UV damage-induced phosphorylation of HBO1 triggers CRL4DDB2-mediated degradation to regulate cell proliferation

AMERICAN SOCIETY FOR MICROBIOLOGY | Molecular and Cellular Biology

UV Damage-Induced Phosphorylation of HBO1 Triggers CRL4^{DDB2}-Mediated Degradation To Regulate Cell Proliferation

Ryoichi Matsunuma,[a,b] Hiroyuki Niida,[a] Tatsuya Ohhata,[a] Kyoko Kitagawa,[a] Satoshi Sakai,[a] Chiharu Uchida,[a,c] Bunsyo Shiotani,[d] Masaki Matsumoto,[e] Keiichi I. Nakayama,[e] Hiroyuki Ogura,[b] Norihiko Shiiya,[b] Masatoshi Kitagawa[a]

Department of Molecular Biology,[a] First Department of Surgery,[b] and Research Equipment Center,[c] Hamamatsu University School of Medicine, Shizuoka, Japan; Department of Cellular and Molecular Biology, Hiroshima University, Hiroshima, Japan[d]; Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan[e]

Histone acetyltransferase binding to ORC-1 (HBO1) is a critically important histone acetyltransferase for forming the prereplicative complex (pre-RC) at the replication origin. Pre-RC formation is completed by loading of the MCM2-7 heterohexameric complex, which functions as a helicase in DNA replication. HBO1 recruited to the replication origin by CDT1 acetylates histone H4 to relax the chromatin conformation and facilitates loading of the MCM complex onto replication origins. However, the acetylation status and mechanism of regulation of histone H3 at replication origins remain elusive. HBO1 positively regulates cell proliferation under normal cell growth conditions. Whether HBO1 regulates proliferation in response to DNA damage is poorly understood. In this study, we demonstrated that HBO1 was degraded after DNA damage to suppress cell proliferation. Ser50 and Ser53 of HBO1 were phosphorylated in an ATM/ATR DNA damage sensor-dependent manner after UV treatment. ATM/ATR-dependently phosphorylated HBO1 preferentially interacted with DDB2 and was ubiquitylated by CRL4^{DDB2}. Replacement of endogenous HBO1 in Ser50/53Ala mutants maintained acetylation of histone H3K14 and impaired cell cycle regulation in response to UV irradiation. Our findings demonstrate that HBO1 is one of the targets in the DNA damage checkpoint. These results show that ubiquitin-dependent control of the HBO1 protein contributes to cell survival during UV irradiation.

Tight regulation of genome maintenance processes, including DNA repair, checkpoints, apoptosis, and cell cycle control, prevents DNA instability after DNA damage. Mammalian cells coordinately operate these systems for organism survival, in part through ataxia telangiectasia mutated (ATM) and ATM- and RAD3-related protein (ATR), two critical kinases that function as regulators of major checkpoint pathways. ATM is primarily activated by DNA double-strand breaks (DSBs) (1), and ATR is activated in response to inhibition of DNA replication (2). Activated ATM and ATR phosphorylate histone H2AX to recruit DNA repair proteins (3) and also checkpoint kinase 1 (Chk1) to suppress cell cycle progression (4, 5). Chk1 indirectly inhibits dephosphorylation of Tyr15 of cyclin-dependent kinase 2 (CDK2) (6) and CDC2 via Cdc25A degradation (7). ATM and ATR also phosphorylate the p53 tumor suppressor to increase its protein stability (8). p53 is a critical cellular factor that induces apoptosis genes (9) and the p21 CDK inhibitor gene (10, 11). Thus, substrates of ATM and ATR are involved in arresting the cell cycle, repairing DNA, and eliminating damaged cells by apoptosis.

Histone acetyltransferase binding to ORC-1 (HBO1) was originally identified as an ORC1 binding protein (12) and acts as a cofactor in the prereplicative complex (pre-RC) (13). This histone acetyltransferase (HAT) associates with distinct complexes to acetylate histones H3 and H4 (14, 15). HBO1 is also involved in cell proliferation control through regulating the expression of multiple genes in the p53 pathway (16). A previous study demonstrated that HBO1 is a candidate ATM and ATR substrate (17). However, although some data have shown that ATM/ATR phosphorylates HBO1 in response to DNA damage, the physiological significance of this phosphorylation remains elusive.

The ubiquitin-proteasome system is involved in controlling protein levels of many cellular proteins and thus contributes to the regulation of several cellular processes, including cell cycle control and the DNA damage response (18, 19). Ubiquitin E3 ligases selectively recognize their substrates to promote ubiquitylation followed by ubiquitin-dependent degradation in the proteasome. A recent report showed that Fbxw15, an F box protein that is the substrate recognition subunit of the SCF complex, participates in lipopolysaccharide (LPS)-induced degradation of HBO1 (20). However, whether HBO1 stability is affected under DNA damage conditions and the relevant underlying mechanisms have been unclear.

The DDB1-CUL4A-RBX1 (CRL4) E3 ligase is involved in the DNA damage response as well as in cell proliferation, development, and replication (21, 22). DDB2, encoded by the *XPE* gene, also associates with CUL4-DDB1 and serves as the substrate recognition complex of the CRL4 ubiquitin ligase. DDB1 recognizes cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs) that are generated by UV irradiation. CRL4^{DDB2} ubiquitylates histones (23, 24) and XPC (25, 26) in the nucleotide excision repair (NER) pathway after UV damage. CRL4^{DDB2} also ubiquitylates p21 and targets it for ubiquitin-mediated protea-

Received 18 August 2015 **Returned for modification** 22 September 2015
Accepted 9 November 2015

Accepted manuscript posted online 16 November 2015

Citation Matsunuma R, Niida H, Ohhata T, Kitagawa K, Sakai S, Uchida C, Shiotani B, Matsumoto M, Nakayama KI, Ogura H, Shiiya N, Kitagawa M. 2016. UV damage-induced phosphorylation of HBO1 triggers CRL4^{DDB2}-mediated degradation to regulate cell proliferation. Mol Cell Biol 36:394–406. doi:10.1128/MCB.00809-15.

Address correspondence to Hiroyuki Niida, niidah@hama-med.ac.jp.

R.M. and H.N. contributed equally to this article.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/MCB.00809-15.

somal degradation (27), and it also ubiquitylates DDB2 itself (28, 29).

In this study, we demonstrate that CRL4^{DDB2} is a novel ubiquitin ligase of HBO1. We show that Ser50 and Ser53 of HBO1 are robustly phosphorylated after UV irradiation, in an ATM/ATR-dependent manner, and that phosphorylated HBO1 is preferentially ubiquitylated by CRL4^{DDB2}. Inhibition of phosphorylation at Ser50 and Ser53 in HBO1 by mutation of these residues to Ala resulted in a failure to repair DNA damage and suppress cell proliferation after UV exposure. Our findings suggest that negative regulation of HBO1 by the ubiquitin-proteasome system may be involved in genome maintenance in surviving cells after UV irradiation.

MATERIALS AND METHODS

Cells, cell culture, and treatment. HeLa and HEK293 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. UV irradiation was performed with a Funa-UV-Linker FS-800 UV cross-linker (Funakoshi).

Antibodies, small interfering RNAs (siRNAs), and inhibitors. The following antibodies were used for immunoblotting: anti-HBO1 antibody (sc-13284; Santa Cruz Biotechnology), anti-phospho-(Ser/Thr) ATM/ATR substrate antibody (2851; Cell Signaling Technology), anti-Myc antibody (sc-40; Santa Cruz Biotechnology), antihemagglutinin (anti-HA) antibody (sc-57592; Santa Cruz), anti-CUL1 antibody (sc-17775; Santa Cruz), anti-CUL2 antibody (sc-166506; Santa Cruz), anti-CUL4 antibody (sc-8557; Santa Cruz), anti-DDB2 antibody (sc-81246; Santa Cruz), anti-β-actin antibody (sc-47778; Santa Cruz Biotechnology), anti-ATM antibody (sc-23921; Santa Cruz Biotechnology), anti-ATR antibody (sc-1887; Santa Cruz Biotechnology), and antibromodeoxyuridine (anti-BrdU) antibody (347580; BD). Anti-phospho-Ser50 and -Ser53 HBO1 rabbit polyclonal antibodies were generated by immunization with a synthetic phosphopeptide (CSARLpSQSpSQD). Antiserum obtained from an immunized rabbit was purified using column chromatography conjugated with the phosphopeptide and then passed through a column conjugated with nonphosphorylated peptide to eliminate antibodies against nonphosphorylated HBO1. Antibody specificity was confirmed by enzyme-linked immunoabsorbent assay, dot blot assay, and immunoblotting.

The siRNA sequences used were as follows: ATM, 5′-GGAAAUCAG UAGUUUGGUCTT-3′; ATR, 5′-CCUCCGUGAUGUUGCUUGATT-3′; negative control, 5′-UUCUCCGAACGUGUCACGUTT-3′; HBO1, 5′-CCCUUCCUGUUCUAUGUUATT-3′; CUL1, 5′-CAGGUUUACCU UCAUGAAATT-3′ (sc-35126); CUL2, 5′-CUCCUUUGUUCAUGUUG AATT-3′ (sc-35128); CUL4A, 5′-GGAAGAGACUAAUUGCUUATT-3′ (sc-44355); and CUL4B, 5′-GCAUUCUUCUCUUGAUUGATT-3′. HBO1 and DDB2 short hairpin RNA (shRNA) plasmids were purchased from Santa Cruz (sc-35530B-SH and sc-37799-SH).

The following ATM and ATR inhibitors were used: an ATM kinase inhibitor (118500; Calbiochem) and ATR inhibitor VE821 (HY14731; MedChem Express).

Plasmids. The Myc-HBO1 expression plasmid was described in a previous report (30). The Myc-CDT1 expression plasmid was a gift from H. Nishitani. HA-CUL4A, FLAG-DDB1, HA-CDT2, and HA-DDB2 were provided by M. Saijo (31).

Establishment of knockdown and TG cells. To establish stable knockdown shHBO1 and shDDB2 cells, HeLa cells were transfected with the shHBO1 and shDDB2 plasmids, respectively. Forty-eight hours after transfection, cells were selected with 10 μM puromycin for 3 days and then cultured in normal medium for 7 days. At least 24 colonies were isolated, and HBO1 and DDB2 expression was checked by Western blotting. To obtain stable Myc-HBO1 wild-type (WT) and S50/53A transgenic (TG) cells, each shHBO1-resistant expression plasmid and neomycin flanked by the lox plasmid were cotransfected into shHBO1 cells, and cells were selected with 0.3 mg/ml G418 for 10 days. At least 24 colonies were isolated, and Myc-HBO1 expression was evaluated by Western blotting with anti-Myc antibody.

Western blotting. For preparation of whole-cell extracts, cells were lysed in IP kinase buffer as previously described (32).

Alkaline phosphatase treatment. To dephosphorylate the HBO1 protein, 10-μg lysates of UV-irradiated HeLa or HEK293 cells were mixed with 1× alkaline phosphatase buffer and 15 U of alkaline phosphatase (New England BioLabs). Mixtures were incubated at 37°C for 2 h, and reactions were terminated by adding sample buffer and boiling for 3 min.

Immunoprecipitation (IP) under denaturing conditions. To avoid detection of ubiquitylation of substrate-associated proteins, we performed a ubiquitylation assay under denaturing conditions as previously described (33). Cells were lysed in IP kinase buffer as previously described (32), and an equal volume of 2× denaturing IP buffer (100 mM Tris-HCl, pH 7.5, 2% SDS, 10 mM dithiothreitol) was added. Samples were incubated at 100°C for 8 min and then centrifuged at 13,000 rpm for 10 min. The supernatants were diluted with 5 volumes of IP kinase buffer and immunoprecipitated with 2 μg of antibodies and protein G-Sepharose 4FF (GE Healthcare) at 4°C for 2 h. Immunoprecipitates were washed four times with IP kinase buffer. Immunoprecipitated samples were separated by SDS-PAGE and transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Millipore), followed by immunoblotting.

Cell cycle analysis by FACS. Myc-HBO1 WT and S50/53A TG cells were irradiated with 0, 8, or 15 J/m^2 UV light. Cells were cultured for 24 and 48 h, and cell numbers were counted to measure cell proliferation. For cell cycle analysis, Myc-HBO1 WT and S50/53A TG cells were irradiated with 15 J/m^2 UV light and cultured for 12 and 36 h. Cells were incubated with 20 μM BrdU for 40 min, harvested, and fixed with ice-cold 70% ethanol (EtOH) overnight. Incorporated BrdU was stained with anti-BrdU antibody. In brief, the chromosome was denatured with 2 N HCl, 0.5% NP-40 on ice for 1 h and then neutralized with 0.1 M sodium borate, pH 8.5, at room temperature for 5 min. Cells were washed with phosphate-buffered saline (PBS) three times and then immunostained with anti-BrdU antibody diluted 1:50 with PBS-TB (0.5% Tween 20, 1% bovine serum albumin [BSA] in PBS) for 1 h. Cells were washed with PBS and immunostained with Alexa Fluor 488-conjugated anti-mouse IgG antibody diluted 1:500 with PBS-TB for 1 h in the dark. Cells were washed with PBS and counterstained with 50 μg/ml propidium iodide, 100 μg/ml RNase A for over 1 h at 37°C. The cell cycle was analyzed by fluorescence-activated cell sorting (FACS) (Gallios; Beckman Coulter).

Apoptosis assay. Cells irradiated with 15 J/m^2 UV light were stained with phycoerythrin (PE)-labeled annexin V (BD Pharmingen) for 15 min at room temperature in annexin V binding buffer (10 mM HEPES, 0.14 M NaCl, 2.5 mM $CaCl_2$). Apoptotic cells were detected by FACS.

Comet assay. Alkaline comet assays were performed using a Trevigen comet assay kit according to the manufacturer's instructions. DNA was stained with SYBR green, and slides were photographed digitally (Biozero BZ-9000; Keyence). Tail moments were analyzed using TriTek CometScore freeware as reported previously (34).

Statistical analysis. Quantitative data are presented as means ± standard deviations (SD) and were analyzed by Student's *t* test.

RESULTS

Ubiquitin-dependent degradation of HBO1 is enhanced by DNA damage. HBO1 is recruited to the replication origin in a CDT1-dependent manner during prereplicative complex formation. CDT1 is degraded by the ubiquitin-proteasome pathway after UV irradiation (35–37). In examining pre-RC formation after UV damage, we found that not only CDT1 but also both ectopic and endogenous HBO1 protein levels decreased after UV irradiation (Fig. 1A; see Fig. S1A in the supplemental material). Furthermore, the decrease in both Myc-tagged HBO1 (Myc-HBO1) and endogenous HBO1 was suppressed by addition of the proteasome inhibitor MG132. We next examined whether HBO1 was ubiqui-

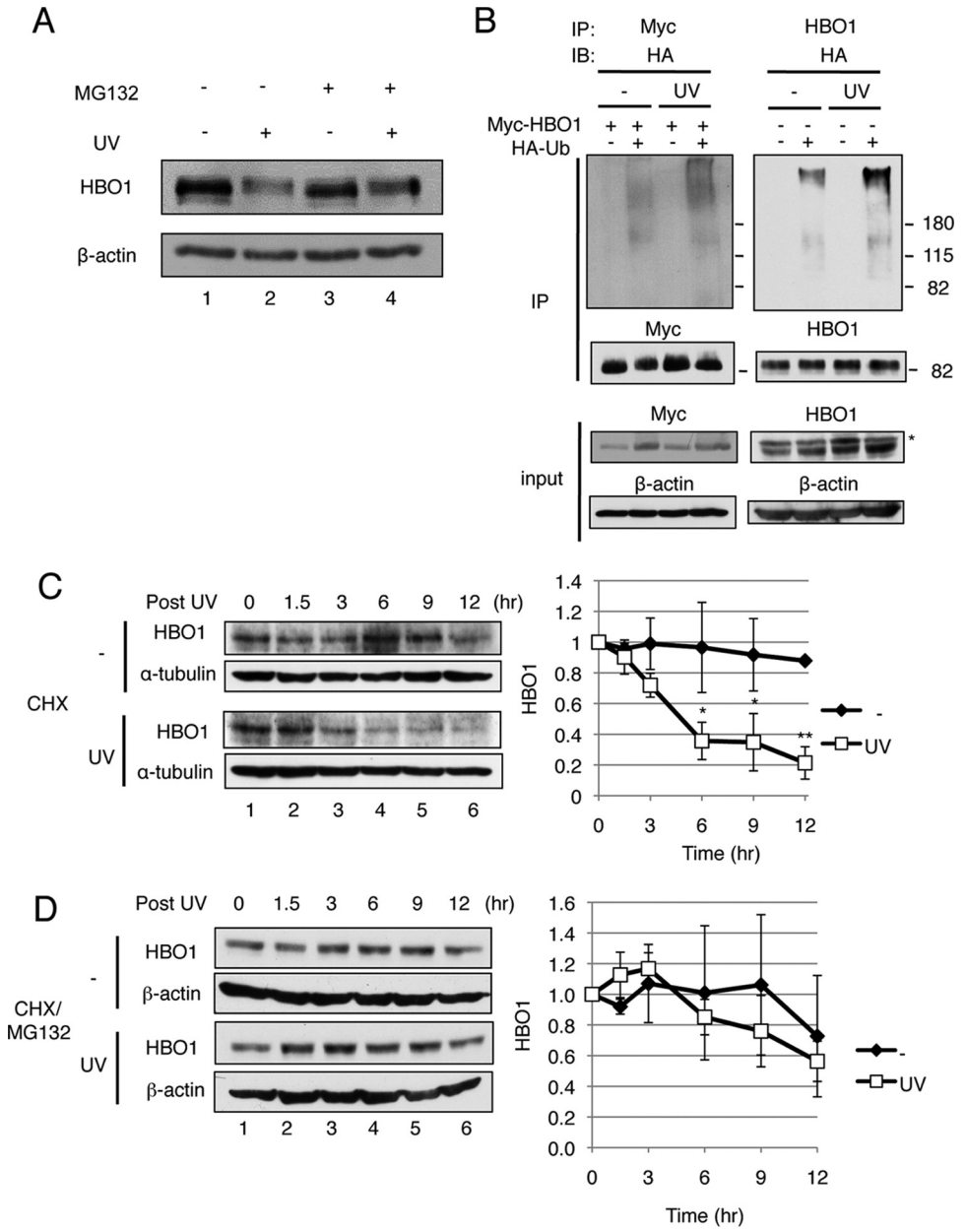

FIG 1 HBO1 is degraded by the ubiquitin-proteasome system in response to UV damage. (A) Endogenous HBO1 was degraded by UV light and protected by MG132. HEK293 cells were treated with or without MG132 (10 μM) for 4 h, followed by irradiation with 40 J/m^2 UV light, and were then cultured for 3 h. Cell lysates were analyzed by Western blotting with the indicated antibodies. (B) UV-induced ubiquitylation of HBO1 was detected under denaturing conditions. HEK293 cells were transfected with Myc-HBO1 WT and HA-Ub (left) or only with HA-Ub (right) and then treated with MG132 for 4 h before UV irradiation at 40 J/m^2 and were then cultured for 2 h. Cell lysates were immunoprecipitated with anti-Myc antibody (left) or anti-HBO1 antibody (right) under denaturing conditions, and the immunoprecipitates were examined by Western blotting (IB) using the indicated antibodies. The asterisk indicates a nonspecific band. (C) Endogenous HBO1 was degraded after UV irradiation. HEK293 cells were irradiated with 100 J/m^2 UV light or not irradiated and were treated with 12.5 μM cycloheximide (CHX) for the indicated times. Cell lysates were analyzed by Western blotting with the indicated antibodies. The graph indicates average quantities of HBO1 proteins for three independent experiments. Error bars indicate standard errors (*, $P < 0.05$; **, $P < 0.01$). (D) UV-induced degradation was suppressed by the proteasome inhibitor. HEK293 cells were treated with 10 μM MG132 for 4 h and then irradiated with 100 J/m^2 UV light or not irradiated. CHX (12.5 μM) was then added, and the cells were cultured for the indicated times. Cell lysates were analyzed by Western blotting with the indicated antibodies. The graph indicates average quantities of HBO1 proteins. Error bars indicate standard errors.

tylated after UV irradiation. HEK293 cells were cotransfected with Myc-HBO1 and HA-tagged ubiquitin (HA-Ub) in the presence or absence of MG132, and Myc-tagged proteins were examined by Western blotting after UV irradiation (see Fig. S1B). Myc-CDT1 was included as the positive control. Significant amounts of slowly migrating Myc-HBO1 were detected in the presence of MG132, suggesting that Myc-HBO1 was ubiquitylated. To confirm this possibility and to avoid the detection of ubiquitylated HBO1-associated proteins, we performed another ubiquitylation assay with immunoprecipitates from Myc-HBO1-expressing cell lysates pre-

pared under denaturing conditions (Fig. 1B, left panel). We also examined ubiquitylation of endogenous HBO1 with only transfected HA-Ub (Fig. 1B, right panel). Although we detected ubiquitylated Myc-HBO1 and endogenous HBO1 in the absence of UV damage, highly ubiquitylated Myc-HBO1 and endogenous HBO1 were detected in immunoprecipitates by immunoblotting with anti-HA antibody at 2 h post-UV irradiation (Fig. 1B). We further examined whether degradation of endogenous HBO1 was facilitated by UV irradiation by using a cycloheximide (CHX) chase assay (Fig. 1C). We performed CHX chase assays with 100 J/m^2 UV light; however, we found that induction of HBO1 degradation by 40 J/m^2 UV light was almost the same as that by 100 J/m^2 UV light (see Fig. S1C). Translation of HBO1 was inhibited by adding CHX at the same time as UV irradiation, and cells were harvested at the indicated times. After UV irradiation, HBO1 levels rapidly decreased, and the half-life of HBO1 was approximately 4.5 h after irradiation (Fig. 1C). In the absence of UV light, the half-life of HBO1 was over 12 h. To confirm that degradation of HBO1 after UV irradiation in the CHX chase assay was proteasome dependent, the CHX chase assay was performed in the presence of the proteasome inhibitor MG132 (Fig. 1D). MG132 treatment blocked the UV-dependent degradation of HBO1 (the half-life of HBO1 after UV irradiation was over 12 h). These results indicate that HBO1 is degraded via the ubiquitin-proteasome pathway after UV damage.

Ser50 and Ser53 of HBO1 are phosphorylated upon induction of DNA damage. Because UV irradiation promotes ubiquitin-proteasome-mediated HBO1 degradation, we speculated that UV treatment might target the HBO1 protein by inducing post-translational modification of HBO1. Previous studies suggested HBO1 as one of the substrates of ATM and ATR, well-known sensor proteins for the DNA damage checkpoint (1, 2, 8). In HBO1, Ser50 and Ser53 are each followed by Gln (SQ), and this corresponds to a consensus sequence for ATM and ATR substrates (Fig. 2A). Furthermore, phosphorylation of these serine residues was previously detected after infrared (IR) irradiation (17).

To identify the type of DNA damage that induces phosphorylation of HBO1 at Ser50 and Ser53, the Myc-HBO1 protein was overexpressed in HEK293 cells to assess HBO1 phosphorylation at Ser/Thr-Gln (S/TQ) sites after various genotoxic stresses by use of a pS/TQ antibody. Twenty-four hours after transfection with the Myc-HBO1 expression plasmid, cells were treated with various genotoxic reagents, including the DNA replication inhibitor aphidicolin, hydroxyurea, the DNA strand breaker bleomycin, phleomycin, and UV irradiation. Immunoblot analysis of immunoprecipitated Myc-HBO1 with the pS/TQ antibody revealed that all genotoxic stresses tested, except aphidicolin, significantly increased phosphorylation of HBO1 (see Fig. S2A in the supplemental material).

We next generated a specific anti-phospho-Ser50 and -Ser53 HBO1 antibody (anti-pS50/53 HBO1). Consistent with the results obtained with the anti-pS/TQ antibody, the anti-pS50/53 HBO1 antibody detected clear signals in cells transiently expressing wild-type HBO1 in the presence of various genotoxic treatments (Fig. 2B). All genotoxic stresses induced significant phosphorylation of Ser50 and Ser53 in HBO1 (Fig. 2B, bottom panel). We established HBO1 knockdown/TG HeLa cells depleted of endogenous HBO1 by use of shRNA (see Fig. S2B and C in the supplemental material), as well as HeLa cells expressing the shRNA-resistant HBO1 WT or S50/53A mutant, as described in Materials and Methods. Expression levels of exogenous proteins were comparable to the levels in the original HeLa cells (Fig. 2C). The anti-pS50/53 HBO1 antibody did not cross-react with UV-irradiated shHBO1 HeLa cells stably expressing Myc-HBO1 with Ser50 and Ser53 replaced by Ala (S50/53A) (Fig. 2C, lanes 2 and 5). We did not observe degradation of Myc-HBO1 WT or endogenous HBO1 in UV-irradiated cells, because cell lysates were prepared at 30 min post-UV treatment. Furthermore, no signals were detected for UV-irradiated HEK293 and HeLa cells treated with alkaline phosphatase, demonstrating the antibody specificity (see Fig. S3A). The anti-pS50/53 HBO1 antibody very weakly detected single mutant HBO1 S50A (Ser50 mutated to Ala) and S53A (Ser53 mutated to Ala) proteins (see Fig. S3B, right graph, lanes 2 and 3). We speculate that the weak signals arose from low phosphorylation levels of the single Ala mutants in HEK293 cells, because the anti-pS50/53 HBO1 antibody was able to detect pSer50, pSer53, and pSer50/53 peptides with similar affinities (see Fig. S3C).

To determine whether the UV-induced phosphorylations of Ser50 and Ser53 were ATM/ATR dependent, we performed IP-Western blot analyses of ATM- and/or ATR-depleted HeLa cell lysates by using the anti-pS50/53 HBO1 antibody (Fig. 2D). In control lysates without UV irradiation, a small portion of endogenous HBO1 was phosphorylated at Ser50 and Ser53 (Fig. 2D, top panel, lane 7, and bottom panel). UV irradiation (40 J/m^2) increased the phosphorylation of Ser50 and Ser53 (Fig. 2D, top panel, lane 8, and bottom panel). ATM and ATR depletion significantly decreased the phosphorylation levels regardless of UV damage (Fig. 2D, top panel, lanes 5 and 6 versus lanes 7 and 8, and bottom panel). Interestingly, quantitative analysis indicated that phosphorylation of HBO1 after UV damage in ATM/ATR-depleted cells was significantly suppressed in comparison with that in singly ATM-depleted cells (Fig. 2D, top panel, lane 2 versus lane 6, and bottom panel). In addition, phosphorylation of HBO1 after UV irradiation was partially reduced in singly ATM-depleted cells compared to that in control siRNA-treated cells (Fig. 2D, top panel, lane 2 versus lane 8, and bottom panel). Although UV irradiation usually activates ATR, our ATM knockdown results suggested a contribution of ATM to the phosphorylation of HBO1. Therefore, we checked the generation of double-strand breaks (DSBs) by 40 J/m^2 UV irradiation (Fig. 2E). HeLa cells covered with an 8-μm-pore-size membrane were irradiated with 40 J/m^2 UV light and then fixed with methanol (MeOH)-acetone after 30 min of incubation. 53BP1, which binds DSB sites, and XPC, which recognizes CPDs and *6,4*-PPs, were costained at damage sites. This result indicated that our experimental conditions for UV irradiation generated not only pyrimidine dimers but also DSBs at damage sites. Consistent with 53BP1 accumulation at damage sites, colocalization of phosphorylated ATM Ser1981, which is an indicator of ATM activation, with XPC was detected (Fig. 2E). Furthermore, to confirm that the phosphorylations of Ser50 and Ser53 of HBO1 were ATM/ATR dependent, HEK293 cells were treated with ATM and ATR inhibitors (Fig. 2F). Inhibitor treatment abolished phosphorylation of Ser50 and Ser53 of HBO1 as well as that of Ser317 of Chk1 and Ser1981 of ATM 30 min after UV irradiation (Fig. 2F, lane 2 versus lane 4). Degradation of HBO1 was not detected under these conditions, as we harvested cells at an earlier time point (30 min post-UV treatment) than that for the experiment shown in Fig. 1A (3 h post-UV treatment). Ser317 is a well-known ATM and ATR phosphorylation site in Chk1 that is targeted by ionizing irradiation and UV irradiation,

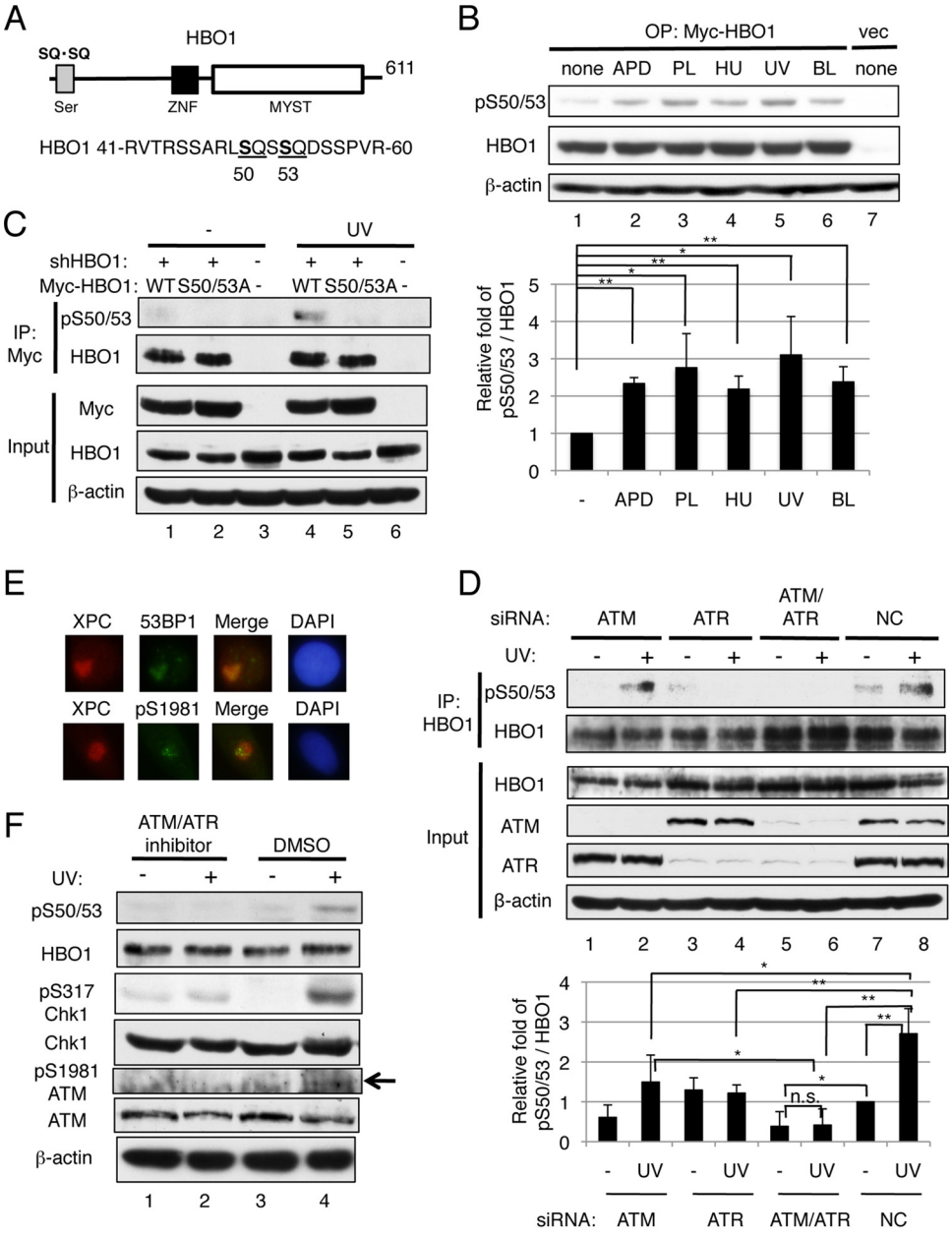

FIG 2 ATM/ATR-dependent phosphorylation of HBO1 at Ser50 and Ser53 is enhanced by DNA damage. (A) Schematic representation of the HBO1 protein. The amino acid sequence of HBO1 from residues 41 to 60 is shown, with the consensus sequences for ATM and ATR substrates underlined. (B) Various DNA damages and replication stresses induced phosphorylation at the Ser50 and Ser53 sites in HBO1. HEK293 cells overexpressing Myc-HBO1 WT were subjected to Western blotting with an anti-Ser50 and -Ser53 HBO1-specific antibody (pS50/53). Cells were exposed to genotoxic stresses for 1 h at the following concentrations: aphidicolin (APD), 50 μM; phleomycin (PL), 100 μM; hydroxyurea (HU), 2.5 mM; and bleomycin (BL), 40 μg/ml. The dose of UV light was 40 J/m^2. Signal intensities were quantitated by use of ImageJ (**, $P < 0.01$). OP, overproduction. (C) Mutation of Ser50 and Ser53 in HBO1 to Ala abolished detection with the anti-pS50/53 antibody. Stably expressed HBO1 WT and S50/53A mutant proteins in HeLa shHBO1 cells and the original HeLa cells were irradiated with 40 J/m^2 UV light. Cell lysates were prepared after 30 min of UV irradiation and subjected to IP-Western blotting with the indicated antibodies. (D) Phosphorylation of Ser50 and Ser53 of endogenous HBO1 was ATM and ATR dependent. ATM and/or ATR siRNAs were transfected into HeLa cells. At 48 h posttransfection, cells were exposed to 40 J/m^2 UV light, cultured for 30 min, and then harvested. Cell lysates were analyzed by IP-Western blotting with the indicated antibodies. pS50/53 signals were quantitated by use of ImageJ (*, $P < 0.05$; **, $P < 0.01$; n.s., not significant). (E) Not only CPDs but also DSBs were generated by 40 J/m^2 UV irradiation. Micropore UV irradiation was performed through an 8-μm micropore filter. CPDs and DSBs were detected indirectly by use of anti-XPC and anti-53BP1 antibodies, respectively. Accumulation of activated ATM at damage sites was detected by use of an anti-ATM pS1981 antibody. (F) Inhibition of ATM and ATR kinase activities suppressed phosphorylation of Ser50 and Ser53 in HBO1. Cells were incubated with ATM and ATR inhibitors for 2 h before UV irradiation at 40 J/m^2. Cell lysates were prepared at 30 min post-UV treatment and analyzed by Western blotting with the indicated antibodies.

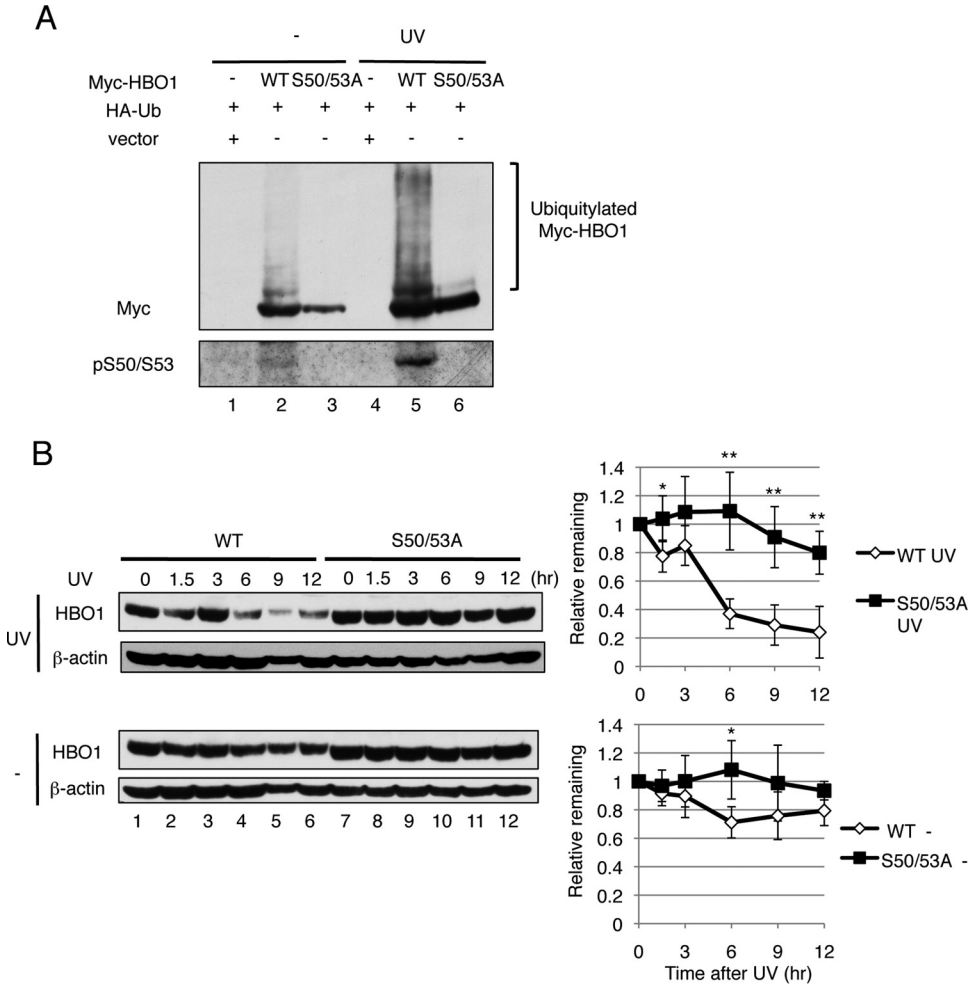

FIG 3 Phosphorylation of Ser50 and Ser53 of HBO1 after UV irradiation promotes ubiquitin-dependent degradation of HBO1. (A) Mutation of Ser50 and Ser53 to Ala suppressed HBO1 ubiquitylation after UV irradiation. HEK293 cells were transfected with Myc-HBO1 WT, Myc-HBO1 S50/53A, or empty vector together with HA-Ub, treated with MG132 for 4 h before UV irradiation at 40 J/m^2, and then cultured for 3 h. Ubiquitylation of HBO1 was analyzed by Western blotting with the indicated antibodies. (B) Mutation of Ser50 and Ser53 to Ala protected HBO1 from degradation after UV irradiation. HEK293 cells were transfected with Myc-HBO1 WT or Myc-HBO1 S50/53A and irradiated with 100 J/m^2 UV light or not irradiated. Cells were then treated with 12.5 μM CHX for the indicated times. The graphs indicate average HBO1 protein expression levels. Error bars indicate standard errors (*, $P < 0.05$; **, $P < 0.01$).

respectively. From these results, we concluded that the phosphorylations of Ser50 and Ser53 in HBO1 were ATM/ATR dependent, although we cannot yet clarify if these phosphorylations are directly or indirectly mediated by ATM/ATR.

Phosphorylation of Ser50 and Ser53 of HBO1 facilitates ubiquitylation, and mutation of Ser50 and Ser53 to Ala increases HBO1 stability after UV damage. Our results showed that phosphorylation of Ser50 and Ser53 of HBO1 was induced in an ATM/ATR-dependent manner after UV damage. Several studies demonstrated that phosphorylation could facilitate ubiquitylation followed by protein degradation (20, 35). We therefore examined whether inhibition of phosphorylation in HBO1 by Ala substitution affected ubiquitylation of HBO1. Consistent with our previous results, the HBO1 WT, but not the S50/53A mutant, was phosphorylated by UV irradiation (Fig. 3A, bottom panel, lane 6). While ubiquitylation of HBO1 was enhanced by UV treatment (Fig. 3A, lanes 2 and 5), it was significantly suppressed by Ala substitution (Fig. 3A, lanes 5 and 6). These results strongly suggest that HBO1 is phosphorylated at S50/53 by UV irradiation and thus targeted for ubiquitylation.

To compare the degradation rates of the Myc-HBO1 WT and Myc-HBO1 S50/53A proteins, we performed a CHX chase assay (Fig. 3B). In contrast to the HBO1 WT, which showed a half-life of 4.5 h after UV irradiation, the Myc-HBO1 S50/53A protein was resistant to UV-induced degradation and showed a half-life of over 12 h (Fig. 3B, top panels). In the absence of UV light, HBO1 WT levels tended to decrease slowly, whereas HBO1 S50/53A levels were stable (Fig. 3B, bottom panels). Together, these results indicate that ATM/ATR-dependent phosphorylation of Ser50 and Ser53 of HBO1 induces ubiquitylation and degradation of HBO1 after UV damage.

CRL4^{DDB2} acts as a ubiquitin ligase for HBO1 in response to UV damage. HBO1 physically interacts with CDT1 and disappears at the replication origin after UV irradiation (13). CDT1 is ubiquitylated by at least two independent ubiquitin ligases. SCF^{SKP2} ubiquitylates CDT1 that is phosphorylated by CDK2

(36). Additionally, $CRL4^{CDT2}$ ubiquitylates CDT1 after DNA damage, in a chromatin-bound PCNA-dependent manner (35, 37). JADE-1, an essential subunit that associates with HBO1 to acetylate chromosomal histone H4, binds to von Hippel-Lindau (VHL) protein (38, 39). $CRL2^{VHL}$ is a ubiquitin ligase of HIF-1 alpha (40). Based on these reports, we speculated that the ubiquitin ligase for HBO1 would be a member of the cullin-RING-ligase (CRL) family. To examine this possibility, we depleted CUL1, CUL2, and CUL4A/B together with cotransfection of Myc-HBO1 and HA-Ub in HeLa cells. Ubiquitylation of HBO1 was examined by immunoblotting with anti-Myc antibody. Knockdown of CUL4A/B mostly suppressed ubiquitylation of HBO1 (Fig. 4A).

To verify this result, we examined the protein level of endogenous HBO1 upon CUL4A/B depletion (Fig. 4B). UV irradiation did not significantly reduce the HBO1 protein level in CUL4A/B knockdown cells, and a significant fraction of HBO1 with phosphorylated Ser50 and Ser53 remained in these cells (Fig. 4B, left panel, lane 4, and right panel). These data suggested that phosphorylation of HBO1 at Ser50 and Ser53 led to CUL4A/B-dependent ubiquitylation and subsequent degradation. Next, we performed an *in vivo* HBO1 ubiquitylation assay (Fig. 4C). To carry out this assay, we first performed a time course analysis of HBO1 ubiquitylation after UV irradiation. Ubiquitylation of HBO1 by endogenous ubiquitin ligases was detected 2 h after UV irradiation, and ubiquitylated HBO1 gradually accumulated until 6 h postirradiation in the presence of MG132 (see Fig. S4 in the supplemental material). Based on these results, we performed the *in vivo* HBO1 ubiquitylation assay with 2 h of incubation after UV irradiation to minimize the effect of endogenous ubiquitin ligases of HBO1. $CRL4^{CDT2}$ is a well-characterized ubiquitin ligase for CDT1, and $CRL4^{DDB2}$ is activated by UV-induced DNA damage and ubiquitylates histones H3 and H4 (23, 24), XPC (25, 26), and p21 (27). We transfected core components of the CRL4 ubiquitin ligase—HA-CUL4A, FLAG-DDB1, and FLAG-HA-CDT2 or HA-DDB2—with Myc-HBO1 and HA-Ub in HEK293 cells. In cells overexpressing CUL4A along with DDB2, we observed more ubiquitylation of Myc-HBO1, with a further increase in response to UV irradiation (Fig. 4C, lane 2), whereas coexpression of CUL4A with CDT2 promoted HBO1 ubiquitylation to the same extent as that with the vector control after UV irradiation (Fig. 4C, lane 1). To confirm the DDB2-dependent ubiquitylation of HBO1, we depleted endogenous DDB2 and prolonged the incubation for 6 h after UV treatment, because endogenous ubiquitylation of HBO1 gradually accumulated until 6 h in the presence of MG132 (see Fig. S4 in the supplemental material). We found that Myc-HBO1 was not ubiquitylated in DDB2 knockdown cells after UV irradiation (Fig. 4D). We further examined the degradation of endogenous HBO1 in DDB2-depleted cells after UV treatment. We found that HBO1 protein levels did not decrease in DDB2-depleted cells after UV treatment (Fig. 4E, left panel, lane 4, and right panel). Furthermore, Ser50- and Ser53-phosphorylated HBO1 accumulated in DDB2-depleted cells after UV treatment (Fig. 4E, left panel, lane 4). Collectively, these results suggest that $CRL4^{DDB2}$ acts as a ubiquitin ligase for HBO1 after UV irradiation.

DDB2 interacts with HBO1 and promotes degradation of HBO1 after UV irradiation. To more closely investigate the effects of DDB2 on HBO1 stability, we subjected DDB2-depleted cells expressing shDDB2 or controls to the CHX chase assay (Fig. 5A). While the half-life of HBO1 in control cells was about 3 h, the half-life of HBO1 in DDB2-depleted cells was over 12 h after UV irradiation (Fig. 5A, top panels). Because some portion of Ser50 and Ser53 of HBO1 was phosphorylated in the absence of UV damage (Fig. 2E), we examined HBO1 degradation without UV treatment in control and shDDB2 cells (Fig. 5A, bottom panels). Even in the absence of UV irradiation, the half-life of HBO1 was shorter in control than in shDDB2 cells.

Next, we examined the interaction of HBO1 and DDB2 *in vivo*. Myc-HBO1 WT or S50/53A was cotransfected with HA-DDB2 in HEK293 cells. Twenty-four hours after transfection, cells were irradiated with UV light and then cultured for 30 min and examined by coimmunoprecipitation assays (Fig. 5B). The interaction of Myc-HBO1 WT and HA-DDB2 was increased by UV irradiation (Fig. 5B, lane 5). In contrast, Myc-HBO1 S50/53A failed to interact with HA-DDB2, even after UV treatment (Fig. 5B, lane 6). Increased interactions of endogenous HBO1 with HA-DDB2 or endogenous DDB2 were also detected after UV treatment (Fig. 5C, lane 8, and D, lane 4). Collectively, these results indicated that phosphorylation of Ser50 and Ser53 of HBO1 induced the interaction of HBO1 and DDB2, and then the DDB2-dependent degradation of HBO1 was carried out after UV irradiation.

Phospho-defective HBO1 mutant cells are defective in suppression of histone H3K14 acetylation and cell proliferation. HBO1 promotes loading of the MCM complex on the chromatin by histone acetylation (41) and is essential for DNA replication. Thus, we next analyzed whether defective HBO1 degradation affected cell proliferation under conditions of UV damage. Cell proliferation experiments were performed twice in triplicate for independent clones (clones 1 and 2) expressing the HBO1 WT and S50/53A proteins. Cells were irradiated at 8 and 15 J/m^2 and then cultured for 24 and 48 h. Relative cell numbers at 48 h were determined for each experiment (Fig. 6A). In both experiments, WT-expressing cells showed reduced cell numbers with 8 and 15 J/m^2 of UV irradiation compared with the numbers of HBO1 S50/53A-expressing cells (Fig. 6A). In these experiments, protein levels of Myc-HBO1 WT but not Myc-HBO1 S50/53A were reduced by UV irradiation (Fig. 6B). Consistent with the Myc-HBO1 levels, the acetylation level for histone H3K14 was significantly higher at 48 h in Myc-HBO1 S50/53A cells irradiated with 15 J/m^2 (Fig. 6B, lanes 11 and 12). In contrast, acetylation levels for histone H4 were not correlated with Myc-HBO1 protein levels.

Because Myc-HBO1 S50/53A cells tended to show continuous cell growth and more acetylation of H3K14 after UV damage, we also measured the activation of the DNA damage checkpoint. Surprisingly, signals of Chk1 Ser317 phosphorylation, an indicator of the DNA damage checkpoint, were stronger in Myc-HBO1 S50/53A cells after UV treatment than in WT cells (Fig. 6B, lanes 3 versus 4, 5 versus 6, 9 versus 10, and 11 versus 12). Although appropriate cell cycle arrest after DNA damage is necessary for completion of DNA repair, Myc-HBO1 S50/53A cells showed continuous cell growth and checkpoint activation. These phenomena indicated that Myc-HBO1 S50/53A cells continued to proliferate with DNA damage. Therefore, we next compared the kinetics of DNA repair in cells that had endogenous HBO1 knocked down and were transfected with Myc-HBO1 WT or S50/53A and then treated with UV irradiation (Fig. 6C). Parental HeLa cells and stable knockdown shHBO1/TG Myc-HBO1 WT cells showed gradual DNA damage repair through 7 h. However, stable knockdown shHBO1/TG Myc-HBO1 S50/53A cells were defective in DNA repair 7 h after UV damage. Furthermore, FACS analysis showed an increase in S phase and a decrease in G_1 phase

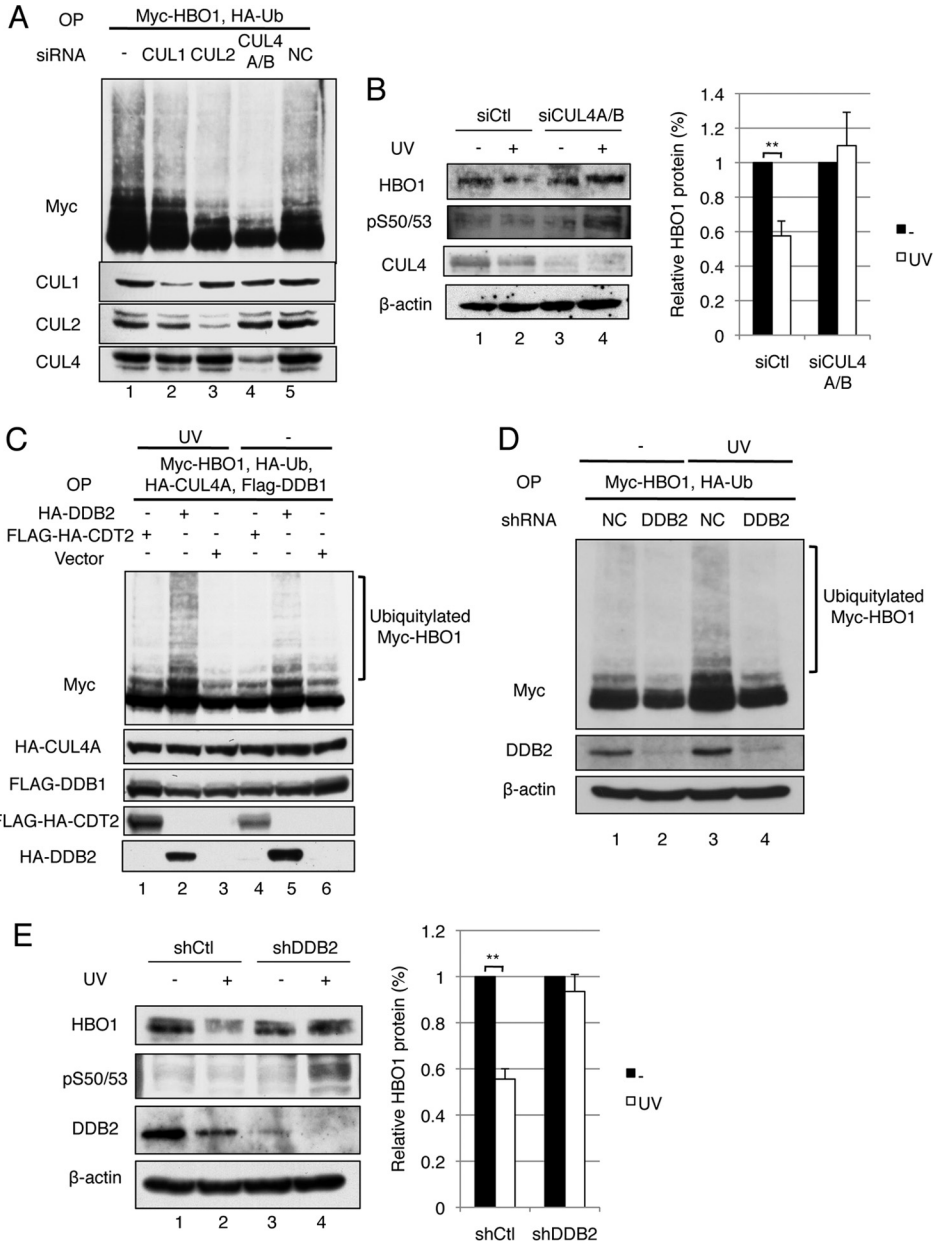

FIG 4 CRL4^{DDB2} ubiquitylates HBO1 in response to UV damage. (A) Depletion of CUL4A/B in HEK293 cells suppressed ubiquitylation of HBO1. CUL1, CUL2, and CUL4A/B were targeted by siRNA transfection for 48 h. Cell lysates were subjected to Western blotting with the indicated antibodies. (B) Depletion of CUL4A/B protected HBO1 phosphorylated at Ser50 and Ser53 from degradation after UV irradiation. CUL4A/B or negative-control siRNA was transfected into HEK293 cells. After 48 h of transfection, cells were exposed to 40 J/m^2 UV light. Cells were incubated for 6 h, and Western blotting was performed with the indicated antibodies. β-Actin was used for normalization of HBO1 expression. The graph indicates the average HBO1 expression levels for three independent experiments. Error bars indicate standard errors (**, $P < 0.01$). (C) Overexpression of DDB2 induced ubiquitylation of HBO1 *in vivo*. Myc-HBO1, HA-Ub, Flag-DDB1, and HA-CUL4A were cotransfected with FLAG-HA-CDT2 or HA-DDB2 into HEK293 cells. Cells were treated with MG132 for 4 h before UV irradiation at 40 J/m^2 and were then cultured for 2 h. Cell lysates were analyzed by Western blotting with the indicated antibodies. (D) Depletion of DDB2 suppressed ubiquitylation of HBO1. shCtl or shDDB2 cells were cotransfected with Myc-HBO1 and HA-Ub. Cells were treated with MG132 for 4 h before UV irradiation at 40 J/m^2 and were then cultured for 6 h. (E) Depletion of DDB2 protected HBO1 phosphorylated at Ser50 and Ser53 from degradation after UV irradiation. shCtl or shDDB2 cells were exposed to 40 J/m^2 UV light. Cells were incubated for 6 h, and Western blotting was performed with the indicated antibodies. β-Actin was used for normalization of HBO1 expression. The graph indicates the average HBO1 expression levels for three independent experiments. Error bars indicate standard errors (**, $P < 0.01$).

for Myc-HBO1 S50/53A cells compared with Myc-HBO1 WT cells after 24 h of UV damage (Fig. 6D; see Fig. S5 in the supplemental material). These data might indicate that Myc-HBO1 S50/53A cells progressed from G_1 into S phase after UV damage and accumulated damaged S50/53A cells in S phase. In addition to continuous cell proliferation after UV damage, we observed an increase in the apoptotic population in Myc-HBO1 S50/53A cells (Fig. 6E).

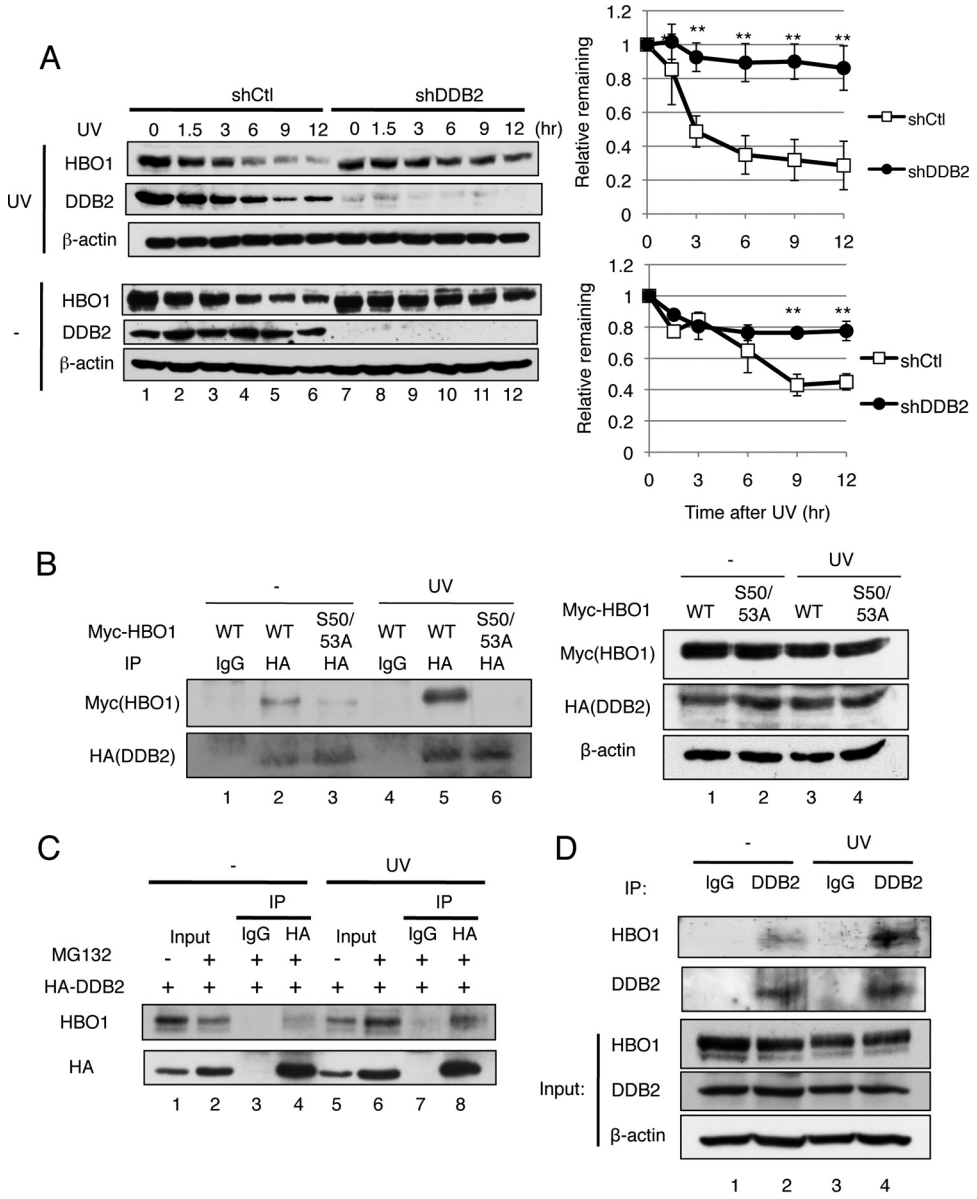

FIG 5 HBO1 interacts with DDB2 in response to UV damage. (A) Depletion of DDB2 suppressed degradation of HBO1 with or without UV treatment. shCtl or shDDB2 cells were exposed or not to 100 J/m^2 UV light and treated with 12.5 μM CHX for the indicated times. β-Actin was used for normalization of HBO1 expression. The graphs indicate the average HBO1 expression levels for three independent experiments. Error bars indicate standard errors (**, $P < 0.01$). (B) The Myc-HBO1 WT but not S50/53A mutant protein interacted with HA-DDB2 after UV irradiation. HEK293 cells were transfected with Myc-HBO1 WT or S50/53A and HA-DDB2. Cells were irradiated with 40 J/m^2 UV light and cultured for 30 min. Immunoprecipitation was performed with anti-HA antibody, and the immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. (C) HA-DDB2 interacted with endogenous HBO1 after UV irradiation. HA-DDB2 was expressed in HEK293 cells. After 48 h of transfection, cells were exposed or not to 40 J/m^2 UV light and then harvested 30 min after UV treatment. HA-DDB2 was immunoprecipitated by use of anti-HA antibody, and immunoprecipitates were subjected to immunoblotting with the indicated antibodies. (D) Endogenous HBO1 interacted with DDB2 after UV irradiation. HEK293 cells were irradiated with 40 J/m^2 UV light and cultured for 10 min. Immunoprecipitation was performed with anti-DDB2 antibody, and the immunoprecipitates were analyzed by immunoblotting with the indicated antibodies.

Together, these data suggest that CRL4^{DDB2}-dependent HBO1 degradation is one of the regulatory mechanisms for controlling cell proliferation and maintaining homeostasis of cells after UV irradiation.

DISCUSSION

Cell proliferation is controlled by multiple regulatory pathways in response to DNA damage, including checkpoints, which sense DNA abnormality and arrest the cell cycle. ATM and ATR are activated at the early steps of the checkpoint by perturbed DNA structures. Several substrates of ATM and ATR, including Chk1/Chk2, p53, and BRCA1, arrest the cell cycle, eliminate damaged cells, and execute DNA repair (4, 5, 42–44). Such events are coordinated to maintain genome integrity. A previous study indicated HBO1 as a candidate ATM/ATR substrate. Although Ser50 and Ser53 of HBO1 were suggested to be targeted by ATM/ATR after

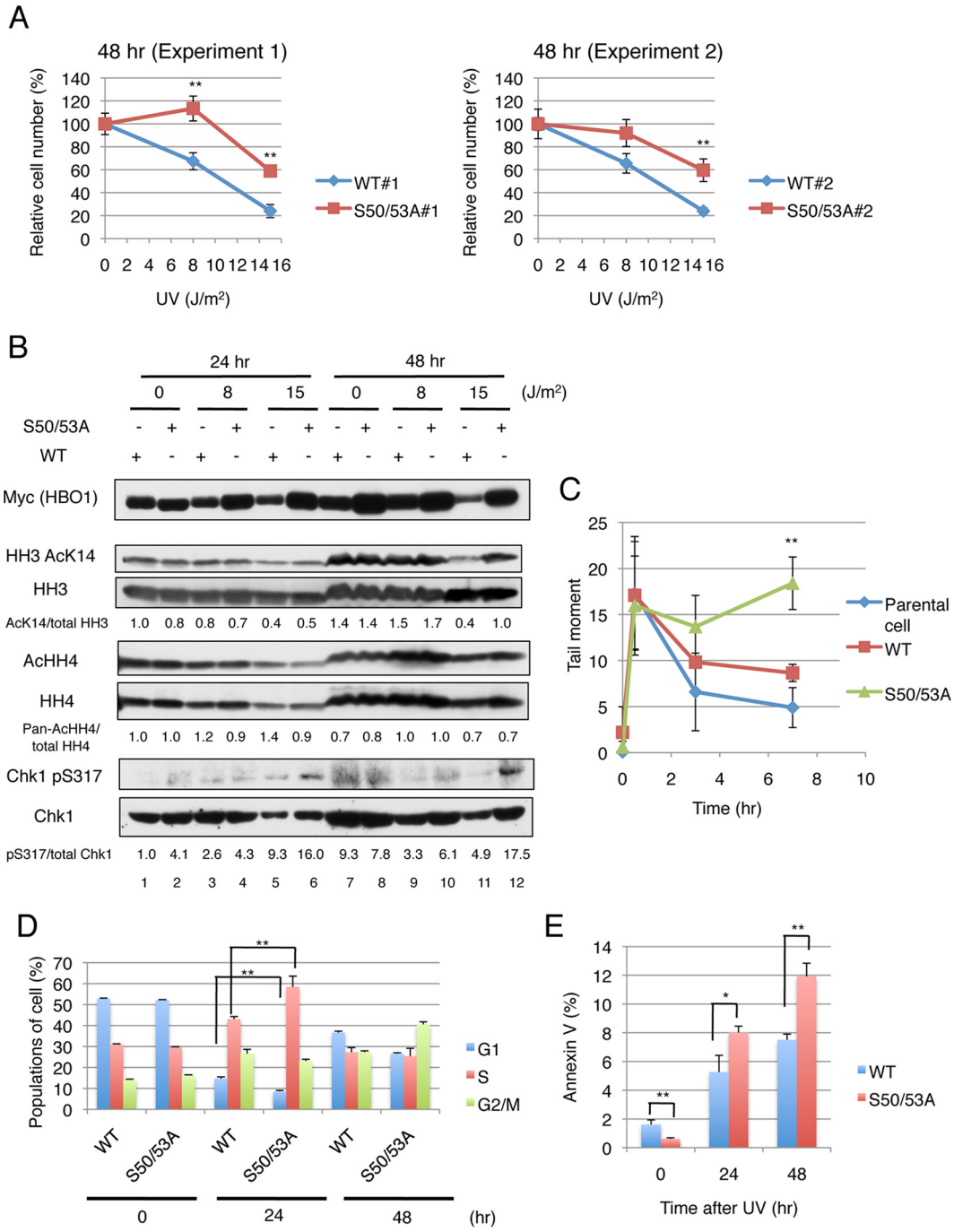

FIG 6 HBO1 S50/53A cells fail to suppress cell proliferation and DNA repair and show increased apoptosis after UV irradiation. (A) HBO1 S50/53A cells failed to suppress proliferation after UV damage. Myc-HBO1 WT and S50/53A TG cells were irradiated with 8 and 15 J/m^2 UV light, and cell numbers were counted 24 and 48 h after UV treatment. Two independent experiments were performed in triplicate with two independent clones and WT and S50/53A cells. Error bars indicate standard errors (**, $P < 0.01$). (B) HBO1 S50/53A cells failed to suppress acetylation of histone H3K14 after UV irradiation. Cell lysates of clone 1 from the experiments for panel A were analyzed for acetylation of histone H3K14, panacetylation of histone H4, and phosphorylation of Chk1 S317 by Western blotting with the indicated antibodies. Signals for histone H3 AcK14/total HH3, pan-acetyl histone H4/total HH4, and phosphorylated Chk1 S317/total Chk1 were quantitated by use of ImageJ. (C) HBO1 S50/53A cells were deficient in DNA repair after UV irradiation. The DNA repair efficiency in stable knockdown shHBO1/TG Myc-HBO1 WT or S50/53A cells was measured by use of alkaline comet assays. Error bars indicate standard errors (**, $P < 0.01$). (D) Cell cycle analysis of HBO1 WT- and S50/53A-expressing cells. HBO1 WT and S50/53A cells were irradiated with 15 J/m^2 UV light and cultured for the indicated times. Cells were labeled with 20 μM BrdU for 40 min before harvest. Cells were fixed with 70% EtOH, and immunostaining was performed with anti-BrdU antibody. Total DNA content was measured by staining with propidium iodide. Cells were separated by FACS for cell cycle analysis. (E) HBO1 S50/53A cells showed increased apoptosis after UV damage. Myc-HBO1 WT and S50/53A TG cells were irradiated with 15 J/m^2 UV light. Cells were incubated for 24 and 48 h after UV treatment. Apoptotic cells were detected with annexin V and analyzed by FACS. Error bars indicate standard errors (*, $P < 0.05$; **, $P < 0.01$).

IR irradiation by use of a phospho-proteomic approach (17), whether other genotoxic stresses also induced ATM/ATR-dependent phosphorylation of these residues and the physiological importance of these phosphorylation events remained unclear.

In this study, we generated a Ser50- and Ser53-phosphorylated HBO1-specific antibody and demonstrated that Ser50 and Ser53 of HBO1 were ATM/ATR-dependently phosphorylated during the normal cell cycle. Furthermore, ATM/ATR-dependent phosphorylation of these residues was enhanced in response to various genotoxic stresses, including UV irradiation. Phosphorylation in the absence or presence of UV treatment was suppressed by ATM and ATR depletion as well as by inhibitors of ATM and ATR kinases. Chk1 is a substrate of ATM and ATR, and Ser317 and Ser345 of mammalian Chk1 are also phosphorylated during the normal cell cycle (45, 46). These phosphorylation events in the absence of DNA damage are essential for the cell cycle, as mutation of either or both Ser317 and Ser345 to Ala results in an accumulation of cells at S phase and cell death. In our cell proliferation experiment, Myc-HBO1 WT and S50/53A cells in log phase without UV treatment showed relatively strong phosphorylation signals of Chk1 Ser317 at 48 h. We speculate that HBO1 may show similar trends, with some portion of Ser50 and Ser53 of HBO1 being phosphorylated during the normal cell cycle. Spontaneous DNA damage would induce low-level phosphorylation of HBO1 during the normal cell cycle, and this would regulate prereplicative complex formation and cell progression from G_1 to S phase via optimum degradation of HBO1. In response to UV damage, phosphorylation of Ser50 and Ser53 of HBO1 by ATM and ATR was immediately increased. The enhanced phosphorylation of Ser50 and Ser53 after UV irradiation aggressively promoted $CRL4^{DDB2}$-dependent ubiquitylation of HBO1 within a few hours.

We predict that the subsequent degradation of HBO1 influences two cell responses, as follows. (i) Although we did not measure acetylation states at replication origins as an indicator of an early response for degradation of HBO1, previous studies indicated that DNA-damaging treatment results in the disappearance of HBO1 from replication origins within 2 h, thus presumably also resulting in decreased acetylation at replication origins (13), and that acetylation at replication origins by HBO1 is essential for pre-RC formation (41). Therefore, HBO1 degradation would first affect the acetylation states of replication origins. Hypoacetylation at replication origins would reduce pre-RC formation and suppress S-phase progression. Although the precise mechanism is still unknown, insufficient prereplicative complex formation may induce activation of the licensing checkpoint (47). We speculate that cell cycle progression of HBO1 S50/53A cells from G_1 to S phase under conditions of UV damage results in avoidance of the licensing checkpoint. The UV-resistant HBO1 S50/53A cells may provide chromosomal conditions to readily load the MCM complex. Indeed, we observed that HBO1 S50/53A protected CDT1 and led to loading of the MCM complex on the chromatin in response to UV damage (data not shown). Thus, cell proliferation is partially prevented by degradation of HBO1 in the presence of DNA damage. Our cell cycle analysis experiment with HBO1 S50/53A cells showed that the S-phase population increased and the G_1-phase population significantly decreased in S50/53A cells in comparison with WT cells 24 h after UV damage. These results suggested that cells expressing degradation-resistant HBO1 S50/53A continued to progress from G_1 to S phase, in contrast to WT cells. In normal cells, HBO1-dependent cell cycle suppression would reduce the risk of the replication machinery encountering DNA damage at the onset of DNA replication. If this regulatory mechanism is defective, the replication process itself would cause severe DNA damage, such as DSBs, and the cells should be removed by apoptosis. Based on our hypothesis, it will be important to measure acetylation states at replication origins of HBO1 S50/53A cells just after UV irradiation.

(ii) Degradation of HBO1 also maintained hypoacetylation states of histone H3K14 for global nucleosomes in HBO1 WT cells until 48 h after UV irradiation. Recently, an exchange of associated factors of HBO1 for acetylation of histone H4 to histone H3 was reported (14). Therefore, we examined the acetylation levels of HBO1 WT and S50/53A cells after UV irradiation. We observed that the acetylation levels of histone H4 between WT and S50/53A cells were almost similar during the experiment. Conversely, acetylation of histone H3K14 in HBO1 WT cells was continuously decreased until 48 h after UV irradiation, while acetylation in the HBO1 S50/53A cells was decreased to levels similar to those in WT cells at 24 h and then recovered at 48 h. Acetylation of histones in nucleosomes is associated with a state of open chromatin accessibility that promotes loading of DNA replication or transcription machineries. Histone deacetylase (HDAC) catalyzes deacetylation of histones to counter these actions and produces a closed, inaccessible chromatin structure. In general, the state of nucleosome acetylation is decided by the balance of HAT and HDAC activities. For example, HDAC11 is suggested to antagonize acetylation of histone H4 by HBO1 at the replication origin in S phase (51). Our results showed that although the amount of HBO1 S50/53A was higher than that of HBO1 WT in cells irradiated with 15 J/m^2 at 24 h, the acetylation levels of histone H3K14 were similar. In contrast to the observations at 24 h, acetylation levels of histone H3K14 at 48 h were recovered and correlated with protein levels of HBO1 S50/53A. We speculate that UV damage activated HDACs 24 h after UV irradiation at 15 J/m^2 and would overcome acetylation activity even if the HBO1 S50/53A protein was maintained. As a result, global acetylation levels in HBO1 WT and S50/53A cells 24 h after UV irradiation were almost similar. In contrast, activation of HDACs by UV damage would be suppressed and H3K14 acetylation levels correlated to HBO1 protein levels at 48 h. Suppression of acetylation of histone H3K14 would reduce the transcription of genes that are necessary for cell proliferation. Zou et al. reported that another ubiquitin ligase of HBO1, SCF^{FBXW15}, mediated HBO1 degradation and decreased acetylation of histone H3K14 in response to pathogenic stress by LPS (20). LPS activated Mek1 to phosphorylate HBO1, which mediated targeting of SCF^{FBXW15} to HBO1. These previous findings suggest that endotoxin impaired the cell replicative capacity by targeting the abundance of a critical epigenetic modifier by using the Mek1-SCF^{FBXW15} apparatus. In the present study, we propose that $CRL4^{DDB2}$ participates in the degradation of HBO1 by UV damage via Ser50/53 phosphorylation of HBO1. The previous study and our results strongly suggest that HBO1 degradation induced by pathogenic stress or UV damage affects global histone acetylation of the entire genome. Thus, both early and late histone deacetylation events induced by degradation of HBO1 would contribute to maintaining chromatin stability after UV damage. The DDB2 complex is a sensor of CPDs in the genome. This complex is activated by UV irradiation and ubiquitylates XPC to play an important role in nucleotide excision repair. DDB2 also induces apoptosis by de-

grading the CDK inhibitor p21. Our findings suggest an additional role for DDB2 in regulating the cell cycle.

Interestingly, our results indicate that CRL4^{DDB2} is involved not only in DNA repair but also in cell cycle control. In this study, we suggest a role for CRL4^{DDB2} in cell proliferation via HBO1 degradation. This raises the question of whether degradation of HBO1 may be involved in DNA repair. HBO1 is a member of the MYST family, which also includes the histone acetyltransferase Tip60. Tip60 is an essential histone acetyltransferase for DNA DSB repair (48, 49). Tip60 acetylation activity of damaged chromatin is required for loading repair proteins. This may also be the case for HBO1. At the early event of global genomic NER, DDB2 recognizes CPDs and facilitates loading of XPC. It is possible that ATM/ATR-phosphorylated HBO1 is recruited to the sites of CPDs by DDB2 and that the recruited HBO1 acetylates histone to change the conformation of damaged chromatin to accelerate loading of repair proteins, including XPC. Acetylation of histones around damage sites would occur at a relatively early step of the NER pathway, because loading of XPC starts within several seconds and continues over 600 s after UV damage (50). We detected ubiquitylated HBO1 2 h after UV irradiation. Degradation of HBO1 might be required for deacetylation of the repaired chromatin. To clarify this possibility, future studies should analyze the activation kinetics of HBO1 HAT activity, HBO1 localization after UV damage, and histone modification around damage sites. Furthermore, nonubiquitylated HBO1 mutants would be useful tools for examining the importance of HBO1 degradation after UV damage in future analyses.

ACKNOWLEDGMENTS

We thank M. Saijo and H. Nishitani for providing CUL4A, DDB1, DDB2, CDT2, and CDT1 expression plasmids. We thank S. Galande for a critical reading of the manuscript and K. Kinpara and our laboratory members for technical assistance.

FUNDING INFORMATION

Ministry of Education, Culture, Sports, Science, and Technology (MEXT) provided funding to Masatoshi Kitagawa under grant number 25290044. Ministry of Education, Culture, Sports, Science, and Technology (MEXT) provided funding to Hiroyuki Niida under grant number 24390072.

REFERENCES

1. **Shiloh Y.** 2001. ATM (ataxia telangiectasia mutated): expanding roles in the DNA damage response and cellular homeostasis. Biochem Soc Trans **29:**661–666. http://dx.doi.org/10.1042/bst0290661.
2. **Flynn RL, Zou L.** 2011. ATR: a master conductor of cellular responses to DNA replication stress. Trends Biochem Sci **36:**133–140. http://dx.doi.org/10.1016/j.tibs.2010.09.005.
3. **Ward IM, Chen J.** 2001. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. J Biol Chem **276:**47759–47762. http://dx.doi.org/10.1074/jbc.C100569200.
4. **Guo Z, Kumagai A, Wang SX, Dunphy WG.** 2000. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in Xenopus egg extracts. Genes Dev **14:**2745–2756. http://dx.doi.org/10.1101/gad.842500.
5. **Liu Q, Guntuku S, Cui X-S, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA, Elledge SJ.** 2000. Chk1 is an essential kinase that is regulated by Atr and required for the G2/M DNA damage checkpoint. Genes Dev **14:**1448–1459.
6. **Mailand N, Falck J, Lukas C, Syljuåsen RG, Welcker M, Bartek J, Lukas J.** 2000. Rapid destruction of human Cdc25A in response to DNA damage. Science **288:**1425–1429. http://dx.doi.org/10.1126/science.288.5470.1425.
7. **Xiao Z, Chen Z, Gunasekera AH, Sowin TJ, Rosenberg SH, Fesik S, Zhang H.** 2003. Chk1 mediates S and G2 arrests through Cdc25A degradation in response to DNA-damaging agents. J Biol Chem **278:**21767–21773. http://dx.doi.org/10.1074/jbc.M300229200.
8. **Abraham RT.** 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev **15:**2177–2196. http://dx.doi.org/10.1101/gad.914401.
9. **Yu J, Zhang L.** 2003. No PUMA, no death: implications for p53-dependent apoptosis. Cancer Cell **4:**248–249. http://dx.doi.org/10.1016/S1535-6108(03)00249-6.
10. **Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, Vogelstein B, Jacks T.** 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev **9:**935–944. http://dx.doi.org/10.1101/gad.9.8.935.
11. **Brugarolas J, Moberg K, Boyd SD, Taya Y, Jacks T, Lees JA.** 1999. Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G1 arrest after γ-irradiation. Proc Natl Acad Sci U S A **96:**1002–1007. http://dx.doi.org/10.1073/pnas.96.3.1002.
12. **Iizuka M, Stillman B.** 1999. Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. J Biol Chem **274:**23027–23034. http://dx.doi.org/10.1074/jbc.274.33.23027.
13. **Miotto B, Struhl K.** 2008. HBO1 histone acetylase is a coactivator of the replication licensing factor Cdt1. Genes Dev **22:**2633–2638. http://dx.doi.org/10.1101/gad.1674108.
14. **Lalonde M-E, Avvakumov N, Glass KC, Joncas F-H, Saksouk N, Holliday M, Paquet E, Yan K, Tong Q, Klein BJ, Tan S, Yang X-J, Kutateladze TG, Côté J.** 2013. Exchange of associated factors directs a switch in HBO1 acetyltransferase histone tail specificity. Genes Dev **27:**2009–2024. http://dx.doi.org/10.1101/gad.223396.113.
15. **Foy RL, Song IY, Chitalia VC, Cohen HT, Saksouk N, Cayrou C, Vaziri C, Cote J, Panchenko MV.** 2008. Role of Jade-1 in the histone acetyltransferase (HAT) HBO1 complex. J Biol Chem **283:**28817–28826. http://dx.doi.org/10.1074/jbc.M801407200.
16. **Avvakumov N, Lalonde ME, Saksouk N, Paquet E, Glass KC, Landry AJ, Doyon Y, Cayrou C, Robitaille GA, Richard DE, Yang XJ, Kutateladze TG, Cote J.** 2012. Conserved molecular interactions within the HBO1 acetyltransferase complexes regulate cell proliferation. Mol Cell Biol **32:**689–703. http://dx.doi.org/10.1128/MCB.06455-11.
17. **Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, III, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP, Elledge SJ.** 2007. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science **316:**1160–1166. http://dx.doi.org/10.1126/science.1140321.
18. **Hershko A, Ciechanover A.** 1998. The ubiquitin system. Annu Rev Biochem **67:**425–479. http://dx.doi.org/10.1146/annurev.biochem.67.1.425.
19. **Kitagawa K, Kotake Y, Kitagawa M.** 2009. Ubiquitin-mediated control of oncogene and tumor suppressor gene products. Cancer Sci **100:**1374–1381. http://dx.doi.org/10.1111/j.1349-7006.2009.01196.x.
20. **Zou C, Chen Y, Smith RM, Snavely C, Li J, Coon TA, Chen BB, Zhao Y, Mallampalli RK.** 2013. SCFFbxw15 mediates histone acetyltransferase binding to origin recognition complex (HBO1) ubiquitin-proteasomal degradation to regulate cell proliferation. J Biol Chem **288:**6306–6316. http://dx.doi.org/10.1074/jbc.M112.426882.
21. **Petroski MD, Deshaies RJ.** 2005. Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol **6:**9–20.
22. **Jackson S, Xiong Y.** 2009. CRL4s: the CUL4-RING E3 ubiquitin ligases. Trends Biochem Sci **34:**562–570. http://dx.doi.org/10.1016/j.tibs.2009.07.002.
23. **Kapetanaki GM, Guerrero-Santoro J, Bisi DC, Hsieh CL, Rapić-Otrin V, Levine AS.** 2006. The DDB1-CUL4ADDB2 ubiquitin ligase is deficient in xeroderma pigmentosum group E and targets histone H2A at UV-damaged DNA sites. Proc Natl Acad Sci U S A **103:**2588–2593. http://dx.doi.org/10.1073/pnas.0511160103.
24. **Wang H, Zhai L, Xu J, Joo H-Y, Jackson S, Erdjument-Bromage H, Tempst P, Xiong Y, Zhang Y.** 2006. Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage. Mol Cell **22:**383–394. http://dx.doi.org/10.1016/j.molcel.2006.03.035.
25. **Sugasawa K, Okuda Y, Saijo M, Nishi R, Matsuda N, Chu G, Mori T, Iwai S, Tanaka K, Tanaka K, Hanaoka F.** 2005. UV-induced ubiquitylation of XPC protein mediated by UV-DDB-ubiquitin ligase complex. Cell **121:**387–400. http://dx.doi.org/10.1016/j.cell.2005.02.035.
26. **El-Mahdy MA, Zhu Q, Wang Q-E, Wani G, Prætorius-Ibba M, Wani AA.** 2006. Cullin 4A-mediated proteolysis of DDB2 protein at DNA dam-

age sites regulates in vivo lesion recognition by XPC. J Biol Chem **281:** 13404–13411. http://dx.doi.org/10.1074/jbc.M511834200.
27. **Stoyanova T, Yoon T, Kopanja D, Mokyr MB, Raychaudhuri P.** 2008. The xeroderma pigmentosum group E gene product DDB2 activates nucleotide excision repair by regulating the level of p21Waf1/Cip1. Mol Cell Biol **28:**177–187. http://dx.doi.org/10.1128/MCB.00880-07.
28. **Groisman R, Polanowska J, Kuraoka I, Sawada J-I, Saijo M, Drapkin R, Kisselev AF, Tanaka K, Nakatani Y.** 2003. The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. Cell **113:**357–367. http://dx.doi.org/10.1016/S0092-8674(03)00316-7.
29. **Matsuda N, Azuma K, Saijo M, Iemura S-I, Hioki Y, Natsume T, Chiba T, Tanaka K, Tanaka K.** 2005. DDB2, the xeroderma pigmentosum group E gene product, is directly ubiquitylated by Cullin 4A-based ubiquitin ligase complex. DNA Repair **4:**537–545. http://dx.doi.org/10.1016/j.dnarep.2004.12.012.
30. **Johmura Y, Osada S, Nishizuka M, Imagawa M.** 2008. FAD24 acts in concert with histone acetyltransferase HBO1 to promote adipogenesis by controlling DNA replication. J Biol Chem **283:**2265–2274. http://dx.doi.org/10.1074/jbc.M707880200.
31. **Nishitani H, Shiomi Y, Iida H, Michishita M, Takami T, Tsurimoto T.** 2008. CDK inhibitor p21 is degraded by a proliferating cell nuclear antigen-coupled Cul4-DDB1Cdt2 pathway during S phase and after UV irradiation. J Biol Chem **283:**29045–29052. http://dx.doi.org/10.1074/jbc.M806045200.
32. **Niida H, Tsuge S, Katsuno Y, Konishi A, Takeda N, Nakanishi M.** 2005. Depletion of Chk1 leads to premature activation of Cdc2-cyclin B and mitotic catastrophe. J Biol Chem **280:**39246–39252. http://dx.doi.org/10.1074/jbc.M505009200.
33. **Nakajima T, Kitagawa K, Ohhata T, Sakai S, Uchida C, Shibata K, Minegishi N, Yumimoto K, Nakayama KI, Masumoto K, Katou F, Niida H, Kitagawa M.** 2015. Regulation of GATA-binding protein 2 levels via ubiquitin-dependent degradation by Fbw7: involvement of cyclin B-cyclin-dependent kinase 1-mediated phosphorylation of Thr176 in GATA-binding protein 2. J Biol Chem **290:**10368–10381. http://dx.doi.org/10.1074/jbc.M114.613018.
34. **Park JH, Park EJ, Lee HS, Kim SJ, Hur SK, Imbalzano AN, Kwon J.** 2006. Mammalian SWI/SNF complexes facilitate DNA double-strand break repair by promoting γ-H2AX induction. EMBO J **25:**3986–3997. http://dx.doi.org/10.1038/sj.emboj.7601291.
35. **Jin J, Arias EE, Chen J, Harper JW, Walter JC.** 2006. A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. Mol Cell **23:**709–721. http://dx.doi.org/10.1016/j.molcel.2006.08.010.
36. **Li X, Zhao Q, Liao R, Sun P, Wu X.** 2003. The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. J Biol Chem **278:**30854–30858. http://dx.doi.org/10.1074/jbc.C300251200.
37. **Ralph E, Boye E, Kearsey SE.** 2006. DNA damage induces Cdt1 proteolysis in fission yeast through a pathway dependent on Cdt2 and Ddb1. EMBO Rep **7:**1134–1139. http://dx.doi.org/10.1038/sj.embor.7400827.
38. **Zhou MI, Wang H, Ross JJ, Kuzmin I, Xu C, Cohen HT.** 2002. The von Hippel-Lindau tumor suppressor stabilizes novel plant homeodomain protein Jade-1. J Biol Chem **277:**39887–39898. http://dx.doi.org/10.1074/jbc.M205040200.
39. **Panchenko MV, Zhou MI, Cohen HT.** 2004. von Hippel-Lindau partner Jade-1 is a transcriptional co-activator associated with histone acetyltransferase activity. J Biol Chem **279:**56032–56041. http://dx.doi.org/10.1074/jbc.M410487200.
40. **Clifford SC, Astuti D, Hooper L, Maxwell PH, Ratcliffe PJ, Maher ER.** 2001. The pVHL-associated SCF ubiquitin ligase complex: molecular genetic analysis of elongin B and C, Rbx1 and HIF-1alpha in renal cell carcinoma. Oncogene **20:**5067–5074. http://dx.doi.org/10.1038/sj.onc.1204602.
41. **Miotto B, Struhl K.** 2010. HBO1 histone acetylase activity is essential for DNA replication licensing and inhibited by geminin. Mol Cell **37:**57–66. http://dx.doi.org/10.1016/j.molcel.2009.12.012.
42. **Wang H-C, Chou W-C, Shieh S-Y, Shen C-Y.** 2006. Ataxia telangiectasia mutated and checkpoint kinase 2 regulate BRCA1 to promote the fidelity of DNA end-joining. Cancer Res **66:**1391–1400. http://dx.doi.org/10.1158/0008-5472.CAN-05-3270.
43. **Tibbetts RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh S-Y, Taya Y, Prives C, Abraham RT.** 1999. A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev **13:**152–157. http://dx.doi.org/10.1101/gad.13.2.152.
44. **Gatei M, Sloper K, Sörensen C, Syljuäsen R, Falck J, Hobson K, Savage K, Lukas J, Zhou B-B, Bartek J, Khanna KK.** 2003. Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation. J Biol Chem **278:**14806–14811. http://dx.doi.org/10.1074/jbc.M210862200.
45. **Niida H, Katsuno Y, Banerjee B, Hande MP, Nakanishi M.** 2007. Specific role of Chk1 phosphorylations in cell survival and checkpoint activation. Mol Cell Biol **27:**2572–2581. http://dx.doi.org/10.1128/MCB.01611-06.
46. **Shimada M, Niida H, Zineldeen DH, Tagami H, Tanaka M, Saito H, Nakanishi M.** 2008. Chk1 is a histone H3 threonine 11 kinase that regulates DNA damage-induced transcriptional repression. Cell **132:**221–232. http://dx.doi.org/10.1016/j.cell.2007.12.013.
47. **McIntosh D, Blow JJ.** 2012. Dormant origins, the licensing checkpoint, and the response to replicative stresses. Cold Spring Harb Perspect Biol **4:**a012955. http://dx.doi.org/10.1101/cshperspect.a012955.
48. **Murr R, Loizou JI, Yang YG, Cuenin C, Li H, Wang ZQ, Herceg Z.** 2006. Histone acetylation by Trrap-Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks. Nat Cell Biol **8:**91–99. http://dx.doi.org/10.1038/ncb1343.
49. **Fischle W.** 2009. Tip60-ing the balance in DSB repair. Nat Cell Biol **11:**1279–1281. http://dx.doi.org/10.1038/ncb1109-1279.
50. **Nishi R, Alekseev S, Dinant C, Hoogstraten D, Houtsmuller AB, Hoeijmakers JH, Vermeulen W, Hanaoka F, Sugasawa K.** 2009. UV-DDB-dependent regulation of nucleotide excision repair kinetics in living cells. DNA Repair (Amst) **8:**767–776. http://dx.doi.org/10.1016/j.dnarep.2009.02.004.
51. **Wong PG, Glozak MA, Cao TV, Vaziri C, Seto E, Alexandrow M.** 2010. Chromatin unfolding by Cdt1 regulates MCM loading via opposing functions of HBO1 and HDAC11-geminin. Cell Cycle **9:**4351–4463. http://dx.doi.org/10.4161/cc.9.21.13596.