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BRCA2 Is Ubiquitinated In Vivo and Interacts with USP11, a Deubiquitinating Enzyme That Exhibits Prosurvival Function in the Cellular Response to DNA Damage

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Individuals carrying a germ line mutation of the breast cancer susceptibility gene *BRCA2* are predisposed to breast, ovarian, and other types of cancer. The BRCA2 protein has been proposed to function in the repair of DNA double-strand breaks. Using an immunopurification-mass spectrometry approach to identify novel proteins that associate with the BRCA2 gene product, we found that a deubiquitinating enzyme, USP11, formed specific complexes with BRCA2. Moreover, BRCA2 was constitutively ubiquitinated in vivo in the absence of detectable proteasomal degradation. Mitomycin C (MMC) led to decreased BRCA2 protein levels associated with increased ubiquitination, consistent with proteasome-dependent degradation. While BRCA2 could be deubiquitinated by USP11 in transient overexpression assays, a catalytically inactive USP11 mutant had no effect on BRCA2 ubiquitination or protein levels. Antagonism of USP11 function either through expression of this mutant or through RNA interference increased cellular sensitivity to MMC in a BRCA2-dependent manner. All of these results imply that BRCA2 expression levels are regulated by ubiquitination in the cellular response to MMC-induced DNA damage and that USP11 participates in DNA damage repair functions within the BRCA2 pathway independently of BRCA2 deubiquitination.

Individuals who carry a germ line mutation in the *BRCA2* gene are predisposed to breast, ovarian, and certain other cancers (42, 49). Loss of function of the wild-type *BRCA2* allele is observed in breast tumors that arise in these individuals (10), defining *BRCA2* as a tumor suppressor gene. Additionally, certain biallelic *BRCA2* mutations have been linked to the D1 and B subtypes of the cancer susceptibility disorder Fanconi anemia (19). The *BRCA2* gene encodes a 3,418-amino-acid nuclear protein (42) with an approximate molecular mass of 460 kDa (24). The BRCA2 protein has been shown to bind to the mammalian homolog of the RecA recombinase, Rad51 (9, 26, 37, 48). Hence, a BRCA2 function in the repair of DNA double-strand breaks through homologous recombination has been proposed (37). In support of this notion, mammalian cells lacking functional BRCA2 are sensitive to DNA-damaging agents (9, 32, 37, 52), show genomic instability (32, 45, 52), and are deficient in homology-directed DNA repair (28, 44). Furthermore, BRCA2 has been shown to possess single-stranded DNA binding ability (51) and can influence Rad51 DNA binding and recombination activities (11). However, the precise role of BRCA2 in homologous recombination and/or DNA repair has not been elucidated.

The study of interacting proteins is an important approach toward understanding the biological functions of proteins. Toward this end, we immunopurified a C-terminal region of BRCA2 and subjected copurifying proteins to mass spectrometry analysis. Here we report the identification of a novel BRCA2-interacting protein, USP11, and characterize its role in BRCA2-mediated DNA damage repair.

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MATERIALS AND METHODS

Plasmids. A Flag-green fluorescent protein (GFP) plasmid was created by PCR amplification of the GFP sequence with the primers GCGCGGTACCGG TCGCCACC*ATGGACTACAAGGACGACGATGACAAG*ATGGTGAGCAA GGGCGAGGAGCTG (the AgeI site is underlined; the Flag sequence is in italic type) and CCCGGGGGTACCGGCGGCCGCTCCGGACTTGTACAGCTCG TCCATGC (the underlined sequences are KpnI and NotI sites, respectively). The resulting PCR fragment was digested with AgeI and KpnI and ligated into the cognate sites of similarly digested pEGFP-C1 (Clontech). The Flag-GFP-BRCA2(2281–3418) construct was created by PCR amplification of the BRCA2 sequence (amino acids 2281 to 3418) with the primers GCGCGGTACCGCGG CCGCCATGGGAGAACCCTCAATCAAAAGAAAC (the NotI site is underlined) and CCCGGGCTCGAGTTAGATATATTTTTTAGTTGTAATTGT GTCC (the XhoI site is underlined). The resulting PCR fragment was digested with NotI and XhoI and ligated in frame into the Flag-GFP plasmid, which had been similarly digested. The Flag-GFP-BRCA2 expression vector was created as follows. The BRCA2 coding region from pGFPB2 (24) was subcloned into pcDNA3 (Invitrogen), creating BRCA2-pcDNA3. An N-terminal PCR fragment of BRCA2, generated with the primers CCCGGGGGTACCGCCACCATGCC TATTGGATCCAAAGAGAGGCC (the KpnI site is underlined) and GGATC ATTTTCACACTGTCCTTCCTGCAGGC (the Sse8387I site is underlined), was digested with KpnI and Sse8387I and ligated into the cognate sites of BRCA2-pcDNA3, creating Kpn-BRCA2-pcDNA3. The BRCA2 sequence was excised from Kpn-BRCA2-pcDNA3 with KpnI and ApaI and ligated into the similarly digested Flag-GFP plasmid.

The Myc-USP11 expression construct was produced as follows. An I.M.A.G.E. clone (GenBank accession number BC000350) was obtained from the American Type Culture Collection (Manassas, Va.). The N-terminal USP11 coding sequence was PCR amplified with the primers CCGGCCAGATCTAATGGCGA CGGTCGCAGCAAATCCAGC (the BglII site is underlined) and CCCGGGC TCGAGCCGGTGCTGCTCTGGCTTGCG (the XhoI site is underlined). The resulting PCR fragment was digested with BglII and XhoI and ligated into the cognate sites of a BamHI/XhoI-digested, pBabe-derived retroviral vector (27a) containing an N-terminal Myc epitope tag, creating pCCBS-Myc-USP11(1–470).

The C-terminal portion of the USP11 sequence was PCR amplified with the primers CCCATGGATCCGCGCCGCAAGCCAGAGC (the BamHI site is underlined) and CCCGGGCTCGAGTCAATTAACATCCATGAACTCAGAGC (the XhoI site is underlined). The resulting PCR fragment was directionally cloned into the BamHI and XhoI sites of the subcloned N-terminal USP11 vector, creating pCCBS-Myc-USP11. Mutations in the USP11 coding sequence were introduced by sequential PCR steps with overlapping primers containing the desired point mutations. The Flag-USP11 wild-type vector was created in a manner similar to the Myc-USP11 vector by using pCCBS-Flag. The Myc-USP11 wild-type vector encoding hygromycin resistance was created by excision of the puromycin resistance gene and ligation of a hygromycin resistance cassette into pCCBS-Myc-USP11. Glutathione *S*-transferase (GST)–USP11 expression vectors were created by subcloning USP11 sequences into pGEX-4T2 (Amersham).

The hemagglutinin (HA)-ubiquitin expression construct (43) was obtained from D. Bohmann. The Flag-Mdm2 plasmid was a gift from the laboratory of Z. Ronai. The short-hairpin RNA (shRNA) vectors, pSUPER and pRETRO-SUPER (4, 5), were gifts from R. Agami. shRNA constructs were created by annealing double-stranded oligonucleotides into the BglII/HindIII sites of vector pSUPER as previously described (5). The H1 RNA promoter and adjacent annealed targeting sequences were excised and ligated into vector pRETRO-SUPER as previously described (4). For shRNA targeting of luciferase, the oligonucleotides GATCCCCGTTACGCTGAGTACTTCGATTCAAGAGATCGAAGTACTCAGCGTAACTTTTTGGAAA and AGCTTTTCCAAAAAGTTACGCTGAGTACTTCGATCTCTTGAATCGAAGTACTCAGCGTAACGGG were annealed and ligated into pSUPER (underlining indicates RNA interference target sequences). For shRNA targeting of USP11, the oligonucleotides GATCCCCCCAGTGGCGCCAGATAGAATTCAAGAGATTCTATCTGGCGCCACTGGTTTTTGGAAA and AGCTTTTCCAAAAACCAGTGGCGCCAGATAGAATCTCTTGAATTCTATCTGGCGCCACTGGGGG were used. For shRNA targeting of BRCA2, the oligonucleotides GATCCCCGCTCCACCCTATAATTCTGTTCAAGAGACAGAATTATAGGGTGGAGCTTTTTGGAAA and AGCTTTTCCAAAAAGCTCCACCCTATAATTCTGTCTCTTGAACAGAATTATAGGGTGGAGCGGG were used. All plasmids were verified by DNA sequencing and/or restriction enzyme digestion.

Cell culture, transfection, and retroviral infection. All cells were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum and 100 U of penicillin-streptomycin/ml. Unless otherwise noted, cells were transfected in 35-mm culture dishes with Lipofectamine Plus (Invitrogen) as instructed by the manufacturer. For immunoprecipitation analysis, cells were transferred to 100-mm culture dishes 2 h after transfection and cultured for 3 days prior to cell lysis. To create colonies of 293 cells stably expressing Flag-GFP-BRCA2(2281–3418), cells were transfected as described above and selected in Dulbecco modified Eagle medium containing G418 (1 mg/ml; Invitrogen) 3 days after transfection. To produce retroviral supernatants, 293T cells plated in 100-mm culture dishes were cotransfected with 5 μg of retroviral vector and 5 μg of packaging plasmid pCL-Ampho (29). Medium (5 ml) containing retroviruses was collected 24, 48, and 72 h posttransfection and filtered through a 45-μm-pore-size filter. To create stable pools of MCF7 and Capan-1 cells for clonogenic survival assays, cells in 60-mm culture dishes were incubated overnight in a mixture (1:1) of retroviral supernatant and fresh medium supplemented with Polybrene (10 μg/ml) (6). Two days later, cells were selected with puromycin (0.5 μg/ml) for 10 to 14 days. Cells infected with a second retrovirus were selected with hygromycin (100 μg/ml) for 2 to 3 weeks.

Isolation of proteins and identification by mass spectrometry (MS) analysis. Extracts were prepared from 20 150-mm culture dishes containing either parental 293 cells or 293 cells stably expressing Flag-GFP-BRCA2(2281–3418). All steps were performed at 4°C, except as noted otherwise. Plates were washed twice with phosphate-buffered saline (PBS) and scraped in PBS; cells were collected and pelleted. Cell pellets were resuspended in 4.5 ml of buffer A (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg each of aprotinin, bestatin, and leupeptin/ml). The resulting suspensions were sonicated (three times, 20 s each), and 9 ml of buffer B (20 mM Tris-HCl [pH 7.4], 1 M NaCl, 0.2% NP-40, 1 mM PMSF, 2 μg each of aprotinin, bestatin, and leupeptin/ml) was added. The mixtures were rotated for 60 min at 4°C, followed by centrifugation (100,000 × *g* for 60 min). The supernatants were added to 0.3 ml of Flag-agarose beads (prepared as instructed by the manufacturer [Sigma] and rinsed in wash buffer [a 1:1 mixture of buffers A and B]) and incubated overnight with rotation.

Beads containing bound proteins were washed three times in 30 ml of wash buffer, transferred to a 0.5-cm Flex column (Kontes), and washed sequentially with 50 ml of wash buffer and 10 ml of elution buffer (25 mM Tris [pH 7.5], 0.1 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 2 μg each of aprotinin, bestatin, and leupeptin/ml). Beads were transferred to a microcentrifuge tube, and bound proteins were eluted by three sequential incubations (approximately 6 h each) with 1.3 ml of elution buffer containing 0.5 mg of Flag peptide (Sigma)/ml on a rotator. The eluates were pooled, filtered in a Flex column, and concentrated in a Biomax 5K filter unit (Millipore) to a volume of 1 ml. Concentrated eluates were incubated with 0.1 ml of sodium deoxycholate (0.15%) for 15 min at room temperature. Trichloroacetic acid (72%; 0.1 ml) was added, and the proteins were precipitated by microcentrifugation (30 min). The pellets were resuspended in 20 μl of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer, and the pH was adjusted with 1 M Tris-HCl (pH 8). Proteins then were separated on an SDS–4 to 12% gradient polyacrylamide gel.

Protein bands of interest, visualized by zinc staining (E-Zinc; Pierce), were excised from the gel and destained with acetonitrile–100 mM ammonium bicarbonate (45:55, vol/vol). The resulting gel slice was reduced with 10 mM Tris(2-carboxyethyl)phosphine, alkylated with 50 mM iodoacetamide, and then digested in situ with trypsin (100 ng per band in 50 mM ammonium bicarbonate). The tryptic peptides were extracted by using Poros 20 R2 beads (Applied Biosystems) in the presence of 5% formic acid and 0.2% trifluoroacetic acid (TFA) and dried (with a Speed-vac). The resulting peptides were dissolved in 3 μl of high-pressure liquid chromatography (HPLC) sample solvents containing water-methanol-acetic acid-TFA (70:30:0.5:0.01, vol/vol/vol/vol). Micro-HPLC–MS-MS analysis was conducted by using an LCQ electrospray ionization ion trap mass spectrometer (ThermoFinnigan) coupled to an online MicroPro-HPLC system (Eldex Laboratories). Tryptic peptide solution (2 μl) was injected into a Magic C_{18} column (5 μm, 200 Å, 0.2 by 50 mm; Michrom BioResources) that had been preequilibrated with 70% solvent A (0.5% acetic acid and 0.01% TFA in water-methanol [95:5, vol/vol]) and 30% solvent B (0.5% acetic acid and 0.01% TFA in methanol-water [95:5, vol/vol]). Peptides were separated and eluted from the HPLC column with a linear gradient of 30 to 95% solvent B over 15 min at a flow rate of 2.5 μl/min. The eluted peptides were introduced directly into the LCQ mass spectrometer by electrospray (2.8 kV). The LCQ mass spectrometer was operated in the data-dependent mode for measuring the molecular masses of peptides (parent peptides) and for collecting MS/MS peptide fragmentation spectra. The measured molecular masses of parent peptides and their MS/MS data were used to search National Center for Biotechnology Information nonredundant DNA and protein sequence databases by using the program KNEXUS (Genomic Solutions).

Antibodies. The Flag monoclonal antibody (MAb) (M2) and Flag-agarose beads were purchased from Sigma. The Myc MAb (9E10) and the HA MAb (12CA5) were produced at the Hybridoma Center at Mount Sinai School of Medicine. Antibodies to BRCA2 were previously described (24). Rabbit polyclonal antiserum to the C terminus of USP11 was raised against a peptide consisting of amino acids 906 to 920. An additional polyclonal rabbit antibody to USP11 was generously provided by Yoshiaki Ishigatsubo (20). Rabbit polyclonal antiserum to ubiquitin was purchased from Sigma.

Coimmunoprecipitation and Western blotting analysis. Cells grown in 100-mm culture dishes were rinsed with PBS. All subsequent steps were performed at 4°C. Cells were lysed for 30 min in 500 μl of lysis buffer (50 mM HEPES [pH 7.6], 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 1 mM PMSF, 10 mM *N*-ethylmaleimide, 2 μg each of aprotinin, bestatin, and leupeptin/ml). Lysates were clarified by microcentrifugation for 15 min. Protein concentrations were determined by Bradford assays (Bio-Rad, Hercules, Calif.). Equal amounts of lysate proteins were used in the assays. For Flag immunoprecipitations, lysates were incubated with 10 μl of Flag-agarose beads (prepared as instructed by the manufacturer) for 3 h with rotation. For all other immunoprecipitations, lysates were incubated with primary antibody for 1.5 h, and immune complexes were captured during 1.5 h of incubation with 10 μl of protein G-Sepharose beads (Amersham). Immune complexes were washed four times with lysis buffer and incubated in SDS gel loading buffer for 3 min at 70°C. Proteins were separated by SDS-PAGE and visualized by Western blotting. For Flag-Mdm2 immunoprecipitations, MG132 (50 μM) was added to the culture medium for 3 h prior to lysis. Following lysis and clarification, lysis buffer was supplemented with 0.25% sodium deoxycholate, 0.2% NP-40, and 0.1% SDS, and this formulation was used for the remainder of the immunoprecipitations.

In vitro deubiquitination assay. Wild-type and C275S GST-USP11 fusion proteins were expressed from pGEX-4T2 in BL21(pLysS) bacteria (Novagen) during 3.5 h of induction with 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C. Cells were pelleted and resuspended in 10 ml of 50 mM Tris–2 mM EDTA. Lysozyme (1 mg/ml) and Triton X-100 (0.25%) were added, and the cells were incubated at room temperature for 15 min. The resulting lysates were sonicated and centrifuged for 15 min at 13,000 rpm in a Sorvall SS34 rotor. Glutathione-agarose beads (Sigma) were swelled in water for 30 min, washed once with PBS, washed once with 50 mM Tris–2 mM EDTA, and resuspended as a 50% slurry in 50 mM Tris–2 mM EDTA. A total of 1 ml of glutathione-agarose

slurry was incubated overnight with each bacterial lysate supernatant. Beads were pelleted and washed four times with 50 mM Tris–2 mM EDTA–Triton X-100 (0.25%). GST-USP11 proteins were eluted during three 30-min incubations at 4°C with 10 mM reduced glutathione in 50 mM Tris–2 mM EDTA. The concentrations of purified GST-USP11 proteins were determined by Bradford assays. Purified polyubiquitin containing predominantly Lys-48-linked linear polyubiquitin chains was purchased from Affiniti Research Products (Mamhead, Exeter, United Kingdom) and reconstituted at a concentration of 2 μg/μl in 50 mM Tris (pH 7.6)–50 mM NaCl–1 mM EDTA. For each reaction, 100 ng of polyubiquitin was diluted in 20 μl of deubiquitination buffer (50 mM Tris [pH 7.6], 50 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol). Purified GST-USP11 proteins were added, and the reaction mixtures were incubated for 2 h at 30°C. Reactions were terminated by the addition of 20 μl of SDS-PAGE loading buffer (unheated). Reaction mixtures then were separated on an SDS–14% polyacrylamide gel and subjected to antiubiquitin Western blotting.

Clonogenic survival assay. Mass populations of marker-selected MCF7 or Capan-1 cells, stably infected with various retroviruses, were each plated in 12 culture dishes and grown to subconfluence. Cells were incubated for 1 h in medium containing 0, 100, 200, or 400 ng of mitomycin C (MMC)/ml (each in triplicate for each cell type). Cells were incubated in fresh medium overnight, reseeded at 1,000 cells per 60-mm culture dish (in triplicate for each combination), and allowed to grow for 10 to 14 days. Some experiments with Capan-1 cells included an additional incubation in medium containing 50 ng of MMC/ml, and cells were reseeded at 3,000 cells per 60-mm culture dish. Colonies were fixed in 10% methanol–10% acetic acid (vol/vol) for 10 min, stained with 1% (wt/vol) crystal violet in methanol for 10 min, and then rinsed three times with water prior to colony counting.

RESULTS

USP11 copurifies with BRCA2. We attempted to purify novel BRCA2-interacting proteins by attaching a Flag epitope tag to the N terminus of BRCA2 and using Flag immunoaffinity beads. We expressed a C-terminal fragment of BRCA2, BRCA2(2281–3418). This region contains the BRCA2 nuclear localization signals (38) and DNA binding domain (51), and its sequence is well conserved among mammalian species (25, 41). The exogenous BRCA2 product also contained a GFP tag, which was found to confer increased stability (24). Flag-GFP-BRCA2(2281–418) was stably expressed in human embryonic kidney 293 cells, and Flag-GFP-BRCA2(2281–3418) complexes were affinity purified, separated by SDS-PAGE, and visualized by zinc staining of the gel (Fig. 1A). Several proteins that specifically associated with Flag-GFP-BRCA2(2281–3418) were identified. One such protein, p110, when subjected to MS/MS analysis of tryptic peptides (Fig. 1B, C, and D), was determined via a five-peptide match (Fig. 1E) to be the ubiquitin-specific protease USP11 (39). An extensive search of public databases revealed several USP11 cDNA clones, one of which was consistent with a 110-kDa protein (GenBank accession number BC000350). This USP11 cDNA encodes a protein of 920 amino acids, containing 1 residue less than a recently reported full-length clone (20). Of note, the *USP11* gene is localized to chromosome Xp11.3, a region that has been implicated in ovarian cancer (39).

USP11 specifically forms complexes with BRCA2. In order to verify that BRCA2 and USP11 form complexes in vivo, we performed reciprocal coimmunoprecipitation and Western blot experiments. Flag-GFP-BRCA2(2281–3418) and Myc-tagged USP11 were transiently expressed in 293T cells and immunoprecipitated by using their respective epitope tags (Fig. 2A). Myc-USP11 specifically coprecipitated with Flag-GFP-BRCA2(2281–3418), and Flag-GFP-BRCA2(2281–3418) specifically coprecipitated with Myc-USP11. No coprecipitation was detected when nonspecific antibodies were used for the immunoprecipitation or when either of the two constructs was omitted. We also expressed and immunoprecipitated Flag-BRCA2(2281–3418) (which lacks GFP) alongside its GFP-containing counterpart (Fig. 2B). Both of these proteins coprecipitated endogenous USP11, whereas Flag-GFP and an N-terminal BRCA2 fragment, Flag-BRCA2(1–936), were unable to do so. These results indicated a specific USP11 and BRCA2 interaction, which was dependent on sequences contained within BRCA2(2281–3418).

To ensure that the detected USP11-BRCA2 interaction was not a result of the fragmented BRCA2 used, a similar experiment was performed with a full-length BRCA2 construct (Fig. 2C). Again, Myc-USP11 specifically coprecipitated with Flag-GFP-BRCA2, and Flag-GFP-BRCA2 specifically coprecipitated with Myc-USP11. The Western blot signal intensity for Myc-USP11 that coprecipitated with Flag-GFP-BRCA2 from 1 mg of lysate was 5 to 10 times lower than that for total Myc-USP11 contained in 100 μg of lysate. Thus, approximately 1 to 2% of Myc-USP11 was present in the Flag-GFP-BRCA2 immune complex. When adjusted for the observed approximate 20% efficiency of Flag-GFP-BRCA2 immunoprecipitation, it was estimated that 5 to 10% of Myc-USP11 was complexed with Flag-GFP-BRCA2 in the cells. A comparable estimation was obtained from the reciprocal experiment.

To verify that the endogenous BRCA2 and USP11 proteins associate, cell lysates from 293 cells were reciprocally immunoprecipitated with antibodies to BRCA2 and USP11 (Fig. 2D). The Capan-1 cell line, which contains a C-terminally truncated BRCA2 protein (14) that is not recognized by the BRCA2 antibody C15, was used as a control for nonspecific precipitation (acting as a BRCA2-null cell line for purposes of this experiment). As shown in Fig. 2D, USP11 coprecipitation with BRCA2 was seen with 293 cell lysates but not with Capan-1 cell lysates. It was not possible to detect BRCA2 in the USP11 immunoprecipitation, perhaps due to the low signal intensity of endogenous immunoreactive BRCA2. It is also plausible that the USP11-BRCA2 interaction interferes with the epitope recognized by the USP11 antibody, such that it is less efficient in precipitating BRCA2-complexed USP11. The specificity of the USP11 antibody used in Fig. 2D is demonstrated in Fig. 2E.

BRCA2 is a nuclear protein (3). To determine the subcellular localization of USP11, we transiently transfected Cos7 cells with Myc-USP11 and performed anti-Myc immunostaining (Fig. 2F). Myc-USP11 was visualized predominantly in the nucleus, with minor cytoplasmic staining. When coexpressed, GFP-BRCA2(2281–3418) colocalized with USP11 in the nucleus, consistent with the ability of BRCA2 and USP11 to associate in vivo.

BRCA2 is a ubiquitinated protein. By virtue of its interaction with USP11, we reasoned that BRCA2 may be a candidate substrate. However, the ubiquitination of BRCA2 has not been reported. To explore this possibility, we coexpressed Flag-GFP-BRCA2 and HA-ubiquitin in 293T cells and subjected Flag immunoprecipitates to Western blot analysis with HA antibody. As shown in Fig. 3A, an HA-ubiquitinated form of Flag-GFP-BRCA2 was detected. To rule out ubiquitination due to overexpression of BRCA2, we expressed HA-ubiquitin in 293T cells and immunoprecipitated endogenous BRCA2 (Fig. 3B). Again, we observed an HA-ubiquitinated form of

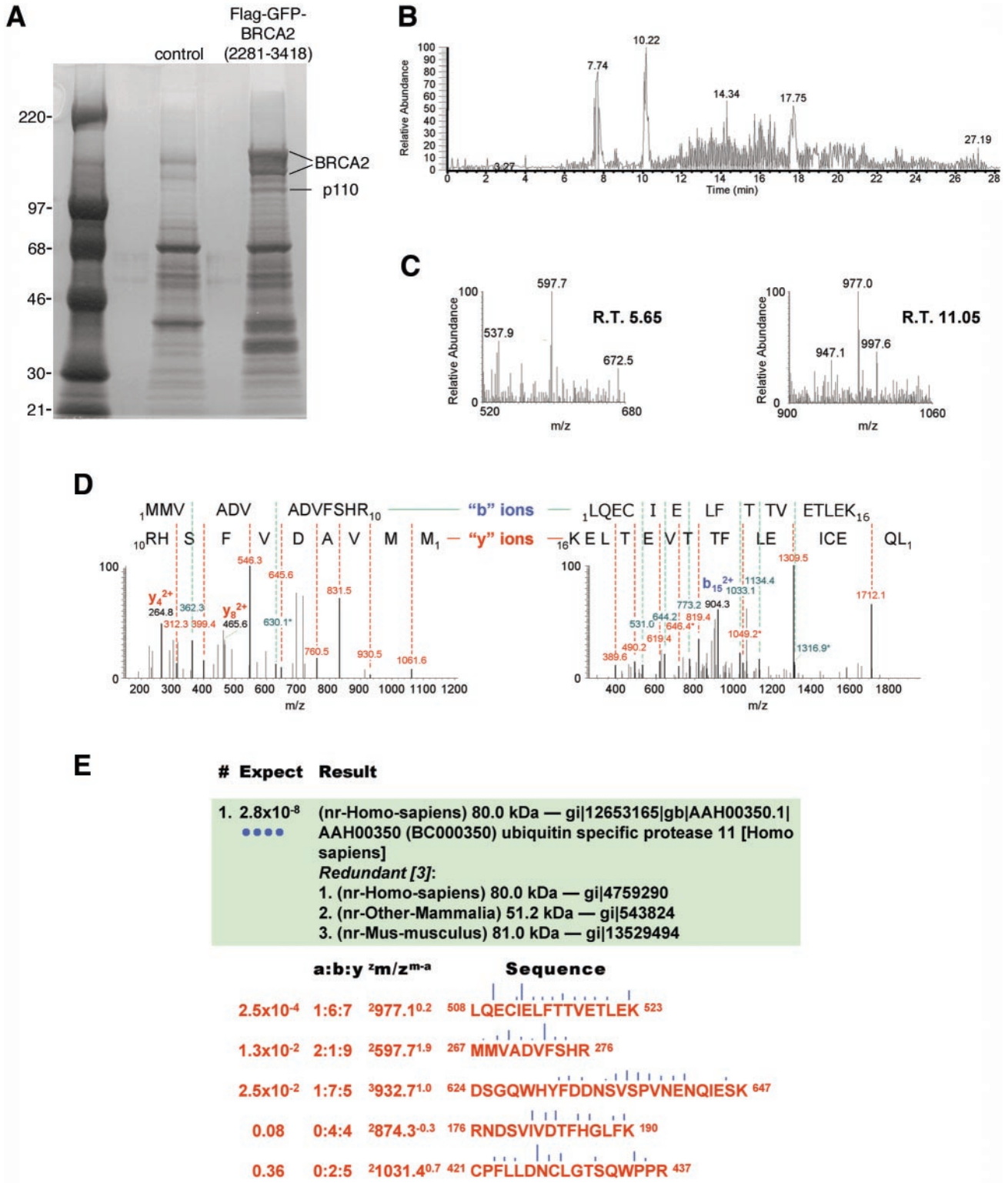

FIG. 1. USP11 is identified as a BRCA2-interacting protein through immunoaffinity purification and micro-HPLC–electrospray ionization ion trap–MS/MS analysis. (A) Flag-GFP-BRCA2(2281–3418) was immunopurified as described in Materials and Methods. Flag-GFP-BRCA2(2281–3418) immune complexes were separated on an SDS–4 to 16% gradient polyacrylamide gel and visualized with E-Zinc. Purification of Flag-GFP-BRCA2(2281–3418) (indicated as BRCA2) was confirmed by MS (data not shown). A 110-kDa copurifying protein is indicated as p110. (B) Ion chromatogram of tryptic peptides obtained from p110 and separated by online micro-C_{18} HPLC. (C) Mass spectra of two peptides from the above chromatogram with mass/charge ratios of 597.7 and 977.0 were detected at retention times (R.T.) of 5.65 and 11.05 min, respectively. (D) Fragment ion mass spectra of the above ions determined by using data-dependent MS/MS. Peaks corresponding to the "y" ions and the "b" ions of the matched USP11 peptides are indicated. (E) A database search with all of the MS/MS data derived from the 110-kDa copurifying protein with KNEXUS resulted in the identification of USP11.

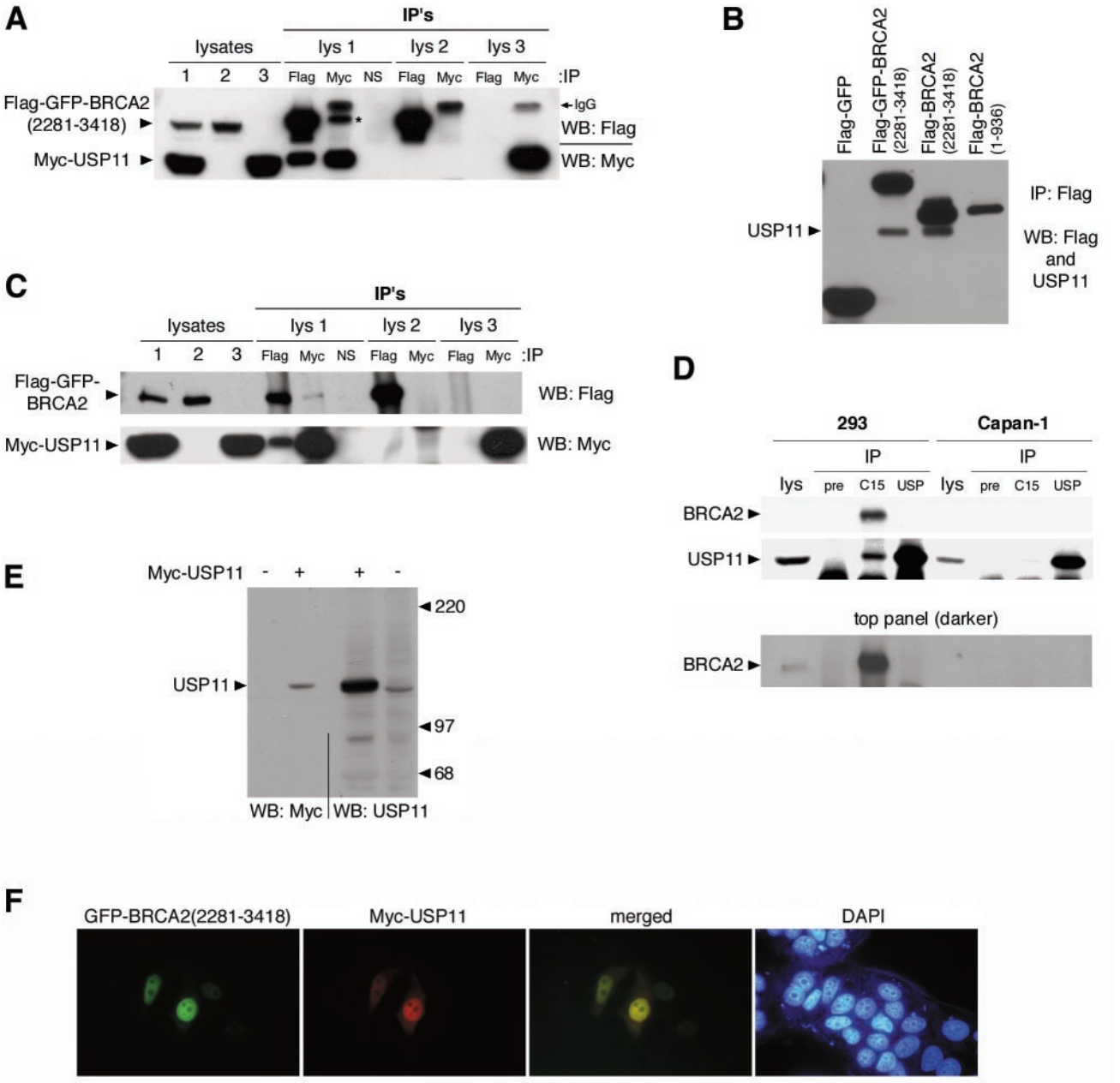

FIG. 2. BRCA2 specifically interacts with USP11. (A) Flag-GFP-BRCA2(2281–3418) and Myc-USP11 were transiently coexpressed (lysate 1) or singularly expressed (lysates 2 and 3) in 293T cells. Immunoprecipitation (IP) was performed with Flag, Myc, or nonspecific (NS) antibodies. Lysates (1/10 IP input) and immunoprecipitates (IP's) from lysates 1, 2, and 3 (lys 1, lys 2, and lys 3, respectively) were separated on an SDS–7% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was cut horizontally and Western blotted (WB) with Flag (top) or Myc (bottom) antibodies. The asterisk indicates the coimmunoprecipitated Flag-GFP-BRCA2 band. Immunoglobulin G (IgG) bands in Myc immunoprecipitates are indicated at the right. (B) Flag-GFP, Flag-GFP-BRCA2, or Flag-BRCA2 constructs containing the indicated BRCA2 sequences were transiently expressed in 293T cells and immunoprecipitated with Flag antibody. Immune complexes were separated on an SDS–7%/14% split polyacrylamide gel. Western blotting was performed by sequential incubations with antibodies to Flag and USP11. (C) Co-immunoprecipitation analysis was performed as described for panel A except that Flag-GFP-BRCA2 (full length) was used and immune complexes were separated on an SDS–5%/7% split polyacrylamide gel. (D) Lysates (2 mg) from 293 and Capan-1 cells were immunoprecipitated with preimmune serum (pre), BRCA2 C-terminal polyclonal antibody (C15) (24), or USP11 polyclonal antibody (USP). Immune complexes and lysates (lys; 1/40 IP input) were separated on an SDS–5%/7% split polyacrylamide gel. The gel was cut horizontally and Western blotted with the BRCA2 antibody (top) and the USP11 antibody (middle). BRCA2 protein was visible in 293 cell lysates upon darker exposure (bottom). (E) Lysates (20 μg) from 293T cells, either untransfected (−) or transfected with Myc-USP11 (+), were separated on an SDS–7% polyacrylamide gel. The membrane was cut vertically and Western blotted with antibodies to Myc (left) and USP11 (right). The left and right membrane halves were lined up by using protein size markers (indicated on the right) prior to Western blot detection. (F) MCF7 cells on coverslips were transfected with GFP-BRCA2(2281–3418) and Myc-USP11. Cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.1% saponin, blocked, and incubated with a MAb to Myc and a tetramethyl rhodamine isothiocyanate-conjugated anti-mouse secondary antibody. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI). Images were captured with a charge-coupled device camera (Sony DKC 5000) mounted on a Nikon Microphot microscope (×60 objective).

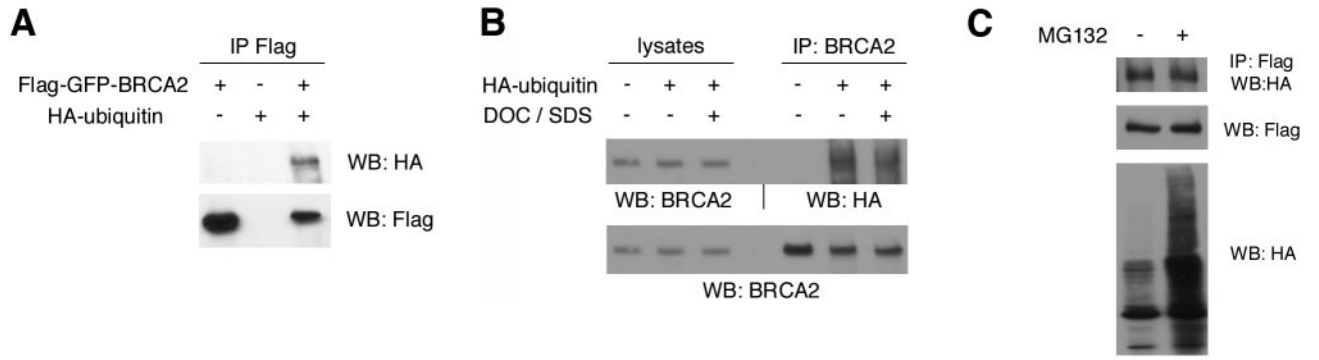

FIG. 3. BRCA2 is a ubiquitinated protein. (A) Flag-GFP-BRCA2 (full length) and HA-ubiquitin were transiently coexpressed or singularly expressed in 293T cells. Lysates were immunoprecipitated (IP) with Flag-agarose beads, and immune complexes were Western blotted (WB) with antibody to HA (top). The blot then was stripped and reprobed with antibody to Flag (bottom). (B) HA-ubiquitin was expressed in 293T cells. Lysates were immunoprecipitated with BRCA2 MAb B2 (IP: BRCA2). Immunoprecipitation in the right lane was performed in the presence of 0.5% sodium deoxycholate (DOC) and 0.1% SDS to dissociate any bound proteins. Lysates (1/20 IP input) and immune complexes (IP: BRCA2) were separated on an SDS–5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was cut vertically and Western blotted with BRCA2 polyclonal antibody C15 (top, left) and HA antibody (top, right). The left and right membrane halves were lined up by using protein size markers prior to Western blot detection (top). The right half of the membrane then was stripped and reprobed with BRCA2 antibody C15 (bottom). Again, the left and right membrane halves were lined up prior to Western blot detection (bottom). (C) Flag-GFP-BRCA2 (full length) and HA-ubiquitin were transiently coexpressed in 293T cells. The cells were incubated with MG132 (50 μM) for 4.5 h prior to lysis. Lysates were immunoprecipitated with Flag-agarose beads, and immune complexes were Western blotted with antibody to HA (top). Lysates (1/20th IP input) were Western blotted with antibodies to Flag (middle) and HA (bottom).

BRCA2 that comigrated with BRCA2 detected in cell lysates. Immunoprecipitation performed with detergents that dissociate bound proteins caused no significant decrease in the amount of HA-ubiquitinated BRCA2 detected (Fig. 3B, right lanes). These results demonstrate that the BRCA2 protein is ubiquitinated in vivo.

Ubiquitination frequently serves as a signal for proteasome-dependent degradation (16). To determine whether ubiquitinated BRCA2 undergoes proteasomal degradation, 293T cells were transfected with Flag-GFP-BRCA2 and HA-ubiquitin and incubated in the presence or absence of the proteasome inhibitor MG132 (33). Proteasomal inhibition did not increase the amount of HA-ubiquitinated BRCA2 detected by immunoprecipitation or the levels of Flag-GFP-BRCA2 in the cell lysates (Fig. 3C). Levels of endogenous BRCA2 were similarly unaffected by MG132 (data not shown). These results indicate that BRCA2 ubiquitinated under physiologic conditions is not rapidly degraded in a proteasome-dependent manner. Whether ubiquitination in this setting serves to target or otherwise modify BRCA2 function (36) remains to be established.

USP11 can deubiquitinate BRCA2. USP11 is a member of the ubiquitin-specific protease family of deubiquitinating enzymes (2) characterized by six conserved regions (47), including Cys and His boxes in the catalytic core of the enzyme (31). To verify that USP11 has intrinsic deubiquitinating activity, polyubiquitin chains were incubated with purified recombinant GST-USP11. As shown in Fig. 4A, a significant decrease in higher-order polyubiquitin chains and an increase in monomeric ubiquitin were observed with wild-type USP11, whereas point mutation of the catalytic cysteine residue (at amino acid 275) to serine (C275S) abolished USP11 deubiquitination activity. Thus, USP11 has the inherent ability to remove covalently linked ubiquitin.

We next tested whether BRCA2 could be deubiquitinated by USP11. USP11, HA-ubiquitin, and Flag-GFP-BRCA2 were coexpressed in 293T cells, and Flag immunoprecipitates were Western blotted for the presence of HA-ubiquitin. As shown in Fig. 4B, overexpressed wild-type USP11 efficiently deubiquitinated Flag-GFP-BRCA2. Mutant USP11, which retained the ability to form complexes with BRCA2, was unable to deubiquitinate Flag-GFP-BRCA2 when expressed at comparable levels. Endogenous BRCA2 was also deubiquitinated in vivo in the presence of exogenous wild-type but not mutant USP11 (Fig. 4C). As a control for substrate specificity, the ubiquitination of Mdm2 was unaffected by USP11 (Fig. 4D). USP11 expression also had no apparent effect on the DNA damage-induced monoubiquitination of Fanconi anemia D2 (FancD2) protein (13) (data not shown), and USP11 was previously shown to be unable to deubiquitinate p53 in vivo (22). These data indicate that USP11 exhibited specificity in its ability to deubiquitinate BRCA2, although another USP11 substrate has been reported (20). It is also important to note that the deubiquitination of BRCA2 in these assays relied on the overexpression of USP11, since ubiquitinated BRCA2 was detected in cells containing endogenous USP11.

Interference with USP11 function increases cell sensitivity to DNA damage. Given the likely role of BRCA2 in homologous recombination and/or DNA damage repair, we investigated whether USP11 has any effect on the cellular response to DNA damage. Since a functional link between BRCA2 and p53 activities has been shown (24), we used MCF7 breast cancer cells, which have an intact p53 pathway, for these analyses. Stable puromycin-selected mass populations of MCF7 cells expressing either wild-type or catalytic mutant (C275S) USP11 proteins were generated and subjected to a clonogenic survival assay following exposure to the DNA interstrand cross-linking agent MMC. As shown in Fig. 5A, the expression of wild-type USP11 had little if any effect on cell survival, whereas the expression of C275S mutant USP11 significantly compromised colony-forming ability following MMC treat-

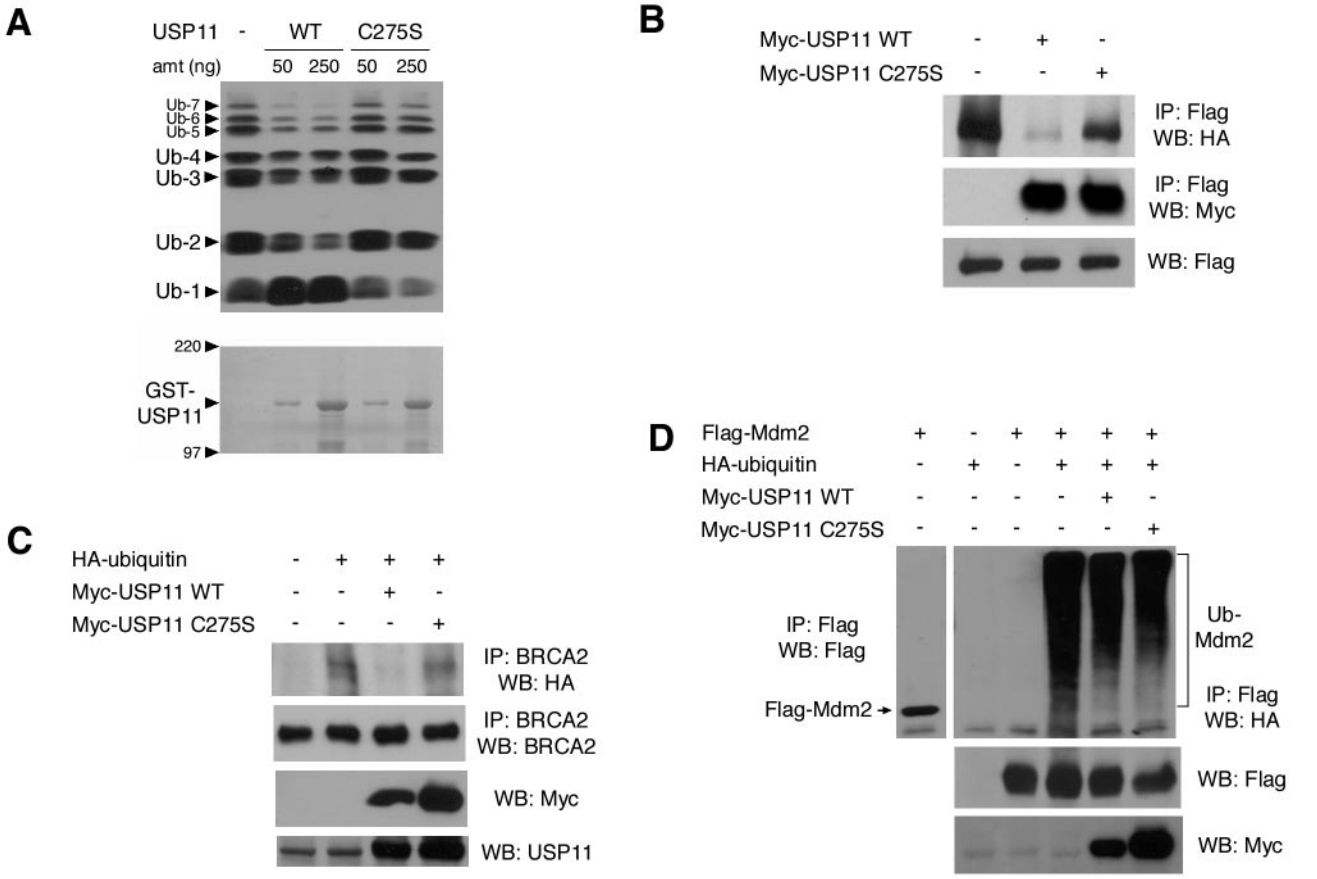

FIG. 4. USP11 can deubiquitinate BRCA2. (A) Polyubiquitin chains were incubated with the indicated amounts of purified recombinant GST-USP11, either wild type (WT) or mutant (C275S), as described in Materials and Methods. Free ubiquitin (Ub-1) and polyubiquitin chains (Ub-2 through Ub-7) were visualized by antiubiquitin Western blotting (top). A Coomassie blue-stained gel containing purified GST-USP11 is also shown (bottom). Each lane contained five times the amount of protein that was used for the top panel. (B) Flag-GFP-BRCA2 (full length) and HA-ubiquitin were coexpressed in 293T cells. Either WT or C275S mutant Myc-USP11 was also coexpressed, where indicated (+). Lysates were immunoprecipitated (IP) with Flag-agarose beads, and immune complexes were Western blotted with antibodies to HA (top) and Myc (middle). The expression of Flag-GFP-BRCA2 is also shown (bottom). (C) HA-ubiquitin and Myc-USP11 (WT or C275S mutant) were coexpressed in 293T cells. Lysates were immunoprecipitated with BRCA2 MAb B2, and Western blotting of immune complexes was performed with MAb to HA (top). The blot then was reprobed with polyclonal antibody to BRCA2 (middle). The expression of Myc-USP11 products is shown in anti-Myc and anti-USP11 Western blots (bottom). (D) Flag-Mdm2 and HA-ubiquitin were coexpressed in 293T cells. Either WT or C275S mutant Myc-USP11 was also coexpressed, where indicated (+). Lysates were immunoprecipitated with Flag-agarose beads, and immune complexes were Western blotted with antibodies to HA (top right) and Flag (top left). The expression of Flag-Mdm2 (middle) and the expression of Myc-USP11 (bottom) are also shown. Ub, ubiquitin.

ment. The expression of C275S mutant USP11 in Capan-1 cells caused no significant change in cell survival (Fig. 5B), indicating that the effect of mutant USP11 on MCF7 cells was both specific and dependent on intact BRCA2. We were unable to express sufficient levels of HA-ubiquitin in these cells to assess the effect of mutant USP11 on BRCA2 ubiquitination. However, since mutant USP11 had no effect on the ubiquitination of BRCA2 in transiently transfected 293T cells (Fig. 4B and C), it is unlikely that stably expressed mutant USP11 acts in a dominant-negative manner in MCF7 cells by blocking the deubiquitination of BRCA2. Nevertheless, these results indicate that mutant USP11 leads to decreased survival in MMC-treated cells and suggest that endogenous USP11 function is important for the cellular response to MMC.

To strengthen the conclusion that endogenous USP11 functions in the DNA damage response, we used an RNA interference approach. The stable expression of shRNA targeting USP11 led to a decrease in USP11 protein levels and reduced colony formation in the MMC survival assay (Fig. 5C). The introduction of exogenous wild-type USP11 into shRNA-targeted cells restored USP11 levels and increased survival following MMC exposure (Fig. 5D), demonstrating the specificity of the cell survival effect. To determine whether the observed effect of USP11 interference was dependent on the BRCA2 pathway, we infected Capan-1 cells with our retrovirus directing the expression of shRNA targeting USP11. We detected a diminution in USP11 levels similar to that seen in MCF7 cells (Fig. 5E, top). However, lower levels of USP11 had no effect on the survival of Capan-1 cells (Fig. 5E, bottom). Comparable results were obtained with EUFA423 fibroblasts (a gift from Markus Grompe), which contain BRCA2 inactivated through biallelic mutations (19) (data not shown). Furthermore, we

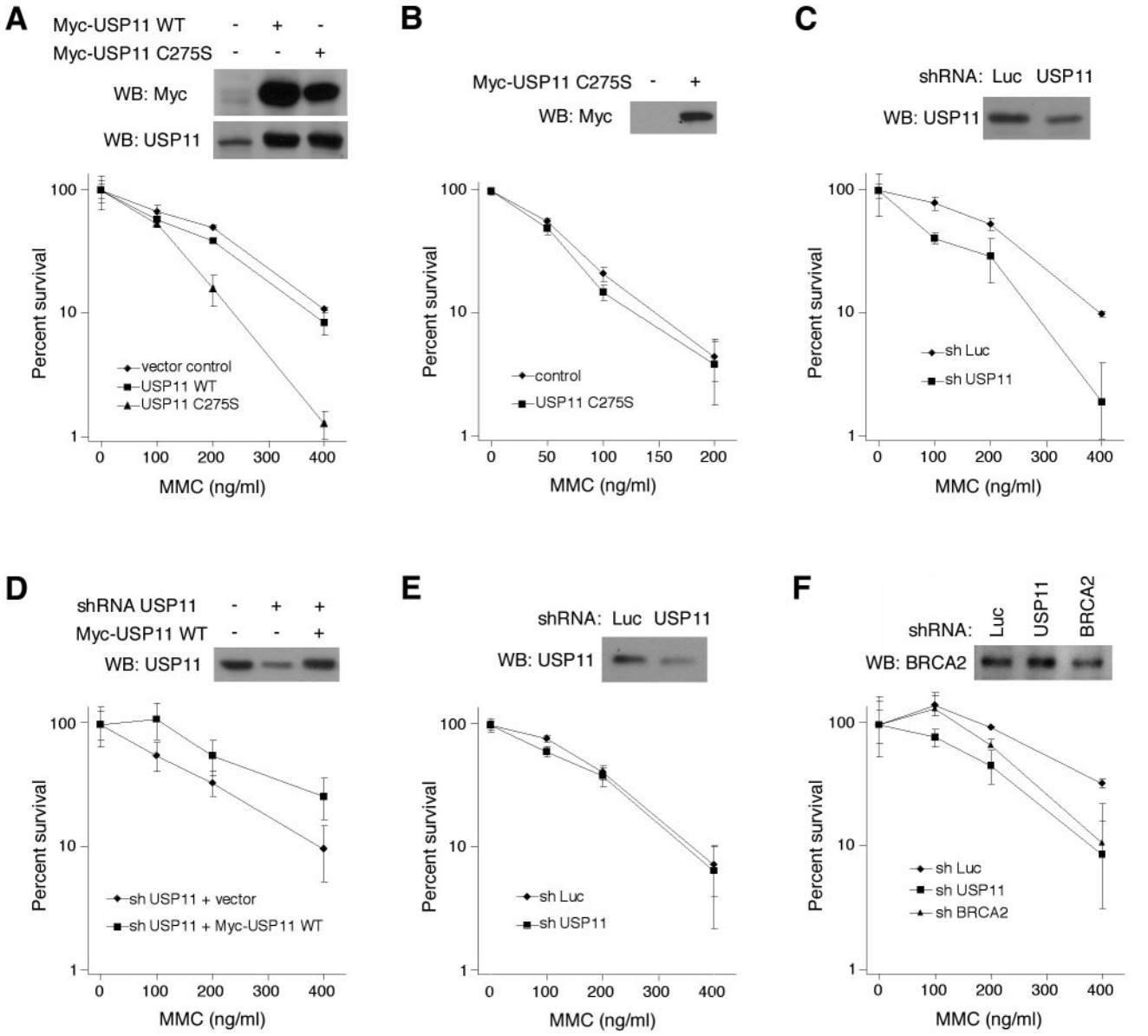

FIG. 5. USP11 exerts prosurvival functions in the cellular response to DNA damage. (A) (Top) Stable pools of MCF7 cells that were retrovirally infected with a control vector, wild-type (WT) Myc-USP11, or Myc-USP11 C275S were subjected to Western blotting (WB) with antibodies to Myc (upper blot) and USP11 (lower blot). (Bottom) A clonogenic MMC survival assay was performed on these cells. Error bars indicate standard deviations. (B) (Top) Capan-1 cells were infected with a retrovirus directing the expression of Myc-USP11 C275S, and a stable pool was selected. Parental Capan-1 cells and those expressing Myc-USP11 C275S were subjected to Western blotting with a Myc antibody. (Bottom) A clonogenic MMC survival assay was performed on these cells. (C) (Top) MCF7 cells were infected with a retrovirus directing the expression of shRNA (4) targeting either luciferase (Luc) or USP11. Stable pools were selected and subjected to Western blotting with antibody to USP11. (Bottom) A clonogenic MMC survival assay was performed on these cells (designated sh Luc or sh USP11, respectively). (D) (Top) MCF7 cells containing USP11 shRNA (used in panel C) were infected with control or WT Myc-USP11-expressing retroviruses. Stable pools were selected with hygromycin and subjected to Western blotting with antibody to USP11 (right two lanes). The USP11 level in uninfected cells is shown for comparison (left lane). (Bottom) A clonogenic MMC survival assay was performed on selected cells. (E) Stable pools of Capan-1 cells infected with a retrovirus directing the expression of shRNA targeting either Luc or USP11 were selected and subjected to Western blotting with antibody to USP11 (top) and a clonogenic MMC survival assay (bottom). (F) (Top) MCF7 cells were infected with shRNA retrovirus targeting BRCA2. Stable pools of cells were selected and analyzed, along with the cells used in panel C, for BRCA2 expression. (Bottom) A clonogenic MMC survival assay was performed on these cells.

observed that a reduction in BRCA2 levels by RNA interference led to a reduction in cell survival similar to that seen with USP11 in the MMC survival assay (Fig. 5F). All of these results support the conclusion that USP11 exerts prosurvival functions through a BRCA2-dependent pathway in response to MMC-induced DNA damage.

MMC up-regulates BRCA2 ubiquitination in a USP11-independent manner. Our analyses revealed that interference

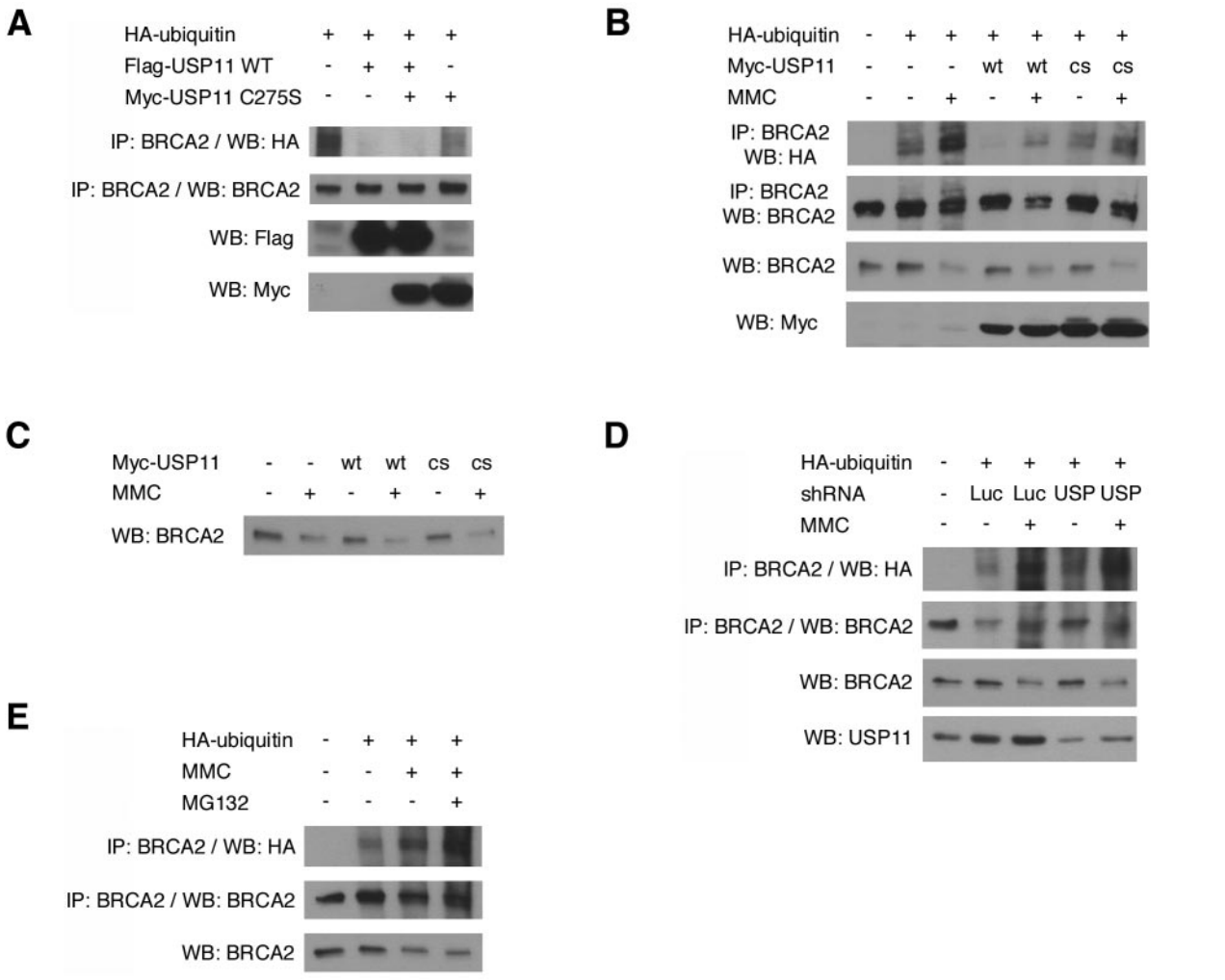

FIG. 6. MMC up-regulates BRCA2 ubiquitination in a USP11-independent manner. (A) (Top) HA-ubiquitin, wild-type (WT) Flag-USP11, and Myc-USP11 C275S were coexpressed in 293T cells, where indicated (+). Lysates were immunoprecipitated (IP) with BRCA2 MAb B2, and Western blotting (WB) of immune complexes was performed with HA MAb. (Middle) The blot was reprobed with polyclonal antibody to BRCA2. (Bottom) The expression of WT Flag-USP11 and Myc-USP11 C275S in the lysates is shown. (B) (Top) HA-ubiquitin and Myc-USP11, either WT (wt) or mutant (C275S) (cs), were coexpressed in 293T cells. Alternate plates of transfected cells were incubated for 72 h with medium containing MMC (160 ng/ml). Lysates were immunoprecipitated with BRCA2 MAb B2, and Western blotting of immune complexes was performed with HA MAb. (Middle) The blot then was reprobed with polyclonal antibody to BRCA2. (Bottom) The expression of endogenous BRCA2 and exogenous Myc-USP11 products was also detected by Western blotting. (C) MCF7 cells stably infected with vector, WT Myc-USP11 (wt), or mutant Myc-USP11 (cs) (same as those used in Fig. 5A) were treated with MMC as described for panel B and assayed for BRCA2 expression. (D) HA-ubiquitin and shRNA vectors targeting luciferase (Luc) or USP11 (USP) were coexpressed in 293T cells, where indicated (+). MMC treatment and analysis of BRCA2 ubiquitination were performed as described for panel B except that endogenous USP11 expression was analyzed in the bottom panel. (E) HA-ubiquitin was expressed in 293T cells. The cells were incubated with MMC (160 ng/ml) for 72 h where indicated (+). MG132 (50 μM) was added to cells in the right lane for 4.5 h prior to lysis. Analysis of BRCA2 ubiquitination was performed as described for panel B.

with USP11 function by overexpression of mutant USP11 or by reduction of USP11 levels compromised cell survival following MMC treatment in a BRCA2-dependent manner. To investigate whether this effect occurs through perturbation of BRCA2 deubiquitination, we performed a mixing experiment assessing the ability of mutant USP11 to block the deubiquitination of wild-type USP11. 293T cells were cotransfected with HA-ubiquitin and with either wild-type Flag-USP11 or C275S mutant Myc-USP11 or both. Endogenous BRCA2 was immunoprecipitated and analyzed for ubiquitination (Fig. 6A). As expected, the expression of wild-type Flag-USP11 led to efficient deubiquitination of BRCA2. However, the coexpression of mutant USP11 did not affect the deubiquitination of BRCA2 by wild-type USP11.

We next investigated the influence of MMC-induced DNA damage on BRCA2 ubiquitination and the ability of mutant USP11 to negatively affect BRCA2 deubiquitination under these conditions. 293T cells were cotransfected in duplicate with HA-ubiquitin and either control plasmid, wild-type USP11, or C275S mutant USP11. One set of each was incubated with MMC (160 ng/ml; 72 h), and endogenous BRCA2 was immunoprecipitated from all cells and analyzed for ubiquitination. As shown in Fig. 6B, MMC treatment led to an increase in BRCA2 ubiquitination and a concomitant decrease

in cellular BRCA2 levels. Although the deubiquitination of BRCA2 was observed in untreated cells expressing wild-type USP11, MMC treatment seemed to override USP11 deubiquitination activity, resulting in increased BRCA2 ubiquitination relative to that in untreated cells. An increase in BRCA2 ubiquitination and a decrease in cellular BRCA2 levels upon MMC treatment were also seen in cells expressing mutant USP11. Thus, mutant USP11 had no effect on changes in BRCA2 ubiquitination or stability following MMC treatment in 293T cells. We extended these findings to the MCF7 cells stably expressing wild-type or mutant USP11, which were used in the MMC survival assays. As shown in Fig. 6C, MMC treatment resulted in a reduction in cellular BRCA2 levels that was independent of exogenously expressed USP11.

We next sought to determine whether endogenous USP11 acts to deubiquitinate BRCA2 in response to MMC treatment. 293T cells were cotransfected in duplicate with HA-ubiquitin and either a control shRNA vector or an shRNA vector targeting USP11. One set of each was incubated with MMC. Endogenous BRCA2 then was immunoprecipitated from all cells and analyzed for ubiquitination (Fig. 6D). While endogenous USP11 levels were increased in response to MMC treatment, shRNA targeting USP11 markedly decreased the expression of USP11 in both untreated and MMC-treated cells. However, USP11 levels appeared to have no influence on either the MMC-induced increase in BRCA2 ubiquitination or the associated decrease in cellular BRCA2 levels. These results further support the conclusion that endogenous USP11 does not deubiquitinate BRCA2 in response to MMC. Instead, these findings indicate that MMC treatment causes increased BRCA2 ubiquitination in a manner independent of USP11. Thus, USP11 prosurvival functions, although shown to be BRCA2 dependent, appear to be mediated through a USP11 substrate other than BRCA2.

The increased ubiquitination and decreased cellular levels of BRCA2 observed after MMC treatment were suggestive of proteasomal BRCA2 degradation. To address this possibility, we transfected 293T cells with HA-ubiquitin and incubated MMC-treated cells in the absence or presence of the proteasomal inhibitor MG132. Endogenous BRCA2 was immunoprecipitated and analyzed for ubiquitination. As previously observed in Fig. 6D, MMC treatment led to increased BRCA2 ubiquitination (Fig. 6E). Moreover, proteasomal inhibition caused a significant additional increase in the amount of ubiquitinated BRCA2 detected (Fig. 6E). These results are consistent with the conclusion that BRCA2 undergoes proteasome-mediated degradation in response to MMC-induced DNA damage.

DISCUSSION

The BRCA2 protein has been proposed to function in the repair of DNA double-strand breaks by homologous recombination (37). BRCA2 binds to Rad51 (9, 26, 37, 48) and can influence Rad51 recombinational activities (11). BRCA2-deficient cells are sensitive to DNA-damaging agents (9, 32, 37, 52), show genomic instability (32, 45, 52), and are deficient in homology-directed DNA repair (28, 44). Recent reports have shown that BRCA2 has DNA binding capabilities (51) and that specific BRCA2 mutations are responsible for the D1 subtype of Fanconi anemia (19), further supporting a role in the repair of DNA damage.

In the present studies, we identified USP11, a deubiquitinating enzyme initially cloned as a candidate gene for X-linked retinal disorders (39), as a component of in vivo complexes with an exogenously expressed C-terminal fragment of BRCA2. This interaction was shown to be specific, in that USP11 was not detected in complexes with another exogenously expressed BRCA2 fragment or with the GFP moiety used as a tag. We demonstrated stable complexes involving endogenous USP11 and wild-type BRCA2, although the nature of these interactions, whether direct or mediated by other members of a multiprotein complex, remains to be resolved. Consistent with the functional interaction, USP11 was shown to localize to the nucleus, the same subcellular compartment where BRCA2 is known to function (3).

A majority of BRCA2-inactivating mutations lead to C-terminal truncations, which not only impair BRCA2 nuclear localization (38, 40) but also would inhibit its ability to form complexes with USP11. Mouse cells containing a targeted C-terminal truncation of BRCA2 have been shown to be hypersensitive to MMC (52), as are Fanconi anemia subtype D1 cells, which harbor biallelic truncating mutations in BRCA2 (19, 35). We showed that overexpression of catalytically inactive USP11 or reduction of USP11 levels via RNA interference increased cellular sensitivity to MMC. Moreover, these effects were only seen in cells containing wild-type BRCA2, indicating that USP11 prosurvival effects in the cellular response to MMC-induced DNA damage were dependent on the BRCA2 pathway.

We demonstrated that the BRCA2 protein is ubiquitinated in vivo and that overexpressed USP11 can deubiquitinate BRCA2. However, BRCA2 was ubiquitinated at physiologic levels of BRCA2 and USP11, and its ubiquitination level was not increased as a result of USP11 antagonism either by the overexpression of mutant USP11 or by a targeted decrease in USP11 levels. These findings suggest that BRCA2 is not a physiologic substrate of USP11 and that BRCA2 ubiquitination observed as a consequence of USP11 overexpression likely relates to the ability of USP11 to form complexes with BRCA2. Instead, our results are consistent with the concept that USP11 functions in the BRCA2 pathway through its effects on another substrate and that BRCA2 may play a role in recruiting USP11 to its physiologic substrate.

USP11 was previously isolated in a yeast two-hybrid screen with the Ran binding protein (RanBPM) and was shown to catalyze the deubiquitination of RanBPM in vitro (20). In addition to roles at the nuclear pore complex, Ran influences the process of chromatin condensation and is required for coordinating the onset of mitosis with S-phase completion in mammalian cells (27). Targeting of Ran by viral oncoproteins results in centrosome amplification, a hallmark of tumor cells (21) that is also observed in BRCA2-deficient mouse embryo fibroblasts (45). The overexpression of a fragment of RanBPM, RanBP2, inhibited the formation of Rad51 foci (34), another BRCA2-dependent cellular event associated with DNA damage repair (15, 53). Whether USP11 interactions with the Ran pathway or with other, as-yet-unidentified targets underlie its functions within the BRCA2 tumor suppressor network remains to be elucidated.

A number of proteins involved in DNA damage repair, including FancD2, PCNA, BRCA1, and histone H2AX, have been shown to be ubiquitinated (7, 13, 17). Moreover, for FancD2 and PCNA, ubiquitination has been linked with activation and/or regulation of their DNA damage repair functions (13, 17). We observed that under physiologic conditions, BRCA2 was constitutively ubiquitinated without detectable evidence of proteasomal degradation. Previous studies have indicated that monoubiquitination or Lys-63 polyubiquitination may serve as a subcellular trafficking signal or as a binding site or recognition domain for other interacting proteins (reviewed in reference 36). Lys-63 polyubiquitination, as observed with PCNA (17), is conjugated by RAD6 and the MMS2-UBC13 heterodimer and plays a role in the error-free branch of postreplicative DNA repair (18). Of note, the BRCA1-BARD1 heterodimer contains E3 ubiquitin ligase activity and was recently shown to catalyze a Lys-6 polyubiquitin linkage (23, 30, 50). Since BRCA1 is found in a cellular complex with BRCA2 (8, 12), it is possible that BRCA1-BARD1 mediates Lys-6 polyubiquitination of BRCA2 in untreated cells, although the nature of BRCA2 ubiquitination under these conditions remains to be resolved.

BRCA2 ubiquitinated in vivo following MMC exposure was shown to exhibit a different fate. We observed increased BRCA2 ubiquitination associated with decreased BRCA2 levels, and proteasome inhibition further increased BRCA2 ubiquitination. All of these findings imply that BRCA2 ubiquitination under these conditions is associated with proteasomal degradation. BRCA2 in MMC-treated cells presumably contains Lys-48 polyubiquitin, since Lys-48 polyubiquitination is known to be a signal for proteasome-dependent degradation (16). Previous studies indicated that BRCA2 is down-regulated at the mRNA level in response to UV irradiation or adriamycin (1), and UV radiation was shown to cause depletion of the BRCA2 protein by a nonproteasomal mechanism (46). These findings imply that distinct mechanisms may exist for BRCA2 down-regulation in response to different types of genotoxic stress. It remains to be established whether the increased ubiquitination and subsequent degradation of BRCA2 in response to MMC reflect its coupled activation and degradation or whether the removal of BRCA2 is necessary for the cell to respond to MMC-induced DNA damage. In either case, our findings that BRCA2 is ubiquitinated in vivo and undergoes proteasomal degradation in response to MMC treatment, in concert with the emerging role of ubiquitination in DNA damage repair, provide new insights into posttranslational modifications that may regulate the function of this important tumor suppressor protein.

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REFERENCES

1. **Andres, J. L., S. Fan, G. J. Turkel, J. A. Wang, N. F. Twu, R. Q. Yuan, K. Lamszus, I. D. Goldberg, and E. M. Rosen.** 1998. Regulation of BRCA1 and BRCA2 expression in human breast cancer cells by DNA-damaging agents. Oncogene **16:**2229–2241.
2. **Baker, R. T., J. W. Tobias, and A. Varshavsky.** 1992. Ubiquitin-specific proteases of Saccharomyces cerevisiae. Cloning of UBP2 and UBP3, and functional analysis of the UBP gene family. J. Biol. Chem. **267:**23364–23375.
3. **Bertwistle, D., S. Swift, N. J. Marston, L. E. Jackson, S. Crossland, M. R. Crompton, C. J. Marshall, and A. Ashworth.** 1997. Nuclear location and cell cycle regulation of the BRCA2 protein. Cancer Res. **57:**5485–5488.
4. **Brummelkamp, T. R., R. Bernards, and R. Agami.** 2002. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cells **2:**243–247.
5. **Brummelkamp, T. R., R. Bernards, and R. Agami.** 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science **296:**550–553.
6. **Cepko, C. L.** 1996. Transient transfection methods for preparation of high-titer retroviral supernatants, p. 9.10.5–9.10.6. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, K. Struhl, and K. Janssen (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
7. **Chen, A., F. E. Kleiman, J. L. Manley, T. Ouchi, and Z. Q. Pan.** 2002. Autoubiquitination of the BRCA1*BARD1 RING ubiquitin ligase. J. Biol. Chem. **277:**22085–22092.
8. **Chen, J., D. P. Silver, D. Walpita, S. B. Cantor, A. F. Gazdar, G. Tomlinson, F. J. Couch, B. L. Weber, T. Ashley, D. M. Livingston, and R. Scully.** 1998. Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. Mol. Cell **2:**317–328.
9. **Chen, P. L., C. F. Chen, Y. Chen, J. Xiao, Z. D. Sharp, and W. H. Lee.** 1998. The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment. Proc. Natl. Acad. Sci. USA **95:**5287–5292.
10. **Collins, N., R. McManus, R. Wooster, J. Mangion, S. Seal, S. R. Lakhani, W. Ormiston, P. A. Daly, D. Ford, D. F. Easton, et al.** 1995. Consistent loss of the wild type allele in breast cancers from a family linked to the BRCA2 gene on chromosome 13q12–13. Oncogene **10:**1673–1675.
11. **Davies, A. A., J. Y. Masson, M. J. McIlwraith, A. Z. Stasiak, A. Stasiak, A. R. Venkitaraman, and S. C. West.** 2001. Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. Mol. Cell **7:**273–282.
12. **Dong, Y., M. A. Hakimi, X. Chen, E. Kumaraswamy, N. S. Cooch, A. K. Godwin, and R. Shiekhattar.** 2003. Regulation of BRCC, a holoenzyme complex containing BRCA1 and BRCA2, by a signalosome-like subunit and its role in DNA repair. Mol. Cell **12:**1087–1099.
13. **Garcia-Higuera, I., T. Taniguchi, S. Ganesan, M. S. Meyn, C. Timmers, J. Hejna, M. Grompe, and A. D. D'Andrea.** 2001. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol. Cell **7:**249–262.
14. **Goggins, M., M. Schutte, J. Lu, C. A. Moskaluk, C. L. Weinstein, G. M. Petersen, C. J. Yeo, C. E. Jackson, H. T. Lynch, R. H. Hruban, and S. E. Kern.** 1996. Germline BRCA2 gene mutations in patients with apparently sporadic pancreatic carcinomas. Cancer Res. **56:**5360–5364.
15. **Haaf, T., E. I. Golub, G. Reddy, C. M. Radding, and D. C. Ward.** 1995. Nuclear foci of mammalian Rad51 recombination protein in somatic cells after DNA damage and its localization in synaptonemal complexes. Proc. Natl. Acad. Sci. USA **92:**2298–2302.
16. **Hochstrasser, M.** 1996. Protein degradation or regulation: Ub the judge. Cell **84:**813–815.
17. **Hoege, C., B. Pfander, G. L. Moldovan, G. Pyrowolakis, and S. Jentsch.** 2002. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature **419:**135–141.
18. **Hofmann, R. M., and C. M. Pickart.** 1999. Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell **96:**645–653.
19. **Howlett, N. G., T. Taniguchi, S. Olson, B. Cox, Q. Waisfisz, C. De Die-Smulders, N. Persky, M. Grompe, H. Joenje, G. Pals, H. Ikeda, E. A. Fox, and A. D. D'Andrea.** 2002. Biallelic inactivation of BRCA2 in Fanconi anemia. Science **297:**606–609.
20. **Ideguchi, H., A. Ueda, M. Tanaka, J. Yang, T. Tsuji, S. Ohno, E. Hagiwara, A. Aoki, and Y. Ishigatsubo.** 2002. Structural and functional characterization of the USP11 deubiquitinating enzyme, which interacts with the RanGTP-associated protein RanBPM. Biochem. J. **367:**87–95.
21. **Lavia, P., A. M. Mileo, A. Giordano, and M. G. Paggi.** 2003. Emerging roles of DNA tumor viruses in cell proliferation: new insights into genomic instability. Oncogene **22:**6508–6516.
22. **Li, M., D. Chen, A. Shiloh, J. Luo, A. Y. Nikolaev, J. Qin, and W. Gu.** 2002. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature **416:**648–653.
23. **Lorick, K. L., J. P. Jensen, S. Fang, A. M. Ong, S. Hatakeyama, and A. M. Weissman.** 1999. RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. Proc. Natl. Acad. Sci. USA **96:**11364–11369.
24. **Marmorstein, L. Y., T. Ouchi, and S. A. Aaronson.** 1998. The BRCA2 gene product functionally interacts with p53 and RAD51. Proc. Natl. Acad. Sci. USA **95:**13869–13874.
25. **McAllister, K. A., A. Haugen-Strano, S. Hagevik, H. A. Brownlee, N. K. Collins, P. A. Futreal, L. M. Bennett, and R. W. Wiseman.** 1997. Characterization of the rat and mouse homologues of the BRCA2 breast cancer susceptibility gene. Cancer Res. **57:**3121–3125.
26. **Mizuta, R., J. M. LaSalle, H. L. Cheng, A. Shinohara, H. Ogawa, N. Cope-**

land, N. A. Jenkins, M. Lalande, and F. W. Alt. 1997. RAB22 and RAB163/mouse BRCA2: proteins that specifically interact with the RAD51 protein. Proc. Natl. Acad. Sci. USA **94:**6927–6932.

27. **Moore, J. D.** 2001. The Ran-GTPase and cell-cycle control. Bioessays **23:** 77–85.

27a. **Morgenstern, J. P., and H. Land.** 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. **18:**3587–3596.

28. **Moynahan, M. E., A. J. Pierce, and M. Jasin.** 2001. BRCA2 is required for homology-directed repair of chromosomal breaks. Mol. Cell **7:**263–272.

29. **Naviaux, R. K., E. Costanzi, M. Haas, and I. M. Verma.** 1996. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. J. Virol. **70:**5701–5705.

30. **Nishikawa, H., S. Ooka, K. Sato, K. Arima, J. Okamoto, R. E. Klevit, M. Fukuda, and T. Ohta.** 2004. Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase. J. Biol. Chem. **279:**3916–3924.

31. **Papa, F. R., and M. Hochstrasser.** 1993. The yeast DOA4 gene encodes a deubiquitinating enzyme related to a product of the human tre-2 oncogene. Nature **366:**313–319.

32. **Patel, K. J., V. P. Yu, H. Lee, A. Corcoran, F. C. Thistlethwaite, M. J. Evans, W. H. Colledge, L. S. Friedman, B. A. Ponder, and A. R. Venkitaraman.** 1998. Involvement of Brca2 in DNA repair. Mol. Cell **1:**347–357.

33. **Rock, K. L., C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, and A. L. Goldberg.** 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell **78:**761–771.

34. **Saitoh, H., M. D. Pizzi, and J. Wang.** 2002. Perturbation of SUMOlation enzyme Ubc9 by distinct domain within nucleoporin RanBP2/Nup358. J. Biol. Chem. **277:**4755–4763.

35. **Sasaki, M. S., and A. Tonomura.** 1973. A high susceptibility of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. Cancer Res. **33:**1829–1836.

36. **Schwartz, D. C., and M. Hochstrasser.** 2003. A superfamily of protein tags: ubiquitin, SUMO and related modifiers. Trends Biochem. Sci. **28:**321–328.

37. **Sharan, S. K., M. Morimatsu, U. Albrecht, D. S. Lim, E. Regel, C. Dinh, A. Sands, G. Eichele, P. Hasty, and A. Bradley.** 1997. Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. Nature **386:**804–810.

38. **Spain, B. H., C. J. Larson, L. S. Shihabuddin, F. H. Gage, and I. M. Verma.** 1999. Truncated BRCA2 is cytoplasmic: implications for cancer-linked mutations. Proc. Natl. Acad. Sci. USA **96:**13920–13925.

39. **Swanson, D. A., C. L. Freund, L. Ploder, R. R. McInnes, and D. Valle.** 1996. A ubiquitin C-terminal hydrolase gene on the proximal short arm of the X chromosome: implications for X-linked retinal disorders. Hum. Mol. Genet. **5:**533–538.

40. **Szabo, C., A. Masiello, J. F. Ryan, and L. C. Brody.** 2000. The breast cancer information core: database design, structure, and scope. Hum. Mutat. **16:** 123–131.

41. **Takata, M., S. Tachiiri, A. Fujimori, L. H. Thompson, Y. Miki, M. Hiraoka, S. Takeda, and M. Yamazoe.** 2002. Conserved domains in the chicken homologue of BRCA2. Oncogene **21:**1130–1134.

42. **Tavtigian, S. V., J. Simard, J. Rommens, F. Couch, D. Shattuck-Eidens, S. Neuhausen, S. Merajver, S. Thorlacius, K. Offit, D. Stoppa-Lyonnet, C. Belanger, R. Bell, S. Berry, R. Bogden, Q. Chen, T. Davis, M. Dumont, C. Frye, T. Hattier, S. Jammulapati, T. Janecki, P. Jiang, R. Kehrer, J. F. Leblanc, D. E. Goldgar, et al.** 1996. The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds. Nat. Genet. **12:**333–337.

43. **Treier, M., L. M. Staszewski, and D. Bohmann.** 1994. Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. Cell **78:**787–798.

44. **Tutt, A., D. Bertwistle, J. Valentine, A. Gabriel, S. Swift, G. Ross, C. Griffin, J. Thacker, and A. Ashworth.** 2001. Mutation in Brca2 stimulates error-prone homology-directed repair of DNA double-strand breaks occurring between repeated sequences. EMBO J. **20:**4704–4716.

45. **Tutt, A., A. Gabriel, D. Bertwistle, F. Connor, H. Paterson, J. Peacock, G. Ross, and A. Ashworth.** 1999. Absence of Brca2 causes genome instability by chromosome breakage and loss associated with centrosome amplification. Curr. Biol. **9:**1107–1110.

46. **Wang, S. C., K. Makino, L. K. Su, A. Y. Pao, J. S. Kim, and M. C. Hung.** 2001. Ultraviolet irradiation induces BRCA2 protein depletion through a p53-independent and protein synthesis-dependent pathway. Cancer Res. **61:** 2838–2842.

47. **Wilkinson, K. D., V. L. Tashayev, L. B. O'Connor, C. N. Larsen, E. Kasperek, and C. M. Pickart.** 1995. Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. Biochemistry **34:** 14535–14546.

48. **Wong, A. K. C., R. Pero, P. A. Ormonde, S. V. Tavtigian, and P. L. Bartel.** 1997. RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. J. Biol. Chem. **272:**31941–31944.

49. **Wooster, R., G. Bignell, J. Lancaster, S. Swift, S. Seal, J. Mangion, N. Collins, S. Gregory, C. Gumbs, and G. Micklem.** 1995. Identification of the breast cancer susceptibility gene BRCA2. Nature **378:**789–792.

50. **Wu-Baer, F., K. Lagrazon, W. Yuan, and R. Baer.** 2003. The BRCA1/BARD1 heterodimer assembles polyubiquitin chains through an unconventional linkage involving lysine residue K6 of ubiquitin. J. Biol. Chem. **278:** 34743–34746.

51. **Yang, H., P. D. Jeffrey, J. Miller, E. Kinnucan, Y. Sun, N. H. Thoma, N. Zheng, P. L. Chen, W. H. Lee, and N. P. Pavletich.** 2002. BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure. Science **297:**1837–1848.

52. **Yu, V. P., M. Koehler, C. Steinlein, M. Schmid, L. A. Hanakahi, A. J. van Gool, S. C. West, and A. R. Venkitaraman.** 2000. Gross chromosomal rearrangements and genetic exchange between nonhomologous chromosomes following BRCA2 inactivation. Genes Dev. **14:**1400–1406.

53. **Yuan, S. S., S. Y. Lee, G. Chen, M. Song, G. E. Tomlinson, and E. Y. Lee.** 1999. BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex in vivo. Cancer Res. **59:**3547–3551.