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BRCA1 contributes to transcription-coupled repair of DNA damage through polyubiquitination and degradation of Cockayne syndrome B protein

Leizhen Wei,[1,2,8] Li Lan,[3] Akira Yasui,[3,4] Kiyoji Tanaka,[5] Masafumi Saijo,[5] Ayako Matsuzawa,[1] Risa Kashiwagi,[1] Emiko Maseki,[1] Yiheng Hu,[6] Jeffrey D. Parvin,[6] Chikashi Ishioka[2] and Natsuko Chiba[1,7]

Departments of [1]Molecular Immunology, [2]Clinical Oncology and [3]Molecular Genetics, and [4]Division of Dynamic Proteome in Aging and Cancer, Institute of Development, Aging and Cancer, Tohoku University, Sendai; [5]Human Cell Biology Group, Graduate School of Frontier Biosciences, Osaka University, Osaka; [6]Department of Biomedical Informatics and the Ohio State University Comprehensive Cancer Center, Ohio State University, Columbus, Ohio, USA

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BRCA1 **is an important gene involved in susceptibility to breast and ovarian cancer and its product regulates the cellular response to DNA double-strand breaks. Here, we present evidence that BRCA1 also contributes to the transcription-coupled repair (TCR) of ultraviolet (UV) light-induced DNA damage. BRCA1 immediately accumulates at the sites of UV irradiation-mediated damage in cell nuclei in a manner that is fully dependent on both Cockayne syndrome B (CSB) protein and active transcription. Suppression of BRCA1 expression inhibits the TCR of UV lesions and increases the UV sensitivity of cells proficient in TCR. BRCA1 physically interacts with CSB protein. BRCA1 polyubiquitinates CSB and this polyubiquitination and subsequent degradation of CSB occur following UV irradiation, even in the absence of Cockayne syndrome A (CSA) protein. The depletion of BRCA1 expression increases the UV sensitivity of CSA-deficient cells. These results indicate that BRCA1 is involved in TCR and that a BRCA1-dependent polyubiquitination pathway for CSB exists alongside the CSA-dependent pathway to yield more efficient excision repair of lesions on the transcribed DNA strand. (*Cancer Sci* 2011; 102: 1840–1847)**

BRCA1 is an important breast and ovarian cancer susceptibility gene.[1,2] *BRCA1* mutations are rare in sporadic breast and ovarian cancers[3,4] and its expression in these cancers is often reduced,[5] suggesting that BRCA1 plays a role in both hereditary and sporadic carcinogenesis. BRCA1 contains a RING domain at the amino (N)-terminus and two BRCA1 carboxy-terminal (BRCT) domains at the carboxy (C)-terminus. RING domain is an essential component of many ubiquitine E3 ligase. BRCA1 associates with BARD1, which also has a RING domain,[6] and the BRCA1/BARD1 heterodimer has ubiquitin ligase activity.[7–9]

BRCA1 has been implicated in a variety of biological processes, including DNA repair, transcription, chromatin remodeling and centrosome duplication.[10] BRCA1 localizes to nuclear foci during S-phase of the cell cycle.[11] Various mediators of DNA damage such as ultraviolet (UV) irradiation disperse the BRCA1 foci, followed by the reappearance of BRCA1 foci.[12] BRCA1 is phosphorylated in response to UV-induced damage.[13] BRCA1 associates with RNA polymerase II (RNAPII)[14] and mediates the ubiquitination of RNAPII following UV irradiation.[15–17]

The main type of DNA damage induced by UV irradiation is the formation of cyclobutane pyrimidine dimers (CPD) and (6-4) photoproduct adducts. These lesions are removed by nucleotide excision repair (NER). The NER operates via two pathways: transcription-coupled repair (TCR) and global genome repair (GGR). The TCR efficiently removes DNA lesions on the transcribed strands of transcriptionally active genes, whereas the GGR repairs DNA lesions throughout the genome. A stalled RNAPII is presumed to trigger the initiation of TCR in harmony with Cockayne syndrome (CS) proteins. Xeroderma pigmentosum (XP) and CS are rare genetic disorders. Xeroderma pigmentosum is characterized by a high incidence of skin cancer and CS is characterized by photosensitivity and neurodevelopmental abnormalities.[18,19] There are seven genes (*XPA–G*) involved in XP and two genes (*CSA* and *CSB*) involved in CS.[18,20] Mutations in *XPA–G* result in defects in both GGR and TCR, with the exception of *XPC* and *XPE*, which are defective in GGR alone. Patients with CS have defects in TCR, but have functional GGR.

Although BRCA1 is known to function in the repair of DNA double-strand breaks (DSB),[21] there have been several reports suggesting roles for BRCA1 in the excision repair of DNA damage. BRCA1 has been reported to function in NER of oxidative DNA damage[22] and in TCR.[23,24] BRCA1-deficient cells are defective with respect to the preferential removal of oxidative base damage from the transcribed DNA strand.[25] These suggest that BRCA1 participates in the TCR pathway. *BRCA1* mutations or reduced expression of BRCA1 might result in the deficiency of TCR, in addition to DSB repair, and cause an increase in cancer risk and contribute to carcinogenesis.

The aim of the present study was to gain insight into the mechanisms involved in the BRCA1-mediated regulation of TCR. Small, restricted areas of cell nuclei were exposed to UV irradiation using an isopore membrane filter and BRCA1 localization was analyzed. The results showed the immediate, Cockayne syndrome B (CSB)-dependent accumulation of BRCA1 at the UV-irradiated sites. A suggested mechanism for BRCA1 function in TCR is also presented.

Materials and Methods

Plasmid construction. pCMV-Myc-ubiquitin and pcDNA3-HA-BRCA1 have been described previously.[15,26] pcDNA3-HA-BRCA1-I26A was generated by site-directed mutagenesis.

Cell lines and transfections. Saos-2, HEK-293T, XP3BRSV, XP12ROSV, XP4PASV, CS3BESV, UV^{s}1KOSV and HA-CSB/UV^{s}1KOSV cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HEK-293T cells were transfected with the vectors using Fugene-6 (Roche, Mannheim, Germany).

Localized UV irradiation. Localized UV irradiation was delivered as previously described.[27] Cells were cultured as

[7]To whom correspondence should be addressed.
E-mail: nchiba@idac.tohoku.ac.jp
[8]Present address: Genome Structure and Stability Group, Beijing Institute of Genomics (BIG), No. 7, Beitucheng West Road, Chaoyang District, Beijing 100029, China.

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monolayers in 35-mm glass-bottomed dishes (Matsunami Glass, Osaka, Japan), covered with a polycarbonate isopore membrane filter containing pores 3 μm in diameter (Millipore, Billerica, MA, USA), and exposed to 254-nm UV irradiation at a dose of 40 J/m^2.

Immunocytochemistry. Immunocytochemistry was carried out as previously described.[28] An anti-CPD antibody (Medical & Biological Laboratories, Nagoya, Japan) and an anti-BARD1 antibody (H-300; Santa-Cruz Biotechnology, Santa Cruz, CA, USA), a polyclonal anti-BRCA1 antibody specific for residues 397–1080 of BRCA1, or an anti-BRCA1 antibody (C-20; Santa-Cruz Biotechnology) were used.

Small interfering RNA (siRNA). A siRNA targeting BRCA1 was synthesized using a Silencer siRNA construction Kit (Ambion, Austin, TX, USA). The siRNA sequence was 5′-AAGGUUUCAAAGCGCCAGUCA-3′.[29] The Silencer negative control siRNA (Ambion) was used as a negative control. Cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA).

Immunoprecipitation and western blot. Immunoprecipitation (IP) was carried out as previously described.[28] Total cell lysates were prepared from CS3BESV cells in 1 × SDS sample buffer (2% SDS, 0.67 M 2-Mercaptoethanol, 50 mM Tris–HCl pH 6.8, 12% glycerol, 1% Bromphenol Blue), sonicated and incubated at 95°C for 10 min. Samples were subjected to electrophoresis in SDS-polyacrylamide gels and immunoblotted using anti-BRCA1, anti-CSB (Santa-Cruz Biotechnology), anti-BARD1 or anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA) as indicated.

Colony formation assay. Cells were transfected with control or BRCA1 siRNA. Forty-eight hours after transfection, the cells were replated. Eight hours later, the cells were exposed to UV irradiation and incubated for 10 days. Colonies were stained with 0.3% crystal violet, and the number of colonies was counted and expressed as a percentage of the non-irradiated colonies as a measure of survival.

Analysis of strand-specific DNA repair. The repair of CPD was examined in the 17.9-kb *Kpn*I fragment within the dihydrofolate reductase (DHFR) gene in XP4PASV cells transfected with control or BRCA1 siRNA and irradiated with 8 J/m^2 using a method previously described.[30] Briefly, DNA was extracted, digested with *Kpn*I and treated with T4 endonuclease V, which generates single-strand breaks at CPD sites. The samples were separated by electrophoresis in 0.65% alkaline agarose gels, transferred onto Hybond N$^+$ membranes (Amersham Biosciences, Little Chalfont, Bucks, UK), and hybridized with strand-specific digoxigenin (DIG)-labeled DNA probes. The strand-specific probes were generated by linear PCR in the presence of DIG-11-dUTP using a PCR DIG Probe Synthesis Kit (Roche). Hybridization with DIG-labeled strand-specific probes was detected using a DIG Detection Kit (Roche).

***In vitro* ubiquitination assay.** Reaction mixtures contained 10 mM HEPES (pH 7.6), 0.5 mM EDTA, 5 mM $MgCl_2$, 2 mM NaF, 2 mM ATP, 60 mM KCl, 14 μM ubiquitin (Sigma), 36 nM E1, 12 μM UbcH5c-His, 10 or 20 nM BRCA1-FLAG/BARD1 and 24 nM CSB. The preparation of E1, UbcH5c-His and BRCA1-FLAG/BARD1 has been described previously.[31] CSB was prepared as described previously.[32] After incubation at 37°C for 1 h, CSB modifications were analyzed using western blotting.

Results

BRCA1 accumulates at UV-irradiated sites. To analyze the response of BRCA1 to UV irradiation, cells covered with an isopore membrane filter were irradiated to generate localized UV damage to the cell nuclei.[27,33] Saos-2 cells were exposed to localized UV irradiation and analyzed by co-immunostaining with antibodies against CPD and BRCA1. Five and 30 min after UV irradiation, BRCA1 was distributed as fine nuclear dots that co-localized with CPD (Fig. 1a).

BRCA1 localizes to nuclear foci within a few hours after UV irradiation, which was explained by the response of BRCA1 to the DSB formed at the sites of stalled replication forks in S-phase.[12] However, in asynchronous cells, BRCA1 accumulation at UV-irradiated sites was observed in almost all cells. To exclude the possibility that we were observing BRCA1 accumulation at UV-induced DSB in S-phase, cells were synchronized in G0/G1. BRCA1 clearly accumulated at the UV-irradiated sites in cells at G0/G1 (Fig. S1a,b). In addition, we analyzed whether phosphorylated H2AX (γH2AX), which is rapidly phosphorylated at DSB, was observed at irradiated sites. Even at a higher dose of 100 J/m^2, no γH2AX was detected at UV-irradiated sites 10 min after irradiation (Fig. S1c). These indicate that DSB are not induced immediately after UV irradiation under these experimental conditions. Therefore, it can be concluded that DSB do not induce immediate BRCA1 accumulation after local UV irradiation.

BRCA1 accumulation at UV-irradiated sites is dependent on CSB. To determine whether BRCA1 accumulation at UV-irradiated sites depends on NER factors, the response of BRCA1 to UV irradiation in several NER-deficient cell lines was examined. The cell lines used were: patient-derived *XPG*-deficient XP3BRSV cells, *XPA*-deficient XP12ROSV cells, *XPC*-deficient XP4PASV cells, *CSA*-deficient CS3BESV cells and *CSB*-deficient UV^{s}1KOSV cells. XPC is involved in the damage recognition step of GGR, whereas CSA and CSB function only in TCR. XPA and XPG are required for both GGR and TCR, and function downstream of XPC, CSA and CSB (Fig. 1b). BRCA1 accumulated at UV-irradiated sites in almost all *XPG*-, *XPA*-, *XPC*- and *CSA*-deficient cells as observed in Saos-2 cells, but not in *CSB*-deficient cells (Fig. 1c). Accumulation of BRCA1 at UV-irradiated sites was observed in a stable transfectant of UV^{s}1KOSV cells expressing full-length hemagglutinin (HA)-tagged CSB (HA-CSB/UV^{s}1KOSV).[34] The localization of BRCA1 following irradiation was also examined in mouse embryonic fibroblasts (MEF). BRCA1 accumulated at UV-irradiated sites in *CSA*-deficient (*CSA*−/−) 6L1030 cells, but not in *CSB*-deficient (*XPA*+/− *CSB*−/−) cells (Fig. S2). This suggests that BRCA1 accumulation at UV-irradiated sites is dependent on CSB.

Inhibition of transcription abolishes BRCA1 accumulation at UV-irradiated sites. Cockayne syndrome B plays an important role in the initiation step of TCR through recognition of a stalled RNAPII.[35] To investigate whether the response of BRCA1 is dependent on active transcription, Saos-2 cells were treated with actinomycin D or α-amanitin prior to UV irradiation. Treatment with either chemical completely abolished the accumulation of BRCA1 at the sites of irradiation (Fig. 1d). Although the expression of BRCA1 was slightly decreased by the treatment with actinomycin D, expression of BRCA1 was clearly detected in cells treated with these transcription inhibitors by western blotting (Fig. S3). Thus, the accumulation of BRCA1 at the UV-irradiated sites is dependent on transcription.

Depletion of BRCA1 impairs TCR but not GGR. To determine whether the loss of BRCA1 expression affects TCR, the UV sensitivity of GGR- and TCR-deficient cells transfected with BRCA1 siRNA was examined. In *XPC*-deficient XP4PASV cells, DNA lesions are repaired by TCR, but not by GGR, whereas in *CSB*-deficient UV^{s}1KOSV cells only the GGR pathway is functional. BRCA1 siRNA efficiently suppressed the expression of BRCA1 in these cells (Fig. 2a). Cells were irradiated with varying doses of UV and their ability to form colonies was assessed (Fig. 2b). When BRCA1 expression was reduced, XP4PASV cells were more sensitive to UV irradiation. By contrast, BRCA1 knockdown did not appear to affect the UV sensitivity of UV^{s}1KOSV cells.

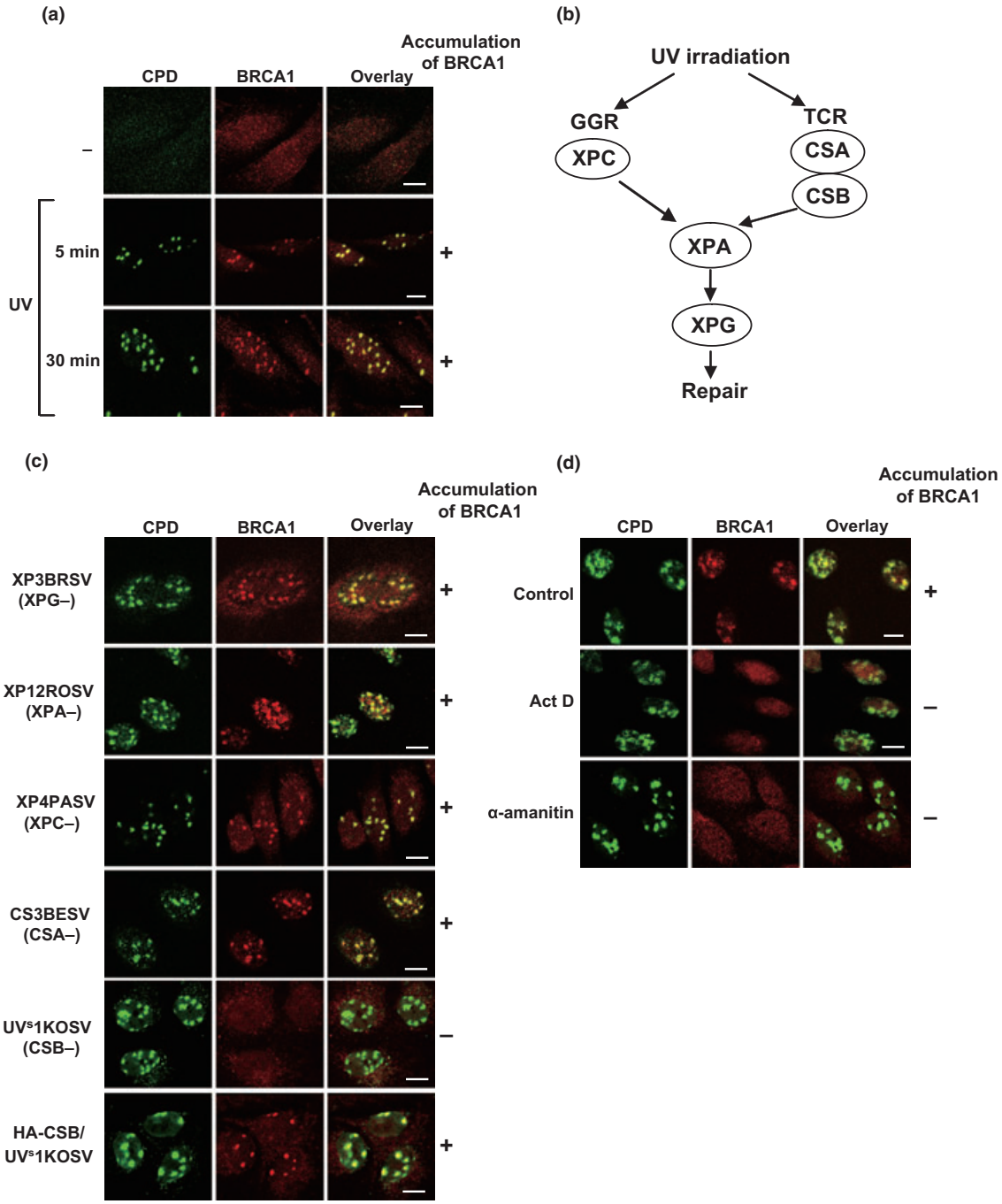

Fig. 1. BRCA1 accumulation at ultraviolet (UV)-irradiated sites is dependent on Cockayne syndrome B (CSB). (a) Saos-2 cells were fixed at the indicated time points after localized UV irradiation and stained with anti-cyclobutane pyrimidine dimers (CPD) and anti-BRCA1 antibodies. (b) Schematic of nucleotide excision repair (NER). (c) XP3BRSV *XPG−/−*, XP12ROSV *XPA−/−*, XP4PASV *XPC−/−*, CS3BESV *CSA−/−*, UV^{s}1KOSV *CSB−/−* and HA-CSB/UV^{s}1KOSV cells were fixed 30 min after UV irradiation and then stained. (d) Saos-2 cells were treated with actinomycin D (Act D) (10 μg/mL) or α-amanitin (100 μg/mL) for 1 h and then exposed to UV irradiation. Scale bars, 10 μm. CSA, Cockayne syndrome A; GGR, global genome repair; TCR, transcription-coupled repair.

Strand-specific DNA probes were also used to examine the removal of CPD from the transcribed and non-transcribed strands of the active DHFR gene in XP4PASV cells transfected with control or BRCA1 siRNA and irradiated. Knockdown of BRCA1 expression reduced the efficiency of CPD removal from the transcribed strand 6 h after UV irradiation (Fig. 2c). This suggests that BRCA1 is important for efficient TCR and mediates resistance to the UV lesion.

Association between BRCA1 and CSB is accompanied by polyubiquitination. To obtain molecular insights into the role of BRCA1 in TCR, the association of BRCA1 with CSB was assessed. HEK-293T cells were UV irradiated and cell extracts were prepared 1 h after exposure. The extracts were then immunoprecipitated with a control IgG or anti-CSB antibody. BRCA1 co-precipitated with CSB (Fig. 3a). In the anti-CSB immune complexes, both BRCA1 and CSB were present as diffuse, slowly migrating bands. Although BARD1 was also detected in anti-CSB immune complexes, it did not show a diffuse pattern. BARD1 accumulated at the UV-irradiated sites (Fig. S4).

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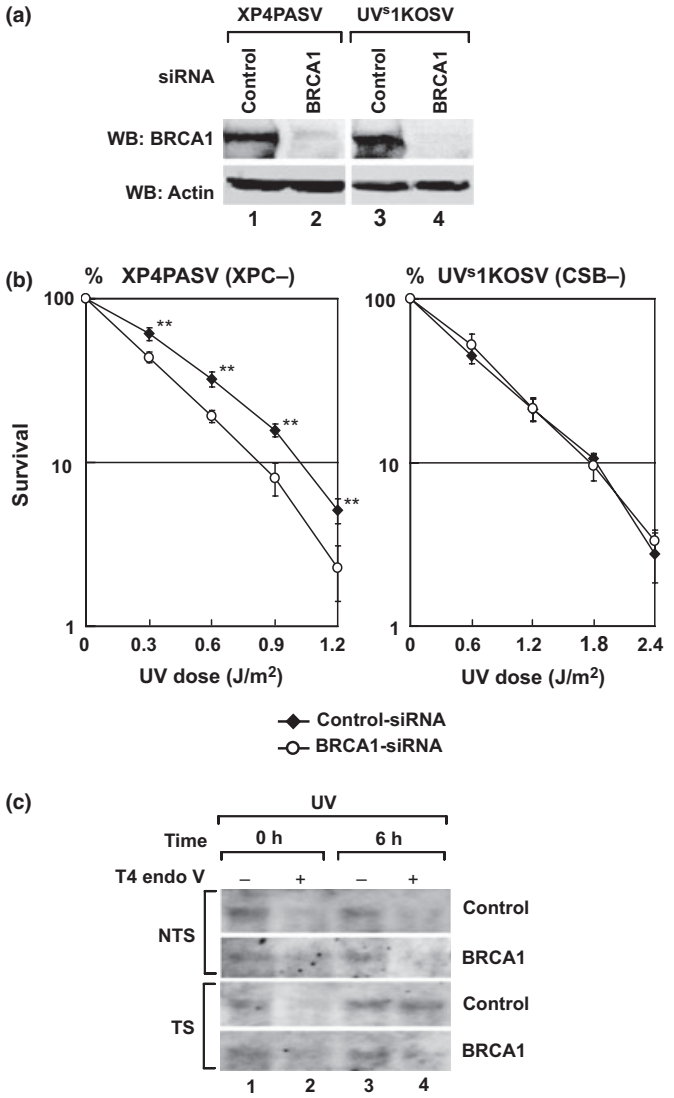

Fig. 2. siRNA knockdown of BRCA1 impairs transcription-coupled repair (TCR). (a) XP4PASV and UV^{s}1KOSV cells were transfected with control or BRCA1 siRNA. Cell lysates analyzed using western blot with anti-BRCA1 and anti-β-actin antibodies. (b) Colony formation assay for XP4PASV and UV^{s}1KOSV cells. XP4PASV and UV^{s}1KOSV cells were transfected with control or BRCA1 siRNA. Data represent the mean ± standard deviations of four independent experiments. $^{**}P < 0.01$ versus the corresponding value for control siRNA. (c) Removal of ultraviolet (UV)-induced CPD from the dihydrofolate reductase (DHFR) fragments in XP4PASV cells immediately or 6 h after UV irradiation. The DHFR fragments were analyzed using strand-specific probes recognizing the transcribed (TS) or non-transcribed (NTS) strand.

Polyubiquitinated proteins show a diffuse pattern on western blots, similar to the behavior observed for BRCA1 and CSB. BRCA1 is ubiquitinated both *in vitro* and *in vivo*, whereas CSB is ubiquitinated *in vitro* only.[36] To determine whether CSB was polyubiquitinated *in vivo*, HEK-293T cells were transfected with a vector expressing a Myc-tagged ubiquitin (Myc-ubiquitin)[15] and then exposed to UV irradiation. Cell lysates were immunoprecipitated with control IgG or anti-Myc antibodies. The slowly migrating form of CSB was clearly precipitated by the anti-Myc antibody (Fig. 3b). Although these slowly migrating bands were observed in non-irradiated cells, the intensity of the bands was enhanced by UV irradiation (Fig. 3c). These indicate that the slowly migrating form of CSB is polyubiquitinated and that UV irradiation increases the polyubiquitination of BRCA1 and CSB.

Polyubiquitinated CSB is processed for proteasomal degradation after UV irradiation. Polyubiquitination is a signal for proteasomal degradation. To determine whether the polyubiquitinated BRCA1 and CSB were targeted for proteasomal degradation following UV irradiation, HEK-293T cells were incubated in the presence or absence of the proteasome inhibitor, MG132, after UV irradiation (Fig. 3d). Treatment with MG132 markedly increased the polyubiquitination of CSB, but not BRCA1. This suggests that CSB is polyubiquitinated and targeted for degradation after UV irradiation, whereas ubiquitination of BRCA1 is unlikely to be coupled to degradation.

BRCA1 polyubiquitinates CSB and is involved in CSA protein-independent resistance to UV irradiation. Cockayne syndrome B is polyubiquitinated *in vitro* by an E3 ubiquitin ligase complex containing CSA and degraded by a proteasomal pathway in a CSA-dependent manner after UV irradiation.[36,37] In contrast, it is reported that CSB expression is downregulated after UV irradiation even in CSA-deficient cells.[38] There might be another pathway for the polyubiquitination and degradation of CSB. Consistent with this expectation, polyubiquitination of CSB was observed in CSA-deficient cells (Fig. 4a). Polyubiquitination of CSB in BRCA1-knockdown cells was significantly lower than that in cells transfected with the control siRNA (Fig. 4b). To test whether the ubiquitin ligase activity of BRCA1 is involved in the polyubiquitination of CSB, HEK-293T cells were transfected with expression vectors for wild-type BRCA1 (HA-BRCA1) or a BRCA1 mutant in which the ubiquitin ligase activity is abolished (HA-BRCA1-I26A).[39,40] In BRCA1-I26A-transfected cells, the polyubiquitination of CSB was markedly lower than that in cells transfected with wild-type BRCA1 (Fig. 4c). These suggest that the ubiquitin ligase activity of BRCA1 is involved in the polyubiquitination of CSB.

Next, ubiquitination assays were performed to determine whether BRCA1 directly ubiquitinates CSB *in vitro*. Purified recombinant CSB protein was incubated with ATP, ubiquitin, E1, UbcH5c and the BRCA1/BARD1 heterodimer and analyzed using western blotting (Fig. 4d). In the complete reaction, polyubiquitinated CSB was observed as slowly migrating diffuse bands and the amount of polyubiquitinated CSB was proportional to the amount of BRCA1/BARD1. This suggests that CSB is a substrate for BRCA1/BARD1.

Next, to assess whether CSB is degraded following UV irradiation in CSA-deficient cells, the amount of CSB was examined in *CSA*-deficient cells following UV irradiation in the presence of cycloheximide (CHX) (Fig. S5a). The amount of CSB protein decreased after UV irradiation in the presence of CHX in CSA-deficient cells. In contrast, CSB protein was not downregulated after UV irradiation in the absence of CHX, as previously reported.[36] The amount of CSB did not alter significantly after treatment with CHX alone (Fig. S5b). Treatment with the proteasome inhibitor together with CHX prevented downregulation of CSB after UV irradiation (Fig. S5c). To examine whether BRCA1 was involved in the downregulation of CSB, CSA-deficient cells were transfected with control or BRCA1 siRNA and the amount of CSB was analyzed after UV irradiation in the presence of CHX. Knockdown of BRCA1 suppressed the downregulation of CSB after UV irradiation in the presence of CHX (Fig. 4e). The amount of BRCA1 in cells transfected with control siRNA decreased following UV irradiation in the presence of CHX, consistent with the report by Hammond-Martel *et al.*[41] These suggest that CSB polyubiquitinated by BRCA1 is degraded via a proteasomal pathway independent of CSA.

Finally, the effect of BRCA1 depletion on the UV sensitivity of *CSA*-deficient cells was analyzed. BRCA1 knockdown

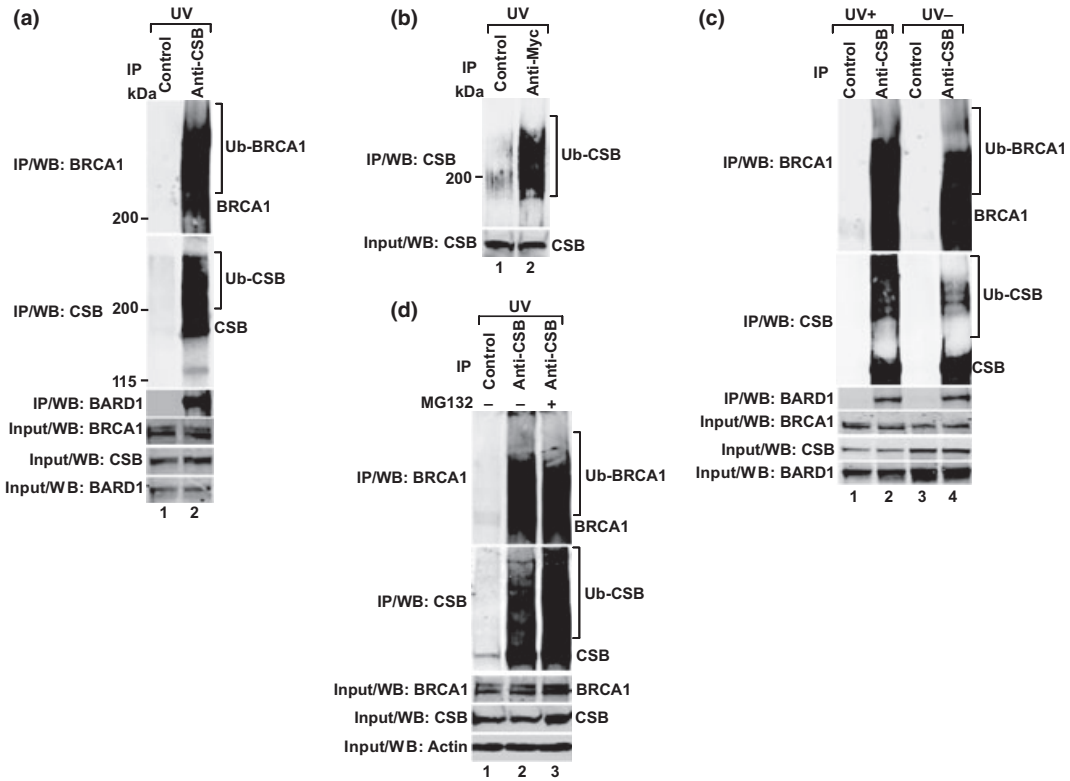

Fig. 3. Cockayne syndrome B (CSB) is associated with BRCA1 and polyubiquitinated for proteasomal degradation after ultraviolet (UV) irradiation. (a) HEK-293T cells were treated with UV irradiation at a dose of 20 J/m^2. One hour after exposure, cell lysates were subjected to immunoprecipitation (IP) with control IgG or anti-CSB antibodies followed by western blotting with anti-BRCA1, anti-CSB or anti-BARD1 antibodies. (b) HEK-293T cells were transfected with Myc-ubiquitin and then UV irradiated. Lysates were subjected to IP using control IgG or anti-Myc antibodies 1 h after UV irradiation. (c) Polyubiquitination of BRCA1 and CSB is enhanced by UV irradiation. (d) CSB polyubiquitination is associated with proteasomal degradation after UV irradiation. HEK-293T cells were treated with or without 50 μM of MG132 after UV irradiation.

increased the UV sensitivity of CSA-deficient cells, supporting a role for BRCA1 in the TCR of UV lesions (Fig. 4f).

Discussion

In the present study we showed that BRCA1 immediately accumulated at locally UV-irradiated sites in a manner that is dependent on CSB and transcription (Fig. 1). Although these are highly suggestive of a role for BRCA1 in TCR, BRCA1 enhances GGR through the transcriptional induction of XPC and DDB2.[42] Therefore, we demonstrated that loss of BRCA1 affects the sensitivity of TCR-proficient and GGR-deficient *XPC*-deficient cells to UV irradiation, but not of GGR-proficient and TCR-deficient *CSB*-deficient cells. Furthermore, the removal of CPD from transcribed strands was suppressed by depletion of BRCAl. Thus, we concluded that BRCA1 is involved in TCR following UV irradiation and increases the survival of cells harboring UV damage.

GFP-tagged CSB protein accumulates in sub-nuclear areas at sites of local UV damage.[43] The amount of CSB protein increases in the chromatin fraction after UV irradiation.[44,45] CSB was responsible for BRCA1 accumulation at UV-irradiated sites. To examine whether BRCA1 moves to chromatin following UV irradiation in a CSB-dependent manner, we fractionated cell lysates from XP4PASV and UV^{s}1KOSV cells into soluble and chromatin-containing fractions (Fig. S6). The amount of CSB protein within the chromatin-containing fractions from XP4PASV cells, increased after UV irradiation. Consistent with Figure 1c, BRCA1 was identified in the chromatin-containing fraction from XP4PASV cells following UV irradiation, but not in that from UV^{s}1KOSV cells.

The CSB-dependent accumulation of BRCA1 at the UV lesions is similar to that seen for other NER factors. Cockayne syndrome A is translocated to the nuclear matrix in an UV- and CSB-dependent manner,[46] and other NER proteins are recruited to TCR sites in a CSB-dependent manner.[44] These suggest that BRCA1 accumulates at UV-irradiated sites and functions in TCR together with other NER factors, and that CSB is an integral factor for recruiting DNA repair factors to TCR sites.

The polyubiquitination of both BRCA1 and CSB was enhanced after UV irradiation. Auto-ubiquitination of BRCA1 enhances its DNA-binding activity.[47] Enhanced polyubiquitination of BRCA1 following UV irradiation might be involved in its function as a DNA repair molecule. However, CSB was polyubiquitinated and processed for proteasomal degradation after UV irradiation. This is consistent with a report that CSB is degraded following UV irradiation.[37] As described above, the amount of CSB increases in the chromatin fraction after UV irradiation. Lake *et al.*[45] reported that the amount of CSB re-appearing in the soluble fraction 7 h after UV irradiation decreases compared with the amount of CSB present before UV irradiation. Cockayne syndrome B might be recruited to the chromatin and then polyubiquitinated for proteasomal degradation after UV irradiation. Since BRCA1 was also recruited to the chromatin fraction after UV irradiation, BRCA1 might ubiquitinate CSB at the chromatin. Phosphorylated RNAPII is also polyubiquitinated by BRCA1 and targeted for proteasomal degradation following UV irradiation.[15,16] BRCA1 might function in TCR through the regulation of protein stability at sites of UV damage.

Depletion of BRCA1 increased UV sensitivity in *CSA*-deficient cells (Fig. 4f), but not in *CSB*-deficient cells (Fig. 2b).

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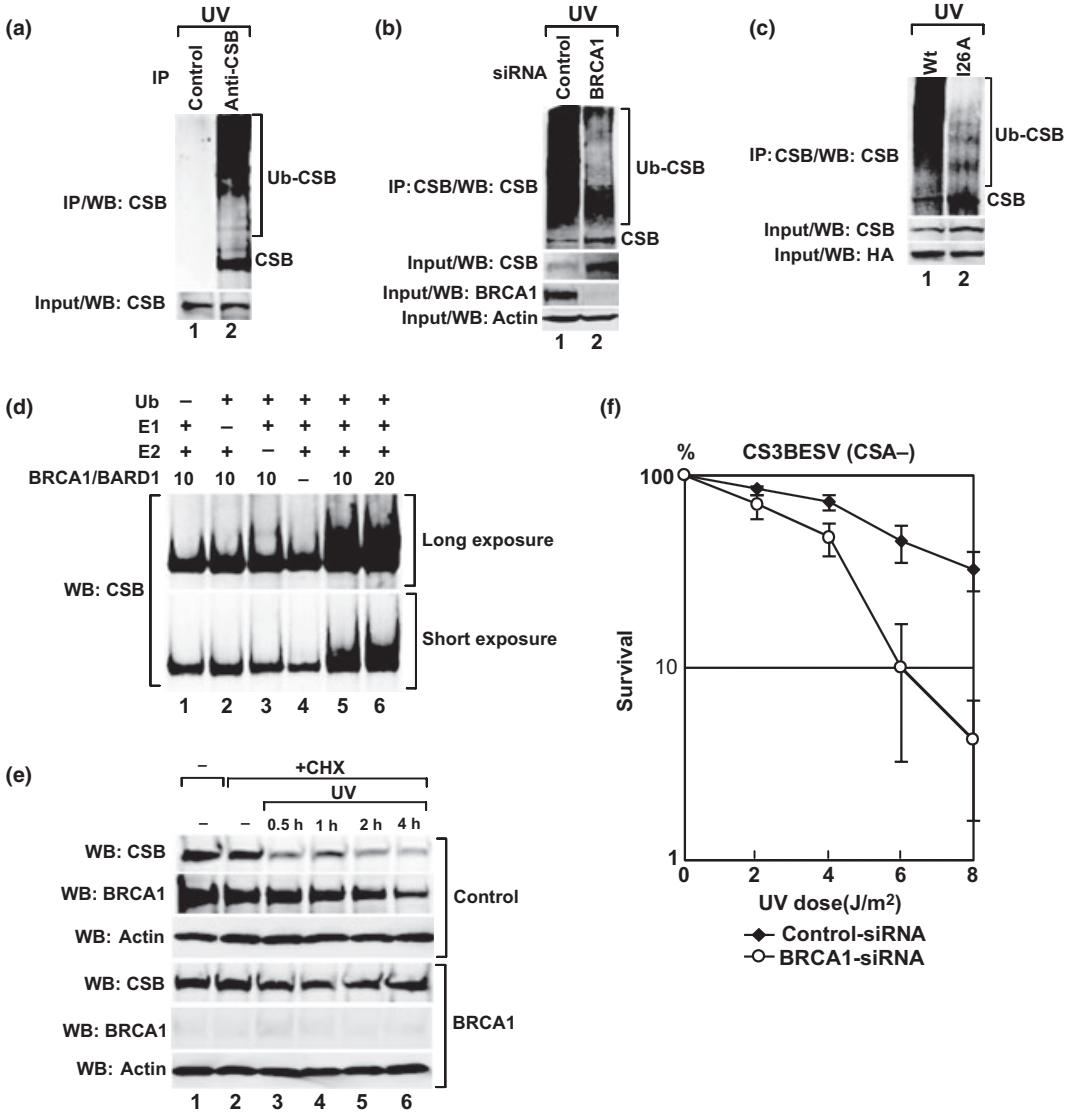

Fig. 4. BRCA1 polyubiquitinates Cockayne syndrome B (CSB) and is involved in the Cockayne syndrome A (CSA)-independent resistance to ultraviolet (UV)-irradiation. (a) CS3BESV cells were treated with UV irradiation. One hour after exposure, cell lysates were subjected to IP. (b) HEK-293T cells were transfected with control or BRCA1 siRNA. One hour after exposure, cell lysates were subjected to immunoprecipitation (IP). (c) HEK-293T cells were transfected with wild-type HA-BRCA1 or HA-BRCA1-I26A. One hour after exposure, cell lysates were subjected to IP. (d) BRCA1 polyubiquitinates CSB *in vitro*. Long and short exposures of the same blot are presented to show that polyubiquitination of CSB is dependent on the amount of BRCA1/BARD1 (10 or 20 nM). (e) CS3BESV cells were transfected with control or BRCA1 siRNA. Cells were pretreated with cycloheximide (CHX) for 1 h and UV irradiated. Cells were incubated with CHX and total cell lysates were prepared at the indicated times for western blot with anti-CSB antibody. (f) Colony formation assay for CS3BESV cells transfected with control or BRCA1 siRNA. The cells were UV irradiatied as indicated. Data represent the mean ± standard deviations of four independent experiments.

This suggests that BRCA1 is involved in TCR for UV damage in a CSB-dependent manner, but independent of CSA. Polyubiquitination of CSB was observed in CSA-deficient cells. Cockayne syndrome B is polyubiquitinated in a BRCA1-dependent manner. Furthermore, CSB was polyubiquitinated by BRCA1/BARD1 *in vitro*, similar to the effect of the CSA complex. Although CSA and BRCA1 might play similar redundant roles in the ubiquitination of CSB, the presence of two independent pathways of CSB polyubiquitination might reflect different roles for CSA and BRCA1 in TCR.

Clinical differences between *CSA*-deficient and *CSB*-deficient patients have not been observed. However, CSA and CSB have different functions in the response to oxidative damage. *CSB−/−* MEF and keratinocytes are hypersensitive to oxidative damage, but *CSA*-deficient cells are not[48]. Cockayne syndrome A functions in the response to oxidative damage, and *CSA*-deficient cell extracts show normal oxidative damage cleavage activity while *CSB*-deficient cell extracts do not.[49] This suggests that downstream pathways that involve CSB exist, one of which might be independent of CSA. Although we identified a function for BRCA1 in TCR of UV lesions in the present study, BRCA1 is also involved in the TCR of oxidative damage[25] and DNA damage induced by ionizing irradiation[23,24]. Therefore, BRCA1 might also be involved in TCR of these DNA damage independent of CSA. Additional studies are needed to gain further understanding of the role played by BRCA1 in TCR pathways.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Immediate BRCA1 accumulation after local ultraviolet (UV) irradiation is not induced by double-strand breaks (DSB).

Fig. S2. Accumulation of BRCA1 at ultraviolet (UV) irradiated sites is dependent on Cockayne syndrome B (CSB).

Fig. S3. Expression of BRCA1 in Saos-2 cells treated with actinomycin D or α-amanitin.

Fig. S4. BARD1 accumulates at sites of ultraviolet (UV) irradiation.

Fig. S5. Cockayne syndrome B (CSB) protein is downregulated after ultraviolet (UV) irradiation in the presence of cycloheximide in Cockayne syndrome A (CSA)-deficient cells.

Fig. S6. Ultraviolet (UV)-irradiation recruits Cockayne syndrome B (CSB) and BRCA1 proteins to the chromatin fraction.

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