were terminated by adding 1 mL cold buffer A containing 25 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 0.01% NP40, 10% glycerol, and 1 mmol/L EDTA. The autoubiquitinated GST-BRCA1$^{1-342}$–containing protein complex was precipitated with glutathione-agarose beads, washed twice with buffer A, and added to 30 μL of deubiquitination reaction buffer containing 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, and 1 mmol/L DTT. Then, 100 nmol/L or the indicated amount of BAP1, UCH-L3, or isopeptidase T was added and incubated at 37°C for 30 min or the indicated time length. Products were resolved by SDS-PAGE and analyzed by Sypro Ruby staining or immunoblotting with FK2 antibody.

Results

BARD1 interacts with BAP1 *in vivo*. It has been reported that BAP1 interacts with the RING finger of BRCA1 (13). Because the RING finger of BRCA1 also interacts with BARD1, we sought to

determine how the three proteins interact with each other. First, HEK-293T cells were transfected with either FLAG-BRCA1 or FLAG-BARD1, and anti-FLAG immunocomplexes were precipitated to examine whether they were capable of coprecipitating endogenous BAP1. Immunoblot of the immunocomplexes with anti-BAP1 antibody showed that the FLAG-BARD1 immunocomplex, but not the FLAG-BRCA1 immunocomplex, coprecipitated BAP1, whereas both complexes coprecipitated endogenous BRCA1 and BARD1, respectively (Fig. 1*A*). We next overexpressed all three proteins (FLAG-BRCA1, Myc-BARD1, and HA-BAP1) and analyzed their interactions by immunoprecipitation and immunoblot. HA-BAP1 was faintly detected in the FLAG-BRCA1 immunocomplex only when Myc-BARD1 was cotransfected (Fig. 1*B, lane 6*). On the other hand, when cotransfected with FLAG-BRCA1, the Myc-BARD1 immunocomplex coprecipitated a larger amount of HA-BAP1 (*lane 10*). In the absence of FLAG-BRCA1, Myc-BARD1 coprecipitated less HA-BAP1 (*lane 12*). These results suggest that BAP1 primarily interacts with BARD1, although BRCA1 plays some role in enhancing the interaction.

BRCA1 interacted with BAP1 via its COOH terminus (13). To test the possibility that BAP1 could interact with BARD1 independent of BRCA1, we next generated BAP1 with an NH_2-terminal or COOH-terminal deletion and tested its binding ability to BARD1. HEK-293T cells were cotransfected with HA-BARD1 and FLAG-BAP1 fragments corresponding to residues 1-490 or 240-729. Anti-HA immunoblot of anti-FLAG immunoprecipitations revealed that deletion of the BAP1 COOH terminus did not disrupt the interaction with BARD1, whereas deletion of the NH_2 terminus abolished the interaction (Fig. 1*C* and *D*). To further map the BARD1 binding site in BAP1, we generated four smaller FLAG-BAP1 fragments composed of residues 1-188, 182-365, 358-537, and 529-729 and tested their ability to bind to BARD1. Whereas FLAG-$BAP1^{182\text{-}365}$, a fragment comprising the region adjacent to the NH_2-terminal UCH domain, coimmunoprecipitated HA-BARD1, other fragments did not (Fig. 1*C* and *D*). Hence, BARD1 interacts with BAP1 through a domain different from the previously known BRCA1 binding site (Fig. 1*D*).

BAP1 interferes with the BRCA1/BARD1 interaction. To further dissect the BRCA1/BARD1/BAP1 interaction, we next used surface plasmon resonance (BIAcore) analysis. His-$BRCA1^{1\text{-}304}$ and His-$BARD1^{14\text{-}189}$, comprising the NH_2-terminal RING domains of BRCA1 and BARD1, and His-FLAG-BAP1 were bacterially purified. His-FLAG-BAP1 was fixed on a sensor tip through an anti-FLAG antibody that had been immobilized onto the tip, and then either His-$BRCA1^{1\text{-}304}$ or His-$BARD1^{14\text{-}189}$ was added. His-$BARD1^{14\text{-}189}$ interacted with His-FLAG-BAP1, and the interaction was stable enough to endure the wash flow (Fig. 2*A*). On the other hand, His-$BRCA1^{1\text{-}304}$ did not interact (Fig. 2*A*). This suggests that BAP1 is capable of interacting with the RING domain of BARD1 independent of BRCA1.

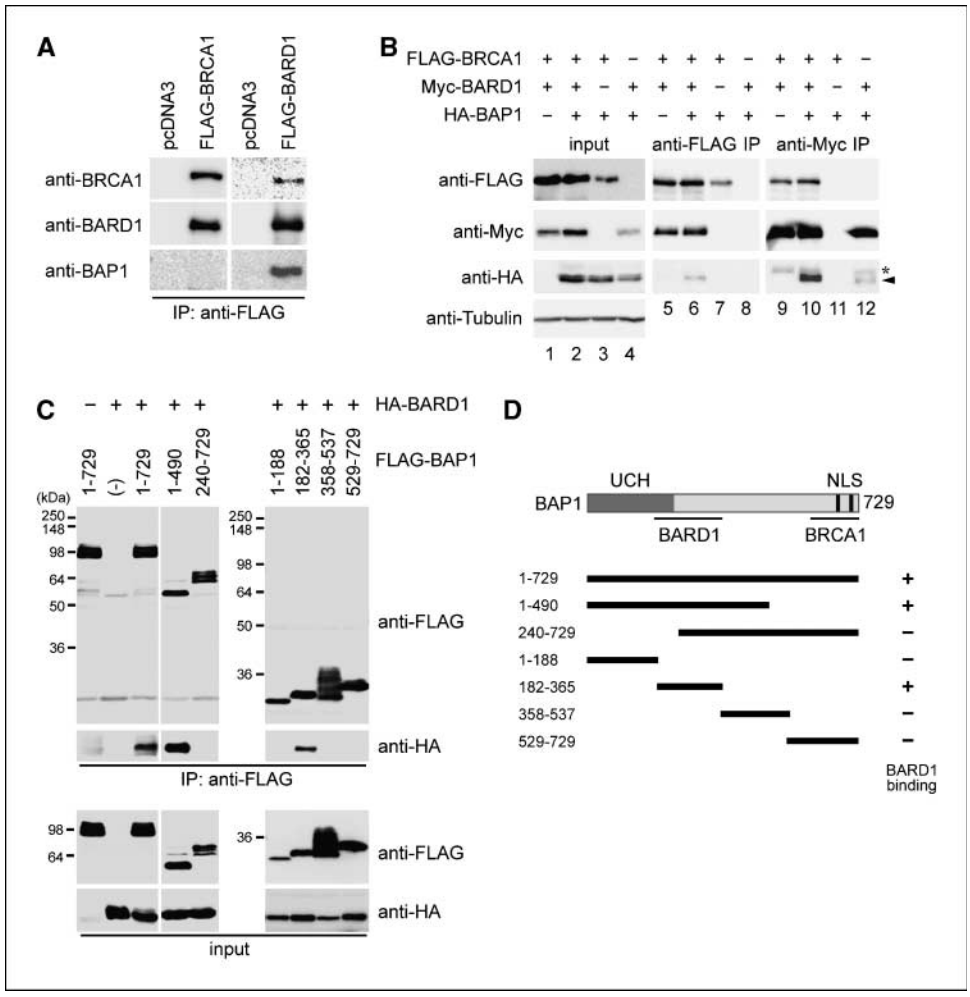

Figure 1. BARD1 interacts with BAP1 at a site different from the previously identified BRCA1 binding site. *A* and *B,* HEK-293T cells were transfected with the indicated expression plasmids or parental pcDNA3 vector (−). Cell lysates were immunoprecipitated (*IP*) and immunoblotted with the indicated antibodies. Three percent of the inputs were also loaded. *Arrowhead,* HA-BAP1; *asterisk,* cross-reaction with Myc-BARD1. *C,* the indicated BAP1 fragments were fused with an NH_2-terminal FLAG-tag and used to examine BARD1 binding as in *A*. *D,* summary of the BARD1 binding of the BAP1 fragments from *C*. The bar at the COOH terminus of BAP1 indicates the previously reported BRCA1 binding site.

To examine whether preformed BRCA1/BARD1 heterodimer complex was capable of binding to BAP1, we next purified His-FLAG-BRCA1$^{1-304}$ and His-BARD1$^{1-320}$ from *E. coli* cells coexpressing both proteins and selected the heterodimer fraction with AKTA purifier (Supplementary Fig. S1). His-FLAG-BAP1 was directly immobilized onto a sensor tip and then serially diluted panels of His-FLAG-BRCA1$^{1-304}$/His-BARD1$^{1-320}$ heterodimer were added. The addition of the heterodimer resulted in higher resonance in a concentration-dependent manner (Fig. 2*B*). This indicates that BRCA1, BARD1, or both could bind to BAP1 under conditions where BRCA1 and BARD1 previously formed a complex.

We next tested whether BAP1 competed with BRCA1 for BARD1 interaction. His-BRCA1$^{1-304}$ was directly immobilized onto a tip. A constant concentration of His-BARD1$^{14-189}$ was added with or without serially diluted panels of His-BAP1 (Fig. 2*C*). The addition of BARD1 without BAP1 resulted in high resonance, consistent with the RING heterodimer formation. Interestingly, addition of His-BAP1 into the flow caused loss of the acquired resonance in a concentration-dependent manner (Fig. 2*C*). This suggests that BAP1 interferes with the interaction between BRCA1 and BARD1.

Finally, we tested whether BAP1 could compete BARD1 away from the BRCA1/BARD1 heterodimer. Preformed His-FLAG-BRCA1$^{1-304}$/His-BARD1$^{1-320}$ heterodimer was immobilized onto a sensor tip through anti-FLAG antibody. Serially diluted panels of His-BAP1 were added. Importantly, the addition of His-BAP1 into the flow caused the loss of resonance in a concentration-dependent manner (Fig. 2*D*). Together, the BIAcore analyses suggest that BAP1 binds to the BRCA1/BARD1 RING heterodimer complex mainly through BARD1, and this binding results in dissociation of BARD1 from BRCA1.

To support the BIAcore analyses, we further analyzed the interaction by GST pull-down assay. GST-BRCA1$^{1-342}$ coprecipitated His-BARD1$^{14-189}$ as expected (Fig. 2*E, lane 8*). Consistent with the

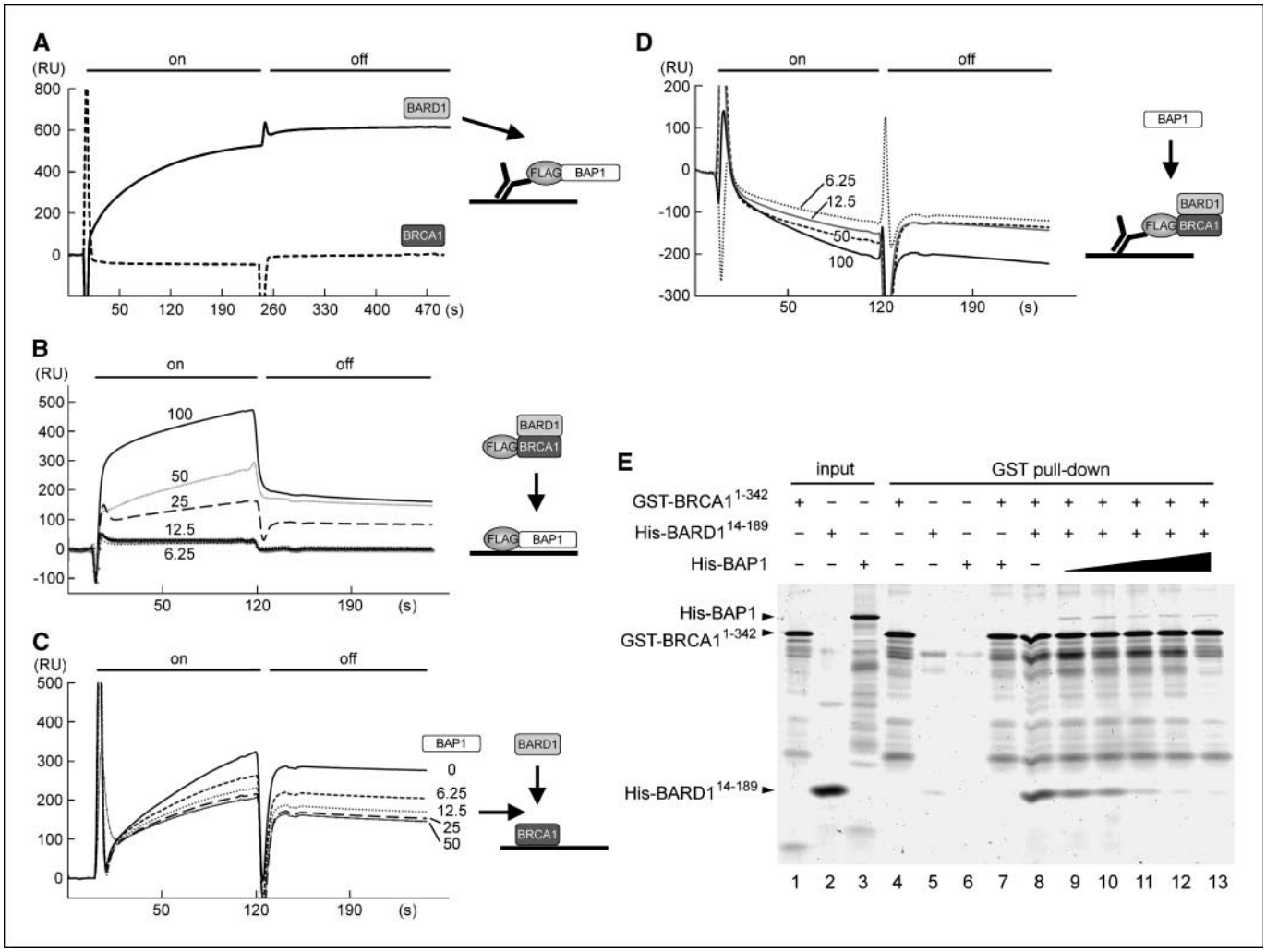

Figure 2. BAP1 interacts with BARD1 and perturbs BRCA1/BARD1 heterodimer formation. BIAcore analyses were carried out with purified His-FLAG-BAP1 (*A* and *B*), His-BRCA1$^{1-304}$ (*C*), or His-FLAG-BRCA1$^{1-304}$/His-BARD1$^{1-320}$ heterodimer (*D*) immobilized on the sensor chip either directly (*B* and *C*) or through anti-FLAG antibody (*A* and *D*). His-BRCA1$^{1-304}$ (100 μg/mL) or His-BARD1$^{14-189}$ (100 μg/mL; *A*), the indicated amount (μg/mL) of His-FLAG-BRCA1$^{1-304}$/His-BARD1$^{1-320}$ (*B*), His-BARD1$^{14-189}$ (100μg/mL) with the indicated amount (μg/mL) of His-BAP1 (*C*), or the indicated amount (μg/mL) of His-BAP1 (*D*) was subsequently injected onto the flow cells (*on*). The protein-containing buffer was substituted with buffer alone at the indicated time point (*off*). *E,* GST-BRCA1$^{1-342}$ (3 μg) was mixed with His-BARD1$^{4-189}$ (3 μg) and His-BAP1 (3 μg or 1, 2, 3, 6, and 8 μg for *lanes 9–13*), as indicated, and incubated with glutathione beads. The proteins bound to the beads were resolved by SDS-PAGE followed by Sypro Ruby staining.

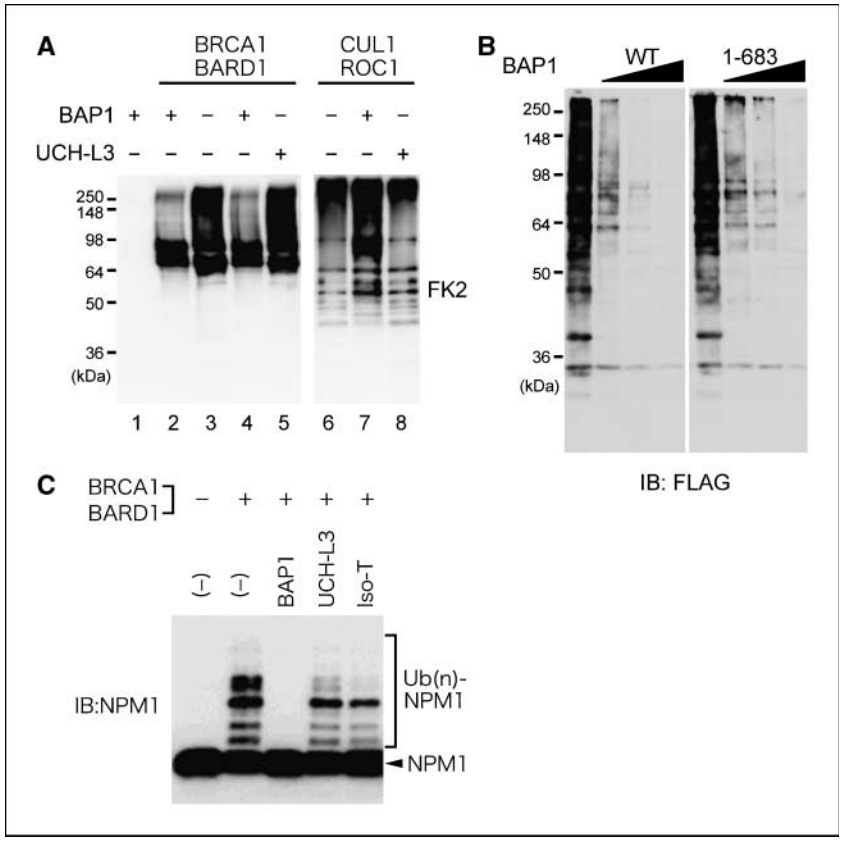

Figure 3. BAP1 inhibits E3 activity of BRCA1/BARD1. *A, in vitro* ubiquitin ligation assays were performed with ubiquitin, E1, UbcH5c, and either His-FLAG-BRCA1$^{1-304}$/His-BARD1$^{1-320}$ or GST-HA-ROC1/His-FLAG-CUL1$^{324-776}$ in the presence of ATP. His-BAP1 or His-UCH-L3 was added to the reaction as indicated. Polyubiquitinated products were detected by immunoblotting with FK2 antibody. *B, in vitro* ubiquitin ligation assays were performed with His-FLAG-BRCA1$^{1-304}$/His-BARD1$^{1-320}$ as in *A*. Either WT or a fragment (1-683) of His-BAP1 (0, 0.5, 1, and 2 μg for *lanes 1–4* and *lanes 5–8*) was added to the reaction. *C, in vitro* ubiquitin ligation assays were performed as in *A* with His-FLAG-NPM1 as a substrate. His-BAP1, His-UCH-L3, or isopeptidase T was added to the reaction. Ubiquitinated products were detected by immunoblotting with anti-NPM1 antibody.

BIAcore data, addition of His-BAP1 resulted in a decreased amount of the coprecipitated His-BARD1$^{14-189}$ in dose-dependent manner (*lanes 9–13*). Notably, a relatively small amount of His-BAP1 also coprecipitated with GST-BRCA1$^{1-342}$ only when His-BARD1$^{14-189}$ was present (compare *lane 7* versus *lanes 9–13*). This observation could be ascribed to an interaction between BAP1 and BRCA1 in the presence of BARD1.

BAP1 inhibits the E3 ligase activity of BRCA1. BARD1 interaction is required for the E3 ligase activity of BRCA1 (7, 9). Therefore, the perturbation of the BRCA1/BARD1 heterodimer mediated by BAP1 prompted us to examine whether BAP1 inhibited E3 activity. Purified His-FLAG-BAP1 was added to ubiquitin ligation reactions containing ATP, ubiquitin, E1, E2/UbcH5c, GST-BRCA1$^{1-342}$, and His-BARD1$^{14-189}$. The polyubiquitinated products were detected by anti-conjugated ubiquitin (FK2) antibody. Coupled with the data showing that BAP1 perturbs the BRCA1/BARD1 heterodimer, His-FLAG-BAP1 inhibited the ubiquitin ligation reaction (Fig. 3*A, lanes 2* and *4*). In contrast, His-UCH-L3, another deubiquitination enzyme in the UCH family, did not affect the reaction (*lane 5*). The inhibition was not a general effect of BAP1 on E3 ligases because the ubiquitin ligation reaction mediated by another RING type E3 ligase, ROC1/CUL1, was not inhibited (*lane 7*). The inhibition was not dependent on the above reported BRCA1-BAP1 interaction because deletion of the binding site in BAP1 still inhibited the activity (Fig. 3*B*). BAP1 also abolished NPM1 ubiquitination catalyzed by BRCA1/BARD1, whereas UCH-L3 and isopeptidase T (a COOH-terminal ubiquitin hydrolase involved in the recycling of ubiquitin) did not (Fig. 3*C*). Hence, BAP1 perturbs BRCA1/BARD1 heterodimer formation and inhibits its E3 activity.

Deubiquitination activity of BAP1. The observed inhibition of ubiquitin ligation was consistent with BAP1-mediated BARD1 dissociation from BRCA1 (Fig. 2). However, it was also possible that the absence of the ubiquitinated product was caused by deubiquitination activity of BAP1. Therefore, we examined whether BAP1 has activity to deubiquitinate BRCA1/BARD1-mediated polyubiquitin chains. *In vitro* ubiquitin ligation reactions were carried out with GST-BRCA1$^{1-342}$ and His-BARD1$^{14-189}$. Autoubiquitinated GST-BRCA1$^{1-342}$ was then pulled down with GST-agarose beads, washed, and subjected to a deubiquitination assay. The autoubiquitinated products were only slightly decreased on addition of His-FLAG-BAP1 (Fig. 4*A* and *B, top*). However, single ubiquitin molecules accumulated on addition of His-FLAG-BAP1 in a time-dependent manner (Fig. 4*A, bottom*) or BAP1 concentration-dependent manner (Fig. 4*B, bottom*). This suggests that BAP1 possesses deubiquitination activity toward ubiquitin chains catalyzed by BRCA1/BARD1. Deubiquitination of ubiquitinated BRCA1 was not BAP1 specific because UCH-L3 and isopeptidase T were also capable of deubiquitinating the substrate (Fig. 4*A* and *B*). Mutant BAP1, C91S, which carries an active site mutation (13, 15), did not cause accumulation of single ubiquitins (Fig. 4*C*), indicating that the reaction is mediated by the conserved UCH domain.

Catalytically inactive BAP1 inhibits E3 ligase activity of BRCA1. The notion that BAP1 possesses deubiquitination activity toward ubiquitination mediated by BRCA1/BARD1 prompted us to determine whether the observed decline of polyubiquitination (Fig. 3) was due to inhibition of BRCA1/BARD1 or deubiquitination. To examine this, either wild-type (WT) or the catalytically inactive C91S mutant of His-BAP1 was added to ubiquitin ligation

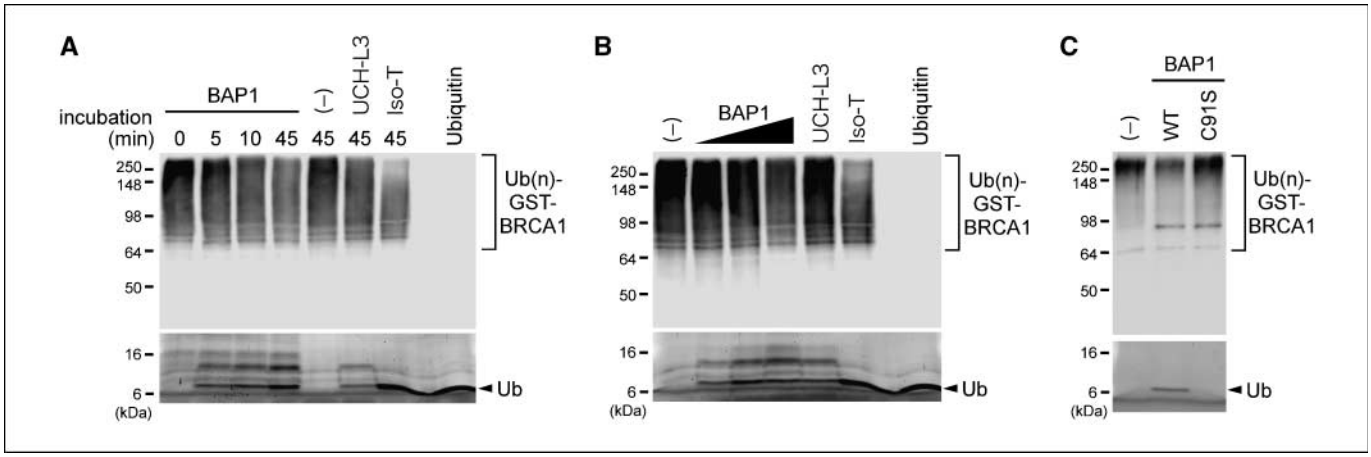

Figure 4. BAP1 exhibits deubiquitination activity toward BRCA1/BARD1-mediated ubiquitinated products. *A, in vitro* ubiquitin ligation assays were performed as in Fig. 3*A* with GST-BRCA1$^{1-342}$ and His-BARD1$^{14-189}$. Autoubiquitinated GST-BRCA1$^{1-342}$ immobilized on glutathione-agarose beads was incubated with 100 nmol/L of His-BAP1, His-UCH-L3, or isopeptidase T for the indicated time length. Autoubiquitinated GST-BRCA1$^{1-342}$ and dissociated free ubiquitin were detected by immunoblotting with FK2 antibody (*top*) and Sypro Ruby stain (*bottom*), respectively. Unreacted free ubiquitin was also loaded as an indicator of the position migrated. *B, in vitro* deubiquitination reactions were performed as in *A* with increasing amounts (25, 50, and 100 nmol/L for *lanes 2–4*) of BAP1. *C, in vitro* deubiquitination reactions were performed as in *A* with catalytically inactive mutant C91S BAP1.

reactions mediated by GST-BRCA1$^{1-342}$ and His-BARD1$^{14-189}$. Both WT and mutant BAP1 inhibited the reaction (Fig. 5*A*), indicating that the observed effect was not due to deubiquitination activity.

We next examined the BAP1 effect on BRCA1/BARD1 *in vivo*. HEK-293T cells were cotransfected with Myc-BRCA1$^{1-772}$, BARD1, and HA-ubiquitin. Cell lysates were boiled and immunoprecipitated with anti-Myc antibody. Autoubiquitinated Myc-BRCA1$^{1-772}$ was detected by immunoblotting with anti-HA antibody (Fig. 5*B, lane 3*). Cotransfection of WT as well as the C91S mutant of FLAG-BAP1 abolished the autoubiquitination (*lanes 4* and *5*). Together, the results indicate that BAP1 inhibits the E3 ligase activity of BRCA1 in a manner independent of its deubiquitination activity.

BAP1 inhibition retards S-phase progression. Loss of the BRCA1/BARD1 complex leads to S-phase retardation (20). To investigate the physiologic significance of BAP1 relative to BRCA1/BARD1 function, we established a HeLa cell line stably expressing shRNA to inhibit BAP1 expression and tested its effect on the cell cycle. BAP1 expression was effectively inhibited in cells expressing BAP1-specific shRNA compared with those expressing control shRNA (Fig. 6*A*). Then, the shRNA-expressing cells were arrested at the G_1-S boundary by a double thymidine block and released to synchronously progress through the cell cycle. Fluorescence-activated cell sorting (FACS) analyses showed that cells repressing BAP1 expression progressed through S phase ~2 hours slower than control cells (Fig. 6*B*). Thus, BAP1 inhibition causes S-phase retardation, a phenotype similar to BRCA1 or BARD1 deficiency. This suggests that BAP1 cooperates with BRCA1/BARD1 in S-phase progression.

BAP1 inhibition leads cells to hypersensitivity to IR. BRCA1/BARD1 is a critical mediator for cell cycle checkpoints and homologous recombination repair in response to DNA damage. Cells defective for BRCA1 exhibit hypersensitivity to IR (21, 22). We therefore tested the effect of BAP1 expression on cell viability after IR. Cells expressing BAP1-specific shRNA or control shRNA were treated with the indicated dose of IR, cultured for 3 days, and evaluated for cell viability. The viability of the BAP1 shRNA knocked down cells was significantly lower than that of control cells at all IR doses examined (Fig. 6*C*), suggesting that BAP1 inhibition causes IR hypersensitivity. Together, the results suggest that BAP1 and BRCA1/BARD1 coordinately regulate ubiquitination during the DNA damage response and the cell cycle.

Discussion

The E3 ligase activity of BRCA1/BARD1 has been thought to have a critical role in its tumor suppressor function, and it may act in multiple cellular processes, including the DNA damage response (6, 23). However, regulation of its activity is poorly understood. In the current study, we show that BAP1 directly interacts with BARD1 and perturbs the BRCA1/BARD1 RING heterodimer, resulting in inactivation of E3 ligase activity. In a previous report, it was shown that BAP1 directly interacts with the RING finger domain of BRCA1 (13). According to our results using recombinant proteins of each RING finger domain *in vitro*, it is likely that the major binding partner of BAP1 is BARD1, rather than BRCA1. In *in vivo* experiments using full-length proteins, however, BRCA1 enhanced the BARD1-BAP1 interaction (Fig. 1*B*, compare *lanes 10 and 12*), with which BAP1 did not interfere (compare *lanes 5* and *6*). This could be, in part, explained by cellular localization of BRCA1 and BARD1. The interaction between BRCA1 and BARD1 masks the nuclear export signals of both BRCA1 and BARD1, causing the BRCA1-BARD1 complex to remain nuclear (24, 25). Because endogenous (13) and transfected (data not shown) BAP1 localize to the nucleus, coexpression of BRCA1 may allow BARD1 the opportunity to encounter BAP1. In addition, because BAP1 interacts with BRCA1 through a domain distinct from the BARD1 binding site, it is possible that BAP1 directly interacts with both proteins through distinct domains to form a trimeric complex *in vivo*. GST pull-down assays also showed that BAP1 exists in the BRCA1 complex when BARD1 is present (Fig. 2*E*), consistent with the possibility of trimeric complex formation. In the case of a trimeric complex, BAP1 may interfere with the BRCA1-BARD1 RING heterodimer and its activity. The inhibition of BRCA1 autoubiquitination by BAP1 *in vivo* (Fig. 5*B*) supports this interpretation.

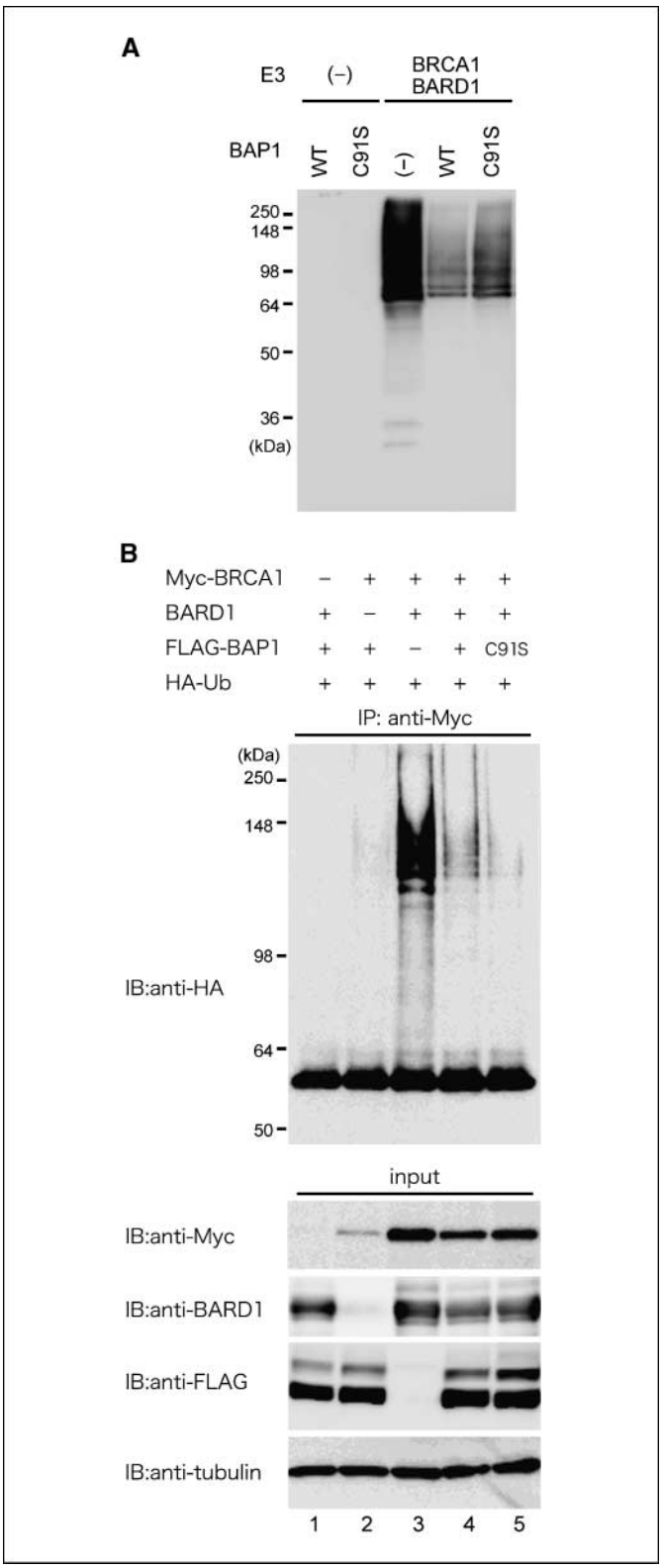

Figure 5. Catalytically inactive BAP1 still inhibits BRCA1/BARD1-mediated ubiquitination. *A, in vitro* ubiquitin ligation assays were performed as in Fig. 3*A*. Either WT or the C91S mutant of His-BAP1 was added to the reaction. *B,* HEK-293T cells were transfected with the indicated plasmids. *Top,* cell lysates were boiled, and autoubiquitinated Myc-BRCA1 was immunoprecipitated followed by immunoblot (*IB*) with anti-HA antibody; *bottom,* inputs of the lysates were also detected with the indicated antibodies.

BAP1 was also capable of deubiquitinating autoubiquitinated BRCA1/BARD1. However, it was previously reported that BAP1 did not deubiquitinate the polyubiquitinated form of BRCA1/BARD1 *in vitro* (15), although it had ubiquitin hydrolase activity on artificial small substrates (13, 15). This discrepancy could be due to the NH_2-terminal structure of BAP1. Substitution of His-FLAG-BAP1 for His-BAP1, the BAP1 protein used in the previous study (15), resulted in no deubiquitination activity of autoubiquitinated BRCA1 (data not shown). Because the catalytic domain exists in the NH_2 terminus of BAP1, the structural change of the BAP1 NH_2 terminus by the use of different tags may affect substrate recognition.

UCH family members prefer to cleave small protein substrates from ubiquitin (14). However, recent biochemical and structural studies of UCH-L3 showed that it also accounts for the hydrolysis of larger ubiquitin conjugates (26), consistent with our observation of BRCA1/BARD1 deubiquitination mediated by BAP1 and UCH-L3. The specificity of the deubiquitination of BRCA1/BARD1 by BAP1 is not clear at present. Because UCH-L3 and isopeptidase T were also capable of deubiquitinating BRCA1/BARD1, the deubiquitination step is likely a nonspecific process. Instead, the interaction between BRCA1/BARD1 and BAP1 could be the critical step for the reaction in cells. Another point that we need to note is that the deubiquitination reaction we tested in this study is that toward polyubiquitin chains mediated by UbcH5c as an E2. Recently, it has been reported that BRCA1/BARD1 is capable of mediating different types of ubiquitination depending on its interacting E2 (27). For example, BRCA1/BARD1 is able to catalyze Lys^{63}-linked polyubiquitin chains in combination with Ube2w/Ubc13/Mms2 (27). Whether, among the UCH family, BAP1 specifically deubiquitinates these types of ubiquitin linkages remains to be determined.

The DUB/E3 interaction has been generally found in cellular pathways, and several types of regulation between the two molecules were reported (14). In the cases of USP8/NRDP1 (28), USP7 (HAUSP)/MDM2 (29, 30), and USP15/Roc1 (Rbx1; ref. 31), the DUB rescues E3 from autoubiquitination and self-inflicted degradation. In the case of VDU2 (USP20)/pVHL, VDU2 can be ubiquitinated by the pVHL E3 ligase for proteasomal degradation, whereas both VDU2 and pVHL interact with hypoxia-inducible factor-1α (HIF-1α) to play a role in HIF-1α stabilization (32). Finally, the A20 protein contains an NH_2-terminal OTU family DUB domain and a COOH-terminal seven C2/C2 zinc finger E3 domain in one molecule. The DUB domain removes Lys^{63}-linked ubiquitin chains from receptor-interacting protein (RIP), an essential mediator of the proximal tumor necrosis factor receptor 1 signaling complex. Then, the E3 domain polyubiquitinates RIP with Lys^{48}-linked ubiquitin chains, thereby targeting RIP for proteasomal degradation (33). BAP1/BRCA1/BARD1 could be a new type of DUB/E3 interaction, where BAP1 inhibits the E3 ligase activity of BRCA1/BARD1 to protect against additional ubiquitination while it deubiquitinates preexisting ubiquitin chains. The dual role of BAP1 toward BRCA1/BARD1 could be important to ensure the inhibition of ubiquitination in cellular pathways. Alternatively, it is also possible that BAP1 uses two different mechanisms in response to different signaling or cellular conditions.

In addition to BAP1, there is another BRCA1-interacting DUB called BRCC36. Interestingly, BRCC36 enhanced the E3 ligase activity of BRCA1/BARD1 when it formed a complex with another BRCC protein, BRCC45 (34). BRCC36 is recruited to the IR-induced nuclear foci by RAP80, the ubiquitin-interacting motif

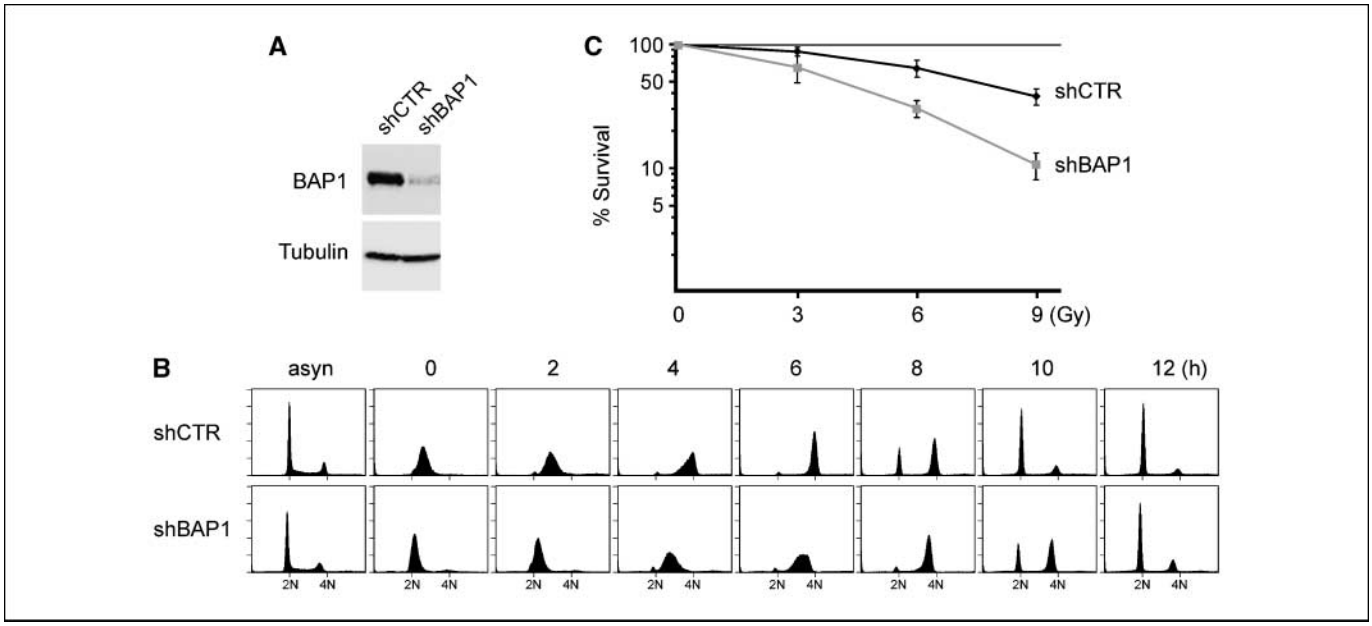

Figure 6. BAP1 inhibition results in S-phase retardation and IR hypersensitivity. *A,* BAP1 protein expression from HeLa cells stably expressing shRNA for BAP1 (*shBAP1*) or control sequence (*shCTR*) was analyzed by immunoblotting. *B,* cells from *A* were arrested by a double thymidine block, and progression through the cell cycle after release from the blocks was monitored by FACS analysis. *asyn,* asynchronous cells. *C,* cells from *A* were irradiated at the indicated doses and viabilities of the cells were determined 3 d after irradiation.

containing protein that also recruits the BRCA1/BARD1/Abraxas complex to nuclear foci (35, 36). This suggests that BRCC36 enhances BRCA1/BARD1 E3 ligase activity in response to DNA damage. Interestingly, BAP1 has also been identified by proteomic analysis as a protein phosphorylated in response to DNA damage on consensus sites recognized by ATM and ATR (37). Together with our results, BAP1 may also affect BRCA1/BARD1-induced ubiquitination in response to DNA damage in a fashion opposing BRCC36. Notably, depletion of BAP1 by shRNA resulted in hypersensitivity to IR (Fig. 6*C*), the same phenotype that was observed in cells with BRCC36 depletion (34) or BRCA1 depletion (21, 22). This suggests that the process mediated by ubiquitination and inhibition of ubiquitination is important for cell survival after IR.

When overexpressed, BRCA1 and BAP1 suppress cell growth (13, 38). On the other hand, depletion of BARD1 suppresses cell proliferation caused by retardation of S phase (20). In addition, our results show that inhibition of BAP1 also causes S-phase retardation. This suggests that precise adjustment of the expression level or function of BRCA1/BARD1 is critical for cell proliferation and that BAP1 contributes to the role of BRCA1/BARD1 in S phase. The observed S-phase retardation could be due to the inability of cells to respond to spontaneous DNA damage occurring in this phase. Together, the results in this study suggest that BAP1 and BRCA1/BARD1 coordinately regulate ubiquitination during the DNA damage response and the cell cycle. These observations reveal one aspect of the regulation mechanism of BRCA1/BARD1 E3 ligase activity and reacknowledge the importance of BAP1 in BRCA1 functions. In turn, the emergent role of BAP1 in breast oncogenesis remains to be determined.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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