BRCA1 Ubiquitinates RPB8 in Response to DNA Damage

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Abstract

The breast and ovarian tumor suppressor BRCA1 catalyzes untraditional polyubiquitin chains that could be a signal for processes other than proteolysis. However, despite intense investigations, the mechanisms regulated by the enzyme activity remain only partially understood. Here, we report that BRCA1-BARD1 mediates polyubiquitination of RPB8, a common subunit of RNA polymerases, in response to DNA damage. A proteomics screen identified RPB8 as a protein modified after epirubicin treatment in BRCA1-dependent manner. RPB8 interacted with BRCA1-BARD1 and was polyubiquitinated by BRCA1-BARD1 *in vivo* and *in vitro*. BRCA1-BARD1 did not destabilize RPB8 *in vivo* but rather caused an increase in the amount of soluble RPB8. Importantly, RPB8 was polyubiquitinated immediately after UV irradiation in a manner sensitive to BRCA1 knockdown by RNA interference. Substitution of five lysine residues of RPB8 with arginine residues abolished its ability to be ubiquitinated while preserving its polymerase activity. HeLa cell lines stably expressing this ubiquitin-resistant form of RPB8 exhibited UV hypersensitivity accompanied by up-regulated caspase activity. Our findings suggest that ubiquitination of a common subunit of RNA polymerases is a mechanism underlying BRCA1-dependent cell survival after DNA damage. [Cancer Res 2007;67(3):951–8]

Introduction

Germ line mutation of the cancer susceptibility gene *BRCA1* causes familial breast and ovarian cancer. BRCA1 acts as a hub protein that coordinates many cellular pathways to prevent tumor progression. Accordingly, down-regulation of this key protein by mechanisms other than *BRCA1* gene mutation causes sporadic breast cancer (1). All cells defective in BRCA1 show genomic instability as evidenced by hypersensitivity to DNA damage, the presence of chromosomal abnormalities, and the loss of heterozygosity at multiple loci. These results are likely to stem from the failure of BRCA1 to function in DNA damage repair, transcriptional regulation, apoptosis induction, intra-S or G_2-M checkpoint function, and regulation of centrosome duplication (2–4).

Involvement of BRCA1 in multiple cellular processes is logical given its enzymatic function as a ubiquitin ligase (E3). In this capacity, it has the potential to interact with numerous protein substrates and subsequently influence the biological response of a cell at many points. BRCA1 contains an NH_2-terminal RING finger domain, a common motif found in ubiquitin ligases. It acquires significant ubiquitin ligase activity when bound to another conformationally similar RING finger protein, BARD1, as a RING heterodimer (5–8). The most common polyubiquitin chain is linked through Lys^{48} of ubiquitin and serves as a signal for rapid degradation of substrates by the proteasome-dependent proteolysis pathway (9). However, BRCA1-BARD1 has the unique capacity to catalyze Lys^6-linked polyubiquitin chains, and the ubiquitination mediated by BRCA1-BARD1 could signal a process other than degradation (10–13). Deleterious missense mutations in the RING finger domain of BRCA1 found in familial breast cancer abolish the E3 ligase activity of BRCA1-BARD1 (6, 7, 14), indicating that the E3 ligase activity is important for role of BRCA1 as a tumor suppressor.

One of the most significant functional features of BRCA1 is that it is a component of the RNA polymerase II holoenzyme (15, 16). BRCA1 specifically interacts with a large fraction of hyperphosphorylated, processive polymerase II (IIO), in preference to the hypophosphorylated polymerase II (IIA) found at promoters (17). It has been proposed that BRCA1 binds polymerase IIO complexes as part of a genome scanning function for DNA damage (18). However, how BRCA1 affects the polymerase II complexes after DNA damage remains partially understood. In this study, we identify RPB8 (also called hRPB17 or *POLR2H*), a common subunit of all three types of RNA polymerases, as a substrate of BRCA1 E3 ligase and show that BRCA1 ubiquitinates RPB8 immediately after DNA damage. HeLa cell lines stably expressing a ubiquitin-resistant form of RPB8 exhibited UV hypersensitivity, a known phenotype of BRCA1 deficiency. These results indicate a significant role of ubiquitin ligase activity of BRCA1 for cell survival after DNA damage and provide a new aspect of a common subunit of RNA polymerases in DNA damage responses.

Materials and Methods

Two-dimensional difference gel electrophoresis. Methods for fluorescence two-dimensional difference gel electrophoresis (DIGE) and mass spectrometric analysis are reported in the Supplementary Data.

Plasmids. cDNA for full-length human RPB8 was amplified by PCR from a MCF10A cell cDNA library using Pfx polymerase (Stratagene, La Jolla, CA). Mammalian expression plasmids for BRCA1, BARD1, ubiquitin, and their mutants were previously described (7, 11). The point mutations to substitute the Lys residue(s) of RPB8 with Arg were produced by site-directed mutagenesis (Stratagene). All plasmids used were verified by DNA sequencing.

Cell cultures and transfections. T47D, HCC1937 breast carcinoma cells, HeLa cervical carcinoma cells, and 293T transformed human kidney cells were cultured in DMEM supplemented with 10% FCS and 1% antibiotic-antimycotic agent (Life Technologies, Inc., Grand Island, NY) in 5% CO_2 at 37°C. MCF10A normal human breast epithelial cells were grown in DMEM/Ham's F12 (1:1) medium supplemented with 2.5% FCS, 100 ng/mL cholera toxin, 20 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone,

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

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doi:10.1158/0008-5472.CAN-06-3187

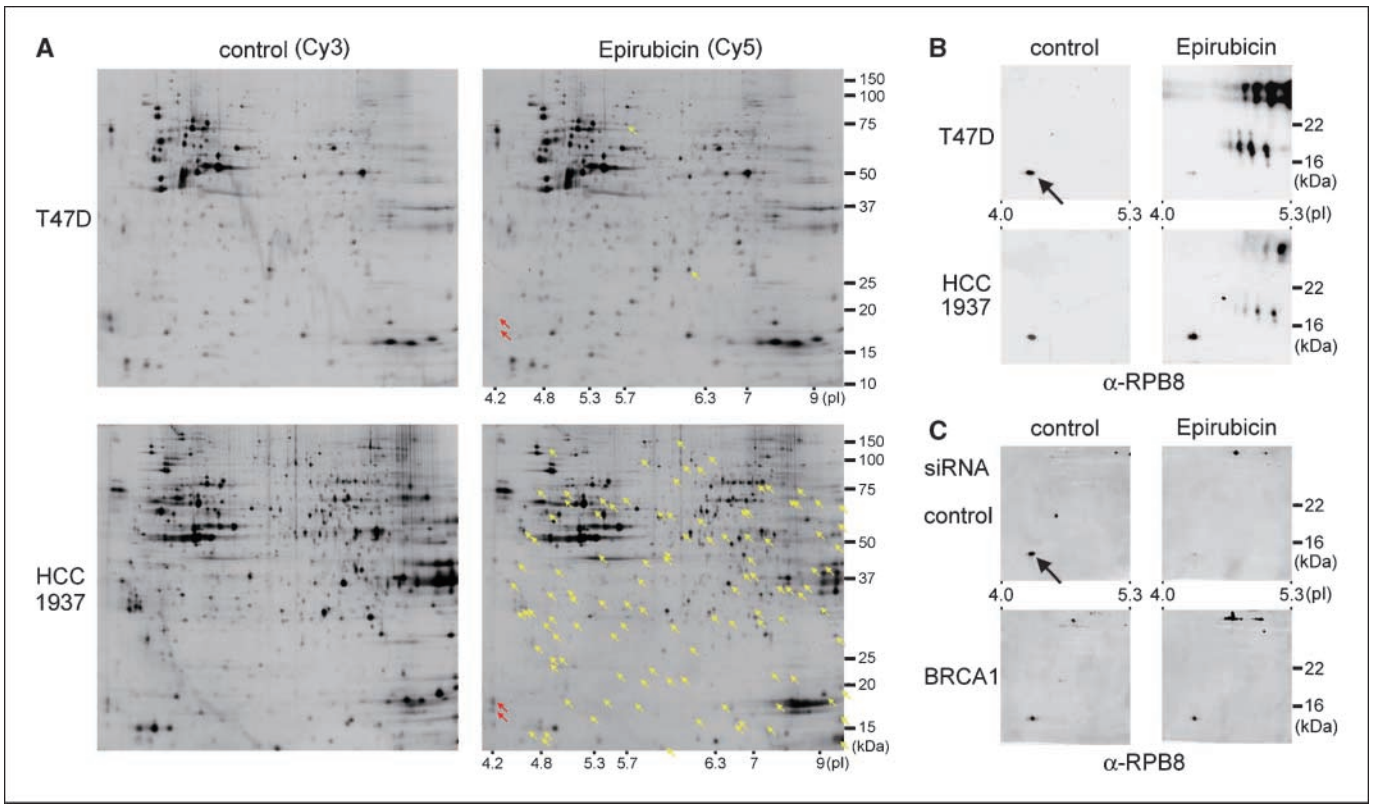

Figure 1. Proteomic screen for proteins affected by epirubicin treatment. *A,* T47D cells (*top*) and HCC1937 cells (*bottom*) were either untreated or treated with 0.2 μg/mL of epirubicin for 3 h and lysed with 7 mol/L urea/2 mol/L thiourea–containing buffer. Protein (50 μg) from untreated and epirubicin-treated cells was labeled with Cy3 (*left*) and Cy5 (*right*), respectively. The differently labeled samples were mixed together, resolved on a two-dimensional gel (pH range 3–10, *left* to *right*), and scanned with a fluorescence image analyzer. *Yellow arrows,* protein spots whose levels were significantly altered after epirubicin treatment. *Red arrows,* proteins that significantly decreased only in T47D cells after epirubicin treatment. The slower-migrating protein was identified as RPB8 and the faster one was myosin light chain. *B,* T47D cells or HCC1937 cells were treated as in *A* and lysed with 7 mol/L urea/2 mol/L thiourea–containing buffer. Lysates (500 μg) were resolved on a two-dimensional gel (pH range 3–10). A part of the gel was subjected to immunoblot with anti-RPB antibody. *Arrow,* RPB8. *C,* T47D cells were transfected either with control siRNA (*top*) or with siRNA for BRCA1 (*bottom*), treated with or without epirubicin, and subjected to anti-RPB8 immunoblotting as in *B*.

10 μg/mL insulin, and 1% antibiotic-antimycotic agent. For epirubicin treatment, cells were incubated in medium containing 0.2 μg/mL epirubicin (Pfizer, New York, NY). To examine the half-life of proteins *in vivo*, cells were incubated with 10 μg/mL cycloheximide (Wako, Osaka, Japan), a protein synthesis inhibitor, for the indicated time periods. 293T cells were transfected using the standard calcium phosphate precipitation method. To generate cell lines that stably expressed either wild-type (WT) or mutant FLAG-RPB8, HeLa cells were transfected using FuGENE6 (Roche, Indianapolis, IN) with pcDNA3 plasmids encoding each protein and selected with G418. For UV irradiation studies, cells were washed with PBS, irradiated with UV light (254 nm; UVP, Inc., Upland, CA) at the indicated doses, and grown in fresh medium for various times.

Antibodies. Mouse monoclonal antibodies to hemagglutinin (HA; Boehringer-Mannheim, Mannheim, Germany), Myc (BabCo, Richmond, CA), FLAG (Sigma, St. Louis, MO), polyubiquitin (Affiniti, Exeter, United Kingdom), conjugated ubiquitin (Affiniti; ref. 10), α- and β-tubulin (Neomarkers, Fremont, CA), and actin (Santa Cruz Biotechnology, Santa Cruz, CA) as well as rabbit polyclonal antibodies to BRCA1 (Santa Cruz Biotechnology), RPB1 (Covance), and cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) were purchased commercially. Anti-FLAG cross-linked agarose beads (Sigma) were used for immunoprecipitation to detect *in vivo* ubiquitinated substrates. Rabbit polyclonal antibodies to BARD1 and RPC155 were generous gifts from Dr. Richard Baer (Columbia University, New York, NY) and Dr. Nouria Hernandez (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), respectively. Rabbit polyclonal antibody to RPB8 was generated against full-length human glutathione *S*-transferase (GST)-RPB8 and purified by protein G agarose chromatography.

RNA interference. SMART pool BRCA1 small interfering RNA (siRNA) mix and control siRNA mix were purchased from Dharmacon Research, Inc. (Lafayette, CO). RNA duplexes (final concentration 50 nmol/L) were transfected into the cells with Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Retrovirus expressing short hairpin RNA (shRNA) that targets BRCA1 mRNA sequence 5′-CUAGAAAUCUGUUGCUAUG-3′ was created by cotransfecting 293T cells with pGP vector, pE-ampho vector, and pSINsi-hU6 retroviral vector that has previously been subcloned with the oligonucleotide 5′-GATCCGCTAGAAATCTGTTGCTATGTTCAAGAGACATAGCAACAGATTTCTAGCTTTTTTAT-3′ according to the manufacturer's protocol (TaKaRa, Otsu, Japan). Oligonucleotide 5′-GATCCGTAAGGCTATGAAGAGATACTTCAAGAGAGTATCTCTTCATAGCCTTACTTTTTTAT-3′ was used for the retrovirus expressing control shRNA. For infection, HeLa cells were incubated with virus supernatants and fresh culture medium containing 8 μg/mL Polybrene (Sigma). Cells were analyzed 48 h after transfection or infection.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting methods were previously described (11). For the immunoblotting analysis after two-dimensional gel electrophoresis, cells were lysed with 7 mol/L urea/2 mol/L thiourea–containing buffer as described above. Soluble fractions were prepared with 0.5% NP40-based buffer as previously described (11). Denatured whole-cell lysates were prepared by boiling in Laemmli SDS-loading buffer with 0.1 mol/L DTT.

***In vitro* ubiquitin ligation assay.** Full-length His-FLAG-RPB8 was obtained from BL21/DE3 bacteria cells with isopropyl-L-thio-β-D-galactopyranoside induction by two-step purification using nickel agarose beads followed by anti-FLAG cross-linked agarose beads (Supplementary Fig. S3). Complexes of WT or I26A mutant of FLAG-BRCA1$^{1\text{-}772}$ with BARD1 were

purified from transfected 293T cells by anti-FLAG affinity chromatography and FLAG peptide elution. Both WT and I26A mutant complexes contained an ~1:1 ratio of BRCA1 and BARD1 proteins (Supplementary Fig. S3). Rabbit E1 (BIOMOL, Plymouth Meeting, PA) and mammalian ubiquitin (Boston Biochem, Cambridge, MA) were purchased commercially. The *in vitro* reaction was done as previously described (11) with a reaction mixture (30 μL) that contained 0.5 μg His-FLAG-RPB8, 40 ng E1, 0.3 μg UbcH5c, and 0.3 μg each of FLAG-BRCA1$^{1-772}$ and BARD1.

Runoff transcription assay. The runoff transcription assay used was described elsewhere (17). Briefly, the runoff template was created by annealing 50 pmol each of a 65-mer oligonucleotide 5′-ATTGGGTAAAGGAGAGTATTTGAGCGGAGGACAGTACTCCGGGTCCCCCCCCCCCCCCCCCCCCCC-3′and a complementary 45-mer oligonucleotide 5′-GACCCGGAGTACTGTCCTCCGCTCTTTTACTCTCCTTTACCCAAT-3′ in a 200 μL annealing mixture containing 20 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, and 0.2 mol/L NaCl. Runoff transcription reactions (20 μL) contained 8.25 mmol/L $MgCl_2$, 5 μg of bovine serum albumin, 250 nmol/L nucleotide triphosphates, 5 units of RNase inhibitor, 50 ng of poly(deoxyinosinic-deoxycytidylic acid), 0.05% NP40, 1 pmol of annealed oligonucleotides, and 0.5 μCi of [α-^{32}P]CTP. Equilibrated FLAG-RPB8 immunocomplexes bound to M2 beads (10 μL) were added to the reactions (20 μL) and incubated for 40 min at 30°C and stopped with 50 μL of PK buffer (300 mmol/L sodium acetate, 0.2% SDS, 10 mmol/L EDTA, 100 ng tRNA, and 10 μg proteinase K). Reactions were then incubated at 55°C for 20 min, extracted with phenol/chloroform, and precipitated with ethanol. Single-stranded RNA transcripts were resolved under denaturing conditions on 12% polyacrylamide/urea gels and scanned with the Typhoon 9400 image analyzer (Amersham, Piscataway, NJ).

Results

Identification of RPB8 as a protein modified in BRCA1-positive cells after epirubicin treatment. To search for candidate substrates for the BRCA1-BARD1 E3 ligase in response to DNA damage, we used two-dimensional DIGE technology. Breast cancer–derived, BRCA1–positive T47D cells and BRCA1-defective HCC1937 cells were incubated for 3 h with epirubicin, a topoisomerase II inhibitor that induces DNA double strand breaks. Cells were lysed with 7 mol/L urea/2 mol/L thiourea–containing buffer, and the proteomes were compared with untreated cells using two-dimensional DIGE. Interestingly, whereas the expression levels of only a few proteins were affected by the epirubicin treatment in T47D cells, that of ~100 proteins were altered in HCC1937 cells (Fig. 1*A*). Conversely and even more interesting, two proteins whose expression levels were dramatically reduced in T47D cells were not changed in HCC1937 cells (Fig. 1*A, red arrows*). Therefore, we speculated that the reduction could depend on the presence of BRCA1. The protein spots were in-gel digested and subjected to nanoscale capillary liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis. LC/MS/MS analysis revealed that the samples were RPB8, a common subunit of three types of RNA polymerases, and myosin light chain. RPB8 is a very acidic, small protein with a calculated molecular mass of 17.1 kDa and an isoelectric point of 4.34 (19). One of the most significant functional features of BRCA1 is that it is a component of the RNA polymerase II holoenzyme (15, 16). Therefore, we focused on RPB8 for further analyses.

To confirm our mass spectrometry data, we generated a rabbit polyclonal antibody to GST-RPB8 for immunoblot analysis. Cells were treated as in Fig. 1*A*, and immunoblot analysis of the proteins resolved by two-dimensional gels verified that the protein spot was indeed RPB8. It was again severely reduced by epirubicin treatment only in T47D cells (Fig. 1*B*). The difference in RPB8 expression in response to epirubicin treatment could be due to the different genetic backgrounds of these two cell lines, not just the absence or presence of BRCA1. Therefore, we next compared RPB8 expression between isogenic cells with and without knockdown of BRCA1

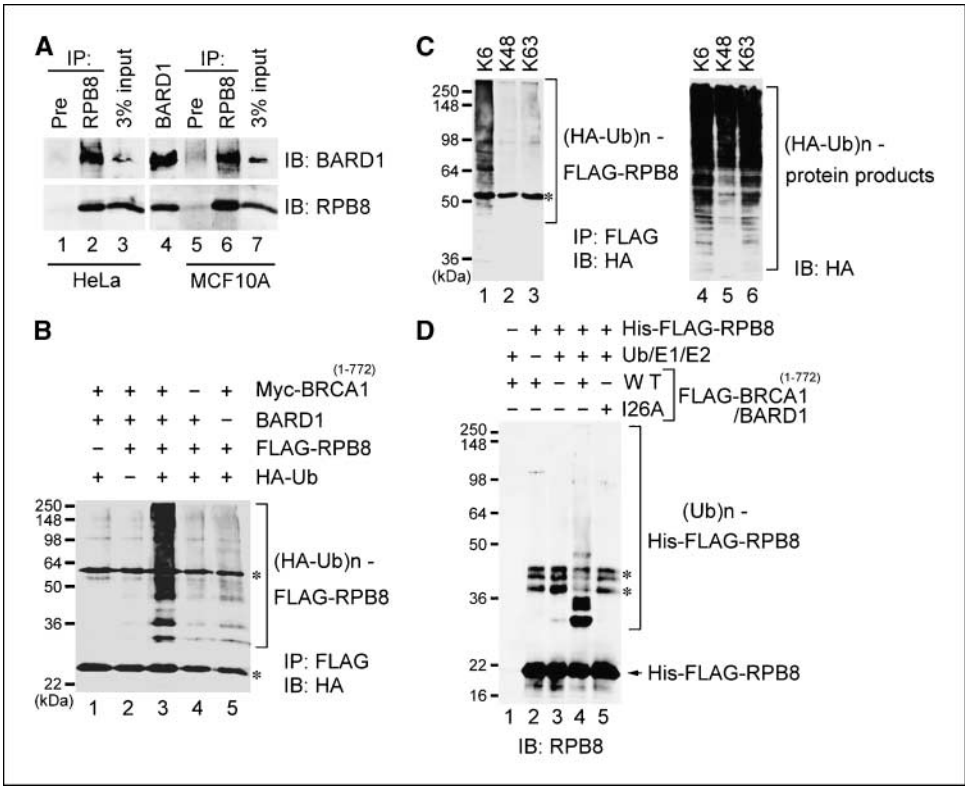

Figure 2. RPB8 and BARD1 interaction, and RPB8 ubiquitination by BRCA1-BARD1. *A,* endogenous RPB8 interacts with BARD1. Lysates prepared from HeLa (*lanes 1–3*) or MCF10A (*lanes 5–7*) cells were immunoprecipitated (*IP*) with anti-RPB8 or preimmune serum (*Pre*) and analyzed by immunoblotting (*IB*) using the indicated antibodies. A portion of the cell lysates corresponding to 3% of the input for immunoprecipitation as well as lysate from 293T cells transfected with BARD1 (*lane 4*) were also loaded. *B,* 293T cells transfected with the indicated plasmids were boiled in 1% SDS lysis buffer, diluted to 0.1% SDS, and immunoprecipitated with anti-FLAG antibody–cross-linked beads. Precipitated FLAG-RPB8 was resolved by 12.5% SDS-PAGE followed by immunoblotting with anti-HA antibody. *, IgG. *C,* polyubiquitination of RPB8 was detected as in *A*, except that HA-ubiquitin (*HA-Ub*) with a single lysine residue was transfected as indicated (*lanes 1–3*). A portion of the cell lysate was subjected to immunoblotting with anti-HA antibody to detect total HA-ubiquitin–conjugated proteins in cells as a control for protein expression (*lanes 4–6*). *, IgG. *D,* bacterially purified His-FLAG-RPB8 was incubated in the presence of ATP with ubiquitin, E1, E2/UbcH5c, and either WT or I26A mutant of FLAG-BRCA1$^{1-772}$/BARD1 complex as indicated and immunoblotted with anti-RPB8 antibody. *, nonspecific products copurified with His-FLAG-RPB8.

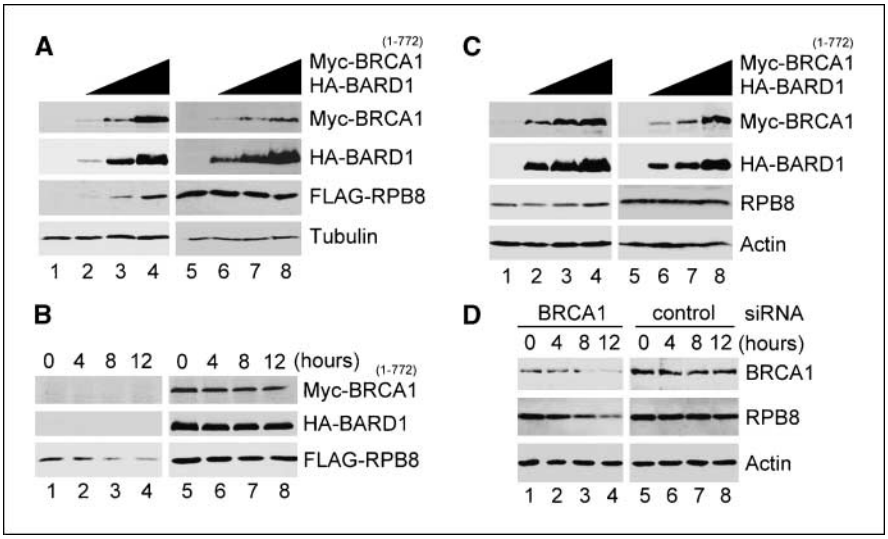

Figure 3. BRCA1-BARD1 did not destabilize RPB8 *in vivo* but rather caused an increase of RPB8 in the soluble fraction. *A,* 293T cells were transfected with plasmids encoding FLAG-RPB8 (*lanes 1–8*, 0.3 μg) and increasing amounts of Myc-BRCA1$^{1-772}$ and HA-BARD1 (*lanes 2* and *6*, 2 μg; *lanes 3* and *7*, 4 μg; *lanes 4* and *8*, 7.35 μg each). Total plasmid DNA was adjusted to 15 μg per plate by adding the parental pcDNA3 vector. The steady-state level of each protein in the soluble fraction (*lanes 1–4*) and whole-cell lysates (*lanes 5–8*) was analyzed by immunoblot using anti-Myc, anti-HA, anti-FLAG, or anti-tubulin antibodies. *B,* 293T cells were transfected with plasmids encoding FLAG-RPB8 (0.2 μg) and either parental pcDNA3 vector (2 μg, *lanes 1–4*) or Myc-BRCA1$^{1-772}$ and HA-BARD1 (1 μg each, *lanes 5–8*). Thirty-six hours after transfection, cells were incubated with cycloheximide (10 μmol/L) and chased for the indicated lengths of time. Soluble fractions of the cell lysates were then immunoblotted with Myc, HA, or FLAG antibody. *C,* steady-state levels of RPB8 were analyzed as in *A*, except that FLAG-RPB8 was not transfected and anti-RPB8 antibody was used to detect endogenous RPB8. *D,* T47D cells were transfected either with siRNA for BRCA1 (*lanes 1–4*) or control siRNA (*lanes 5–8*). Cells were incubated with cycloheximide (10 μmol/L) and chased for the indicated lengths of time. The soluble fraction of the cell lysates was then immunoblotted with the indicated antibodies.

expression. T47D cells were transfected with either control siRNA or BRCA1 siRNA and then treated as in Fig. 1*A*. The siRNA-transfected cells were successfully silenced for BRCA1 expression (Supplementary Fig. S1). Immunoblot analysis of the proteins resolved by two-dimensional gels showed that RPB8 was reduced by epirubicin treatment only in control cells, not in cells with BRCA1 knockdown, supporting the idea that this modification depends on BRCA1 expression (Fig. 1*C*). The reduction of RPB8 at its normal migrating position could be due to protein degradation or to covalent modification.

BRCA1-BARD1 interacts with and ubiquitinates RPB8. The polymerase II holoenzyme interacts with BRCA1 and BARD1 (15, 16). Consistent with the previous reports, a significant amount of endogenous BARD1 coimmunoprecipitated with RPB8 isolated from HeLa cells or MCF10A cells compared with controls (Fig. 2*A*). The same results were observed with MCF7, T47D, and 293T cells (data not shown). Exogenously expressed RPB8 also interacted with BRCA1 and BARD1 (Supplementary Fig. S2). Then, we tested whether RPB8 is ubiquitinated by BRCA1-BARD1 *in vivo*. FLAG-RPB8 was coexpressed in 293T cells with HA-ubiquitin, Myc-BRCA1$^{1-772}$, and BARD1. Cells were collected 36 h after transfection and boiled in 1% SDS–containing buffer, and FLAG-RPB8 was immunoprecipitated. Immunoblotting of the RPB8 precipitates resolved by SDS-PAGE using anti-HA antibody showed a ladder characteristic of polyubiquitinated RPB8 (Fig. 2*B*). Omission of FLAG-RPB8, HA-ubiquitin, Myc-BRCA1$^{1-772}$, or BARD1 all abolished the RPB8 ladders, supporting the idea of BRCA1-BARD1–dependent RPB8 ubiquitination.

BRCA1-BARD1 is the only known E3 ligase to catalyze Lys^6-linked polyubiquitin chains (10, 11, 13). To show that the *in vivo* RPB8 ubiquitin ladders were directly due to BRCA1-BARD1 ligase activity, we verified that RPB8 was modified by ubiquitin through Lys^6 linkages. HA-tagged ubiquitins that have a single lysine residue available for conjugation were used for *in vivo* ubiquitination assays. As expected, BRCA1-BARD1–dependent RPB8 polyubiquitination was predominantly detected when HA-ubiquitin with only Lys^6 available, but not Lys^{48} or Lys^{63}, was coexpressed (Fig. 2*C*). However, it has been suggested that ubiquitin mutants could fold incorrectly and may cause artifacts (20). Recent quantitative analysis of *in vitro* ubiquitination revealed that even for cyclin B1 ubiquitination catalyzed by the anaphase-promoting complex, heterogeneous ubiquitin chains, including Lys^{63}, Lys^{11}, and Lys^{48}, or monoubiquitin attached to multiple lysine residues on the substrate. Further, some types of linkages are dependent on the combination of E2 and E3 enzymes (21). Thus, it is possible that ubiquitination mediated by BRCA1-BARD1 also resulted in multiple polyubiquitin chains, including Lys^6. The preference for Lys^6 ubiquitination observed in the *in vivo* experiment was not enough evidence to support the direct role of BRCA1-BARD1 for RPB8 ubiquitination. Therefore, we further tested whether BRCA1-BARD1 directly catalyzes RPB8 polyubiquitination by *in vitro* ubiquitination using recombinant RPB8 protein (Supplementary Fig. S3). His-FLAG-RPB8 incubated with ubiquitin, E1, E2/His-UbcH5c, and FLAG-BRCA1$^{1-772}$/BARD1 complex (Supplementary Fig. S3) resulted in a ladder and smear detected by anti-RPB8 immunoblot (Fig. 2*D*). Omission of substrate RPB8, ubiquitin/E1/E2, or FLAG-BRCA1$^{1-772}$/BARD1 complex, as well as substitution of BRCA1$^{1-772}$ with the E2-nonbinding mutant I26A, all abolished RPB8 ubiquitination. Hence, the results suggest that the RPB8 polyubiquitination is directly catalyzed by BRCA1-BARD1.

BRCA1-BARD1 does not destabilize RPB8 *in vivo*. Our previous results suggested that BRCA1-BARD1 catalyzed untraditional polyubiquitin chains that served as a signal for a process other than degradation (7, 11, 12). However, the reduced expression of RPB8 after epirubicin treatment detected by two-dimensional DIGE or two-dimensional immunoblot (Fig. 1) suggested the

possibility of BRCA1-mediated RPB8 degradation. Therefore, we tested if BRCA1-BARD1 destabilized RPB8 *in vivo* under several different conditions, including BRCA1-BARD1 overexpression and BRCA1 knockdown by siRNA. FLAG-RPB8 was coexpressed in 293T cells with Myc-BRCA1$^{1-772}$ and HA-BARD1 (Fig. 3*A*). The steady-state level of FLAG-RPB8 increased upon coexpression of BRCA1-BARD1 in a dose-dependent manner in the soluble fraction (*lanes 1–4*) but not in whole-cell lysates (*lanes 5–8*). We then examined protein half-life of FLAG-RPB8 in the soluble fraction using cycloheximide, a protein synthesis inhibitor. The FLAG-RPB8 protein half-life was prolonged by BRCA1-BARD1 overexpression (Fig. 3*B*). We also tested the effect of BRCA1-BARD1 on endogenous RPB8 (Fig. 3*C* and *D*). The steady-state level of RPB8 only slightly increased upon coexpression of BRCA1-BARD1 in the soluble fraction (Fig. 3*C, lane 4*) and no effect was observed when whole-cell lysates were evaluated (*lanes 5–8*). However, RPB8 protein half-life was detectably shortened by BRCA1 knockdown (Fig. 3*D*). This observation was not detected when whole-cell lysates were analyzed (data not shown). Together, analyses of steady-state levels and protein half-lives indicated that only soluble RPB8 was stabilized, whereas that in whole-cell lysate was unchanged (Fig. 3). Alternatively, it was also possible that BRCA1-BARD1 shifted RPB8 from the insoluble fraction, such as the chromatin fraction, to the soluble fraction. However, we could not detect such a shift by fractionation analyses (data not shown). In either case, these findings at least suggest that BRCA1-BARD1–mediated RPB8 ubiquitination is not a signal for its degradation.

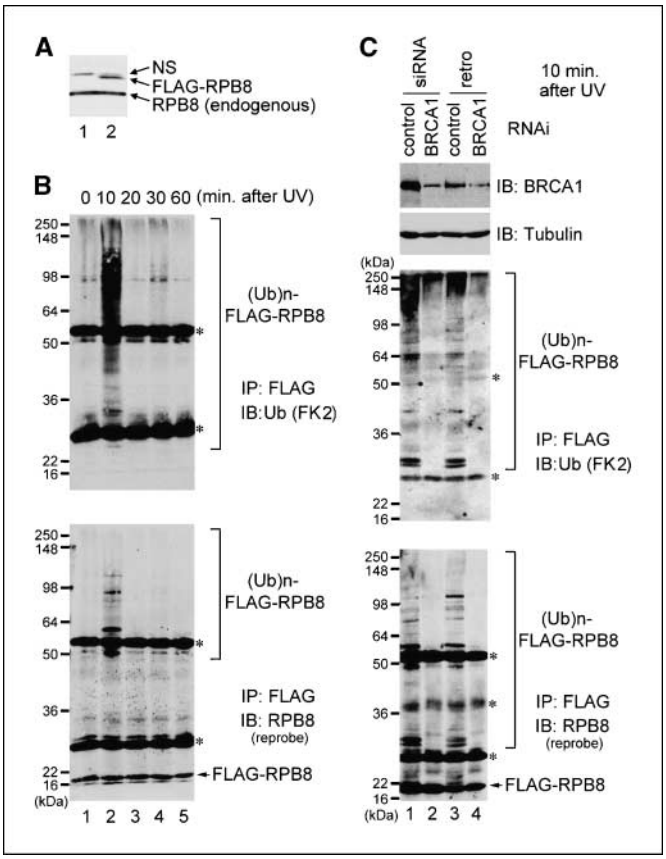

Figure 4. BRCA1-dependent RPB8 polyubiquitination in response to UV irradiation. *A,* parental HeLa cells (*lane 1*) and HeLa cells stably expressing FLAG-RPB8 (*lane 2*) were lysed with SDS-sample buffer and immunoblotted with anti-RPB8 antibody. *NS,* nonspecific products. *B,* HeLa cells stably expressing FLAG-RPB8 were UV irradiated (35 J/m^2) and harvested at the indicated times after irradiation. Ubiquitinated RPB8 was detected as described in Fig. 2*B*, except that anti-ubiquitin antibody (FK2) was used for immunoblotting (*top*). The membrane was reprobed with anti-RPB8 antibody (*bottom*). *C,* HeLa cells stably expressing FLAG-RPB8 were either transfected with control siRNA (*lane 1*), transfected with siRNA for BRCA1 (*lane 2*), infected with retrovirus expressing control shRNA (*lane 3*), or infected with retrovirus expressing shRNA for BRCA1 (*lane 4*). Cells were then UV irradiated (35 J/m^2) and harvested 10 min after irradiation. Cells were boiled in 1% SDS buffer and subjected either to immunoblotting with anti-BRCA1 (*top*) and antitubulin (*upper middle*) or to detection of RPB8 ubiquitination as in *B* (*lower middle* and *bottom*). *, IgG. Note that the different pattern of IgG detection between *B* and *C* is due to different lots of anti-FLAG cross-linked agarose beads.

BRCA1-dependent RPB8 ubiquitination after UV irradiation. BRCA1-mediated RPB8 ubiquitination prompted us to investigate the biological implications of this activity. We examined if RPB8 is ubiquitinated in response to DNA damage. Rather than exposing cells continuously to epirubicin, and because RPB1 is ubiquitinated after UV irradiation, we used UV irradiation to accurately determine the timing of RPB8 ubiquitination after DNA damage (22–25). We established HeLa cell lines that stably express FLAG-RPB8 at a low level (approximately one third of endogenous RPB8; Fig. 4*A*) to avoid artifacts caused by overexpression and analyzed ubiquitination of anti-FLAG immunoprecipitates with anti-ubiquitin (FK2) antibody. Because it has been reported that RPB1 ubiquitination occurs 1 to 2 h after UV irradiation (22–25), we first analyzed these time points. However, we did not detect any ubiquitination of FLAG-RPB8 (Fig. 4*B* and data not shown). Instead, ubiquitinated FLAG-RPB8 readily, and only transiently, appeared 10 min after UV irradiation (Fig. 4*B, top*). Reprobing the membrane with anti-RPB8 antibody verified that the detected ladder was ubiquitinated RPB8 (*bottom*).

To verify that UV irradiation–induced RPB8 ubiquitination requires endogenous BRCA1, RNA interference was used to knock down BRCA1 expression. HeLa cells stably expressing FLAG-RPB8 were transfected with BRCA1-specific siRNA. As a second alternative, we constructed a retrovirus engineered to express shRNA for BRCA1. Forty-eight hours after transfection or infection, cells were irradiated with UV (35 J/m^2) and then harvested 10 min later. Both the siRNA-transfected and the shRNA retrovirus–infected cells were successfully silenced for BRCA1 expression (>90% and >75% reduction, respectively) compared with their controls (Fig. 4*C, top*). As expected, RPB8 ubiquitination after UV irradiation was dramatically reduced by BRCA1 knockdown in both cases (*lower middle*). Reprobing the membrane with anti-RPB8 antibody again verified the ubiquitinated RPB8 that became completely undetectable upon BRCA1 knockdown (*bottom*). These results support the idea that RPB8 is polyubiquitinated by BRCA1-BARD1 in an early phase after UV irradiation.

A ubiquitin-resistant form of RPB8 retains its polymerase activity. For the purpose of studying the physiologic consequences induced by the BRCA1-mediated RPB8 ubiquitination after UV irradiation, we generated a mutant of RPB8 that is incapable of being ubiquitinated by BRCA1-BARD1. RPB8 possesses eight Lys residues in the whole protein (Fig. 5*A*). We first mutated single Lys residues of RPB8 and tested its capacity to be ubiquitinated. However, RPB8 ubiquitination was not dramatically reduced by each single mutation (Fig. 5*B, lanes 2* and *7*; data not shown). Instead, the ubiquitination of RPB8 was reduced as the number of Lys to Arg substitutions increased. This result recapitulates what we observed during studies of BRCA1 auto-ubiquitination and of BRCA1-mediated NPM1/B23 ubiquitination. When five of the eight Lys residues were substituted with Arg (5KR), RPB8 ubiquitination

became undetectable (Fig. 5*B, lane 5*), although its binding capacity to BRCA1-BARD1 was not reduced (data not shown).

To confirm that the many mutations required to make RPB8 resistant to ubiquitination did not impair its fundamental function as a subunit of RNA polymerases, we verified that the 5KR mutant is capable of binding to RPB1 or RPC155 (the largest subunit of polymerase III) *in vivo*. WT FLAG-RPB8 or 5KR was transfected into 293T cells, and anti-FLAG immunocomplexes were isolated. Bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting using anti-RPB1 or anti-RPC155 antibodies. Both RPB1 and RPC155 were detected in the FLAG-5KR immunocomplexes as well as the WT immunocomplexes (Fig. 5*C*). We measured catalytic activity of the anti-FLAG immunoprecipitates using a runoff transcription assay. The 5KR mutant immunocomplexes contained the ability to generate *in vitro* transcripts equal to that of WT immunocomplexes (Fig. 5*D*). Thus, the 5KR mutation of RPB8 constitutes a viable RNA polymerase complex *in vivo* that sustains its polymerase activity. This indicates that RPB8 ubiquitination by BRCA1-BARD1 is not required for RNA polymerase activity.

Ubiquitin-resistant mutant of RPB8 causes UV hypersensitivity. BRCA1 deficiency causes hypersensitivity to DNA damage (14, 26, 27). Because RPB8 is ubiquitinated by BRCA1 after UV irradiation (Fig. 4), it was possible that failure to perform this function could cause the same phenotype. To test this possibility, we established HeLa cell lines that stably express the 5KR mutant of FLAG-RPB8. Two clones each of the WT (WT-1 and WT-2) and of the 5KR (5KR-1 and 5KR-2) cell lines were obtained (Fig. 6*A*). Polyubiquitination of FLAG-RPB8 after UV irradiation was detected in WT cells, but not in mutant cells (Fig. 6*B*). Using these cells, we examined if the expression of the mutant RPB8 affected cell survival after UV irradiation. The cell viabilities of the 5KR clones 48 h after 20 or 35 J/m^2 of UV irradiation were ~38% and 23% of untreated cells at 0 h, respectively, whereas WT clones were ~72% and 53%, respectively (Fig. 6*C*). Parental HeLa cells exhibited viabilities similar to that of WT clones (Fig. 6*C*). Representative data for cells observed by phase contrast microscopy 48 h after UV irradiation (35 J/m^2) and for culture plates stained with Lillie's crystal violet stain are shown (Supplementary Fig. S4). Thus, expression of a ubiquitin-resistant RPB8 form in cells causes UV hypersensitivity.

Because UV-induced cell death is largely ascribable to caspase-induced apoptosis, we next tested whether activation of the caspase pathway by UV irradiation was enhanced in 5KR cells. HeLa cell lines expressing WT or 5KR mutant of FLAG-RPB8 were UV irradiated, and caspase activity was measured by immunoblotting with an antibody to cleaved caspase-3. As shown in Fig. 6*D*, 5KR cells expressed larger amount of cleaved caspase-3 than WT cells did at each time point after UV irradiation. This result suggests that failure to ubiquitinate RPB8 after UV irradiation activates the caspase pathway, resulting in apoptotic cell death.

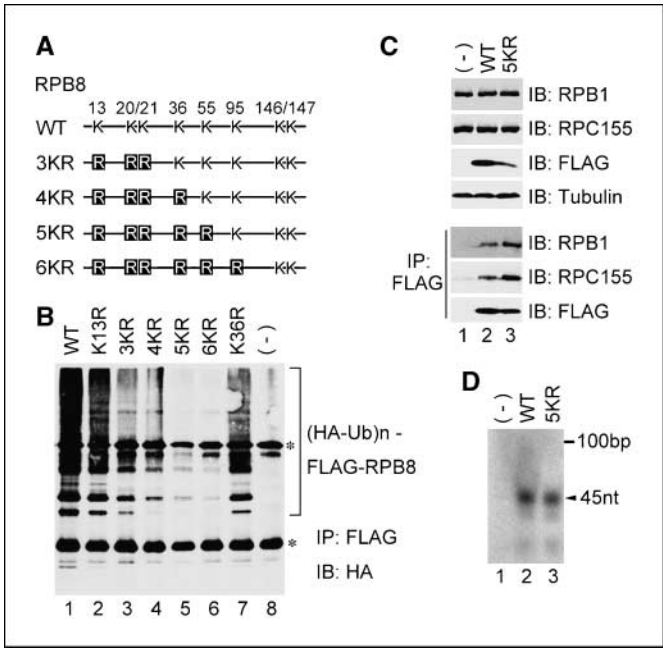

Figure 5. Construction of ubiquitin-resistant RPB8 mutant and assay of its RNA polymerase activity. *A,* the mutant constructs of RPB8. Lys (*K*) residues of RPB8 were substituted with Arg (*R*) as indicated. *B,* Myc-BRCA1$^{1-772}$, BARD1, and HA-ubiquitin were cotransfected into 293T cells either with WT or mutant FLAG-RPB8 as indicated. Polyubiquitination of RPB8 was detected as in Fig. 2*B*. *C,* 293T cells were transfected either with parental pcDNA3 vector (−), WT, or the 5KR mutant of FLAG-RPB8 as indicated. Total cell lysates (*top four panels*) or anti-FLAG immunoprecipitates from equal amounts of total cell lysates (*bottom three panels*) were subjected to immunoblotting with the indicated antibodies. *D,* anti-FLAG immunoprecipitates obtained as in *C* were subjected to an *in vitro* runoff transcription assay using double-stranded DNA templates designed to generate an RNA transcript of 45 nucleotides. Radiolabeled RNA products were resolved by a 12% polyacrylamide/urea gel and scanned with a Typhoon 9400 image analyzer. *, IgG.

Discussion

BRCA1 exists in several different supercomplexes to execute diverse cellular processes. In most of these complexes, BRCA1 exists as a RING heterodimer with BARD1 (28), the form that acquires significant ubiquitin ligase activity (6–8). Revealing the substrates specific for each BRCA1 protein complex is crucial to understand the mechanisms underlying its tumor-suppressor functions.

BRCA1-BARD1 complexes bind to BRCA2 and Rad51 and localize to discrete nuclear foci during S phase. After DNA damage, BRCA1 is phosphorylated by ATM/ATR family kinases (29, 30), and the BRCA1 foci disperse within 30 min (31). The BRCA2-Rad51–containing complex, as well as the BRCA1 complex with Mre11-Rad50-Nbs1, gradually reassemble into different foci (sites of DNA damage) and play important roles in homologous recombination repair. The BRCA1-containing foci begin to appear ~1 h after DNA damage has occurred, reach their peak after 6 to 8 h, and remain until 12 h after damage (31, 32). BRCA1-BARD1 also associates with the RNA polymerase II holoenzyme (15, 16). In contrast to the cases of other complexes described above, BRCA1 dissociates from hyperphosphorylated, processive polymerase II 1 h after DNA damage (17). However, how BRCA1 affects the polymerase II complexes, if at all, during the early stages after DNA damage and before the translocation of BRCA1 to the repair machinery remains to be elucidated. Our results suggest that BRCA1 polyubiquitinates a component of the polymerase II complex, RPB8, at this early stage after DNA damage.

Recently, ubiquitination of phosphorylated RPB1 by BRCA1-BARD1 has been reported (23, 25). Because double knockdown of BRCA1 and BARD1 restored the expression level of the phosphorylated polymerase II that had been repressed by UV irradiation, it was proposed that BRCA1-BARD1 could initiate the degradation of stalled RPB1 (23). However, the BRCA1-BARD1 double knockdown did not detectably affect RPB1 ubiquitination after UV

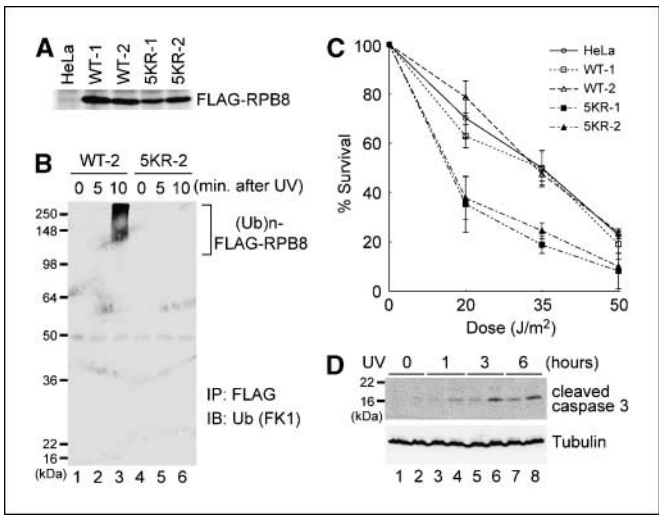

Figure 6. Ubiquitin-resistant RPB8 causes UV hypersensitivity. *A,* cell lysates obtained from two clones each of HeLa cell lines stably expressing either WT (*WT-1* and *WT-2*) or the 5KR mutant (*5KR-1* and *5KR-2*) of FLAG-RPB8 and parental HeLa cells were immunoprecipitated with anti-RPB8 antibody followed by immunoblotting with anti-RPB8 antibody. *B,* HeLa cell lines stably expressing WT (*WT-2, lanes 1–3*) or the 5KR mutant (*5KR-2, lanes 4–6*) of FLAG-RPB8 were UV irradiated (35 J/m^2) and harvested at the indicated times after irradiation. Ubiquitinated RPB8 was detected as described in Fig. 2*B*, except that antipolyubiquitin antibody (*FK1*) was used for immunoblotting. *C,* HeLa cell lines described in *A* were UV irradiated at the indicated doses. Forty-eight hours after irradiation, the cell survival ratio was determined by trypan blue exclusion measurements. The cell number at 0 h (indicated as 0 J/m^2) is 100%. *Points,* mean of measurements carried out in triplicate; *bars,* SD. The experiments were repeated at least twice with similar results. *D,* WT-2 cells (*lanes 1, 3, 5*, and *7*) and 5KR-2 cells (*lanes 2, 4, 6*, and *8*) were UV irradiated (35 J/m^2) and harvested at the indicated times after irradiation. Whole-cell lysates were immunoblotted with anti–caspase-3 antibody or antitubulin antibody.

irradiation. In addition, BRCA1-BARD1–mediated polyubiquitination of other substrates, including NPM1/B23 and phosphorylated CtIP, is not a signal for degradation (12, 33). Therefore, the restored expression level of the phosphorylated polymerase II by BRCA1-BARD1 double knockdown could be due to an indirect effect (23), for example, through the failure to ubiquitinate RPB8. Nonetheless, the clearly shown *in vitro* ubiquitination of phosphorylated RPB1 by BRCA1-BARD1 (23) strongly supports its direct role. The key to solving this discrepancy may be to analyze the timing of RPB1 ubiquitination *in vivo.* RPB1 ubiquitination shown in the previous report occurred 2 h after UV irradiation, when BRCA1 should already be dissociated from polymerase II and relocalized to the Rad50 or Rad51 DNA repair machineries. It is possible that early after DNA damage, RPB1 and RPB8 are transiently ubiquitinated by BRCA1 at the same time, and it may result in dissociation of the polymerase II holoenzyme from the damaged DNA site. RPB1 ubiquitination and degradation occurring in late phases could be mediated by other E3 ligases, such as the CSA-DDB1-CUL4A-ROC1 complex (34, 35).

It is well known that cells with impaired BRCA1 function display hypersensitivity to a range of DNA-damaging agents, including IR and UV irradiation (3, 26). However, the mechanism underlying this phenomenon is not fully understood. Although the failure of checkpoint function is a possible mechanism responsible for the hypersensitivity, it has been reported that neither selective abrogation of the S-phase checkpoint nor the G_2 checkpoint itself results in decreased cell survival after DNA damage (36, 37). Therefore, it has been proposed that some function of BRCA1 other than S-phase or G_2 cell cycle control may affect cell survival after DNA damage (37). The UV hypersensitivity of the cells stably expressing a ubiquitin-resistant mutant of RPB8 shown in this report provides a possible new role for BRCA1 that may compensate for this theoretical defect. Because hyperphosphorylated stalled polymerase II at damaged sites is an extremely cytotoxic ramification of DNA damage (38), the observed UV hypersensitivity could be caused by trapped polymerase II or prolonged polymerase II hyperphosphorylation. In this process, the ubiquitination of RPB8 could be an important step either for polymerase II disassembly, polymerase II dissociation from DNA, or polymerase II dephosphorylation by FCP1. It is interesting that there is considerable expression of endogenous WT RPB8 in the ubiquitin-resistant RPB8 mutant cells (Fig. 4*A*). This indicates that only partial interference of the RNA polymerase recovery is enough to induce cell death, probably by silencing a gene critical for cell survival. Alternatively, polymerase II complexes containing mutant RPB8 could stall at the damaged sites, subsequently causing a gridlock of all polymerase II complexes, including WT complexes. Supporting this idea, induction of local damage by microbeam UV irradiation in the nucleus led to transcription inhibition throughout the nucleus (39).

Lastly, it is noteworthy that RPB8 is shared by all three classes of RNA polymerases (19, 40). Whereas polymerase II synthesizes mRNA, which is only ~5% of all RNAs, polymerase I and polymerase III synthesize the remaining 95% of all RNAs. Therefore, modification of those complexes, rather than polymerase II, might enormously influence cellular conditions. Whereas RPB1 has been intensively studied, the role of RPB8 in the DNA damage response has been poorly understood. The ubiquitination of RPB8 by BRCA1 reported here provides additional evidence for the role of RNA polymerases in the DNA damage response as well as in carcinogenesis.

Acknowledgments

Received 8/31/2006; revised 11/15/2006; accepted 11/30/2006.

Grant support: Japan Society for the Promotion of Science and the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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We thank Drs. Yanping Zhang, Minoru Takata, and Masamichi Ishiai for helpful discussions and critical reading of the manuscript; and Drs. Richard Baer and Nouria Hernandez for their generous contribution of materials.

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BRCA1 Ubiquitinates RPB8 in Response to DNA Damage

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Cancer Res 2007;67:951-958.