The Cullin 4B–Based UV-Damaged DNA-Binding Protein Ligase Binds to UV-Damaged Chromatin and Ubiquitinates Histone H2A

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Abstract

By removing UV-induced lesions from DNA, the nucleotide excision repair (NER) pathway preserves the integrity of the genome. The UV-damaged DNA-binding (UV-DDB) protein complex is involved in the recognition of chromatin-embedded UV-damaged DNA, which is the least understood step of NER. UV-DDB consists of DDB1 and DDB2, and it is a component of the cullin 4A (CUL4A)–based ubiquitin ligase, DDB1-CUL4A^{DDB2}. We previously showed that DDB1-CUL4A^{DDB2} ubiquitinates histone H2A at the sites of UV lesions in a DDB2-dependent manner. Mutations in DDB2 cause a cancer prone syndrome, xeroderma pigmentosum group E (XP-E). CUL4A and its paralog, cullin 4B (CUL4B), copurify with the UV-DDB complex, but it is unclear whether CUL4B has a role in NER as a separate E3 ubiquitin ligase. Here, we present evidence that CUL4A and CUL4B form two individual E3 ligases, DDB1-CUL4A^{DDB2} and DDB1-CUL4B^{DDB2}. To investigate CUL4B's possible role in NER, we examined its subcellular localization in unirradiated and irradiated cells. CUL4B colocalizes with DDB2 at UV-damaged DNA sites. Furthermore, CUL4B binds to UV-damaged chromatin as a part of the DDB1-CUL4B^{DDB2} E3 ligase in the presence of functional DDB2. In contrast to CUL4A, CUL4B is localized in the nucleus and facilitates the transfer of DDB1 into the nucleus independently of DDB2. Importantly, DDB1-CUL4B^{DDB2} is more efficient than DDB1-CUL4A^{DDB2} in mono-ubiquitinating histone H2A *in vitro*. Overall, this study suggests that DDB1-CUL4B^{DDB2} E3 ligase may have a distinctive function in modifying the chromatin structure at the site of UV lesions to promote efficient NER. [Cancer Res 2008;68(13):5014–22]

Introduction

To preserve the integrity and the stability of the genome, cells have developed various mechanisms for repairing DNA damage. In humans, nucleotide excision repair (NER) is the major pathway that removes UV-induced lesions and bulky DNA adducts arising from exposure to carcinogens. Malfunctions of the NER mechanism lead to increased mutations and cancer predisposition. The UV-damaged DNA-binding (UV-DDB) protein complex is involved in the damage recognition step of global genomic NER, and it consists of two proteins, DDB1 and DDB2 (1, 2). Although no mutations of DDB1 have been identified in humans, mutations in DDB2 result in defective NER, as reflected in the heritable sun-sensitive skin cancer phenotype, xeroderma pigmentosum group E (XP-E; refs. 3–6). The finding that certain polymorphisms in DDB2 elevate the risk of primary lung cancer (7, 8) further strengthens the role of DDB2 as a tumor-preventing factor.

UV-DDB is part of a larger protein complex which functions as a cullin-RING ubiquitin ligase (E3 ligase; refs. 9, 10). E3 ligases participate in the ubiquitin-proteasome pathway and their role is to select the substrate and bring it into close proximity to a ubiquitin-conjugating enzyme (E2), a donor of activated ubiquitin (11). Depending on the type of ubiquitination and the site of ubiquitin linkage, the modified substrates are then targeted for degradation by the proteasome or they serve other biological functions (12). Each of the seven cullin molecules (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, and CUL7) expressed in humans forms a base platform for the RING domain protein RBX1, which binds to the COOH-terminal domain of cullin and interacts with E2. The NH_2-terminal domain of the cullin interacts with the substrate-recruiting module, which usually consists of an adaptor and substrate receptor protein. The substrate adaptor protein (assembled on the platform of one cullin molecule) can interact with various substrate receptor proteins, enabling the formation of several E3 ligases with specificity for different substrates (13, 14). In mammals, two closely related types of cullin, CUL4A and CUL4B, have been identified. DDB1 is a substrate adaptor for both forms of CUL4 (15). In addition to DDB2, other WD40-domain proteins have been identified which interact with DDB1 and serve as substrate receptor proteins for DDB1-CUL4–based E3 ligases (13, 15–17). Recent publications have solidified the notion of DDB1-CUL4-RBX1 as a central platform for building E3s involved in various aspects of the UV-damage response, most importantly, cell cycle regulation and NER (refs. 18, 19 and references therein).

CUL4B (~ 104 kDa) is a paralog of CUL4A in humans (~ 87 kDa), and the encoding genes map to *Xq23* and *13q34*, respectively. Alignment of the CUL4 sequences reveals that they are 83% identical. However, CUL4B has a unique NH_2 terminus of 154 amino acids. Mutated CUL4B is the underlying defect in an X-linked mental retardation syndrome in which patients exhibit structural or functional abnormalities in the central nervous system, skeleton, and hematopoiesis (20, 21). The overexpression of CUL4A is linked to breast and hepatocellular cancers (22–24). Studies on the role of CUL4B suggest that it works in cooperation with CUL4A to target certain proteins for degradation (25, 26).

The DDB1-CUL4A^{DDB2} E3 ligase harbors unique properties resulting from the capacity of the substrate-recruiting module (UV-DDB) to bind chromatin-embedded UV-damaged DNA (27, 28), and of the substrate receptor (DDB2) to target multiple substrates (XPC and DDB2) for ubiquitination at the site of photolesions

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(29–31). We recently reported that monoubiquitinated histone H2A (uH2A) in native chromatin coimmunoprecipitates with the endogenous DDB1-CUL4A^{DDB2} ligase in response to UV irradiation. Furthermore, compared with repair-proficient cells, cells with mutations in DDB2 have an altered formation and binding activity of the DDB1-CUL4A^{DDB2} ligase, accompanied by impaired monoubiquitination of histone H2A after UV treatment (32). Another study showed that histone H2A was ubiquitinated during NER by a different complex, Ring2 E3 ligase (33). The monoubiquitination of H2A after a UV insult may allow the relaxation of chromatin needed for generating adequate space to load other NER factors on the damaged DNA and to facilitate repair *in vivo*. A direct link between DDB1-CUL4A^{DDB2} and histones was also confirmed by another group that purified a fraction which contained CUL4A and CUL4B (CUL4-DDB-RBX1), finding that this fraction has an activity that ubiquitinates all core histones *in vitro* (34).

In the present study, we address the role of CUL4B in NER. Therefore, we have performed experiments to test whether CUL4B forms an independent E3 ligase with UV-DDB. Furthermore, we have determined the subcellular localization of CUL4B in repair-proficient cells and repair-deficient XP-E cells, and have examined colocalization with the UV-damaged DNA. We also assessed the capacity of DDB1-CUL4B^{DDB2} E3 ligase to ubiquitinate histone H2A in an *in vitro* assay.

Materials and Methods

Cell lines. Transformed human fibroblasts, WI38-VA13 and HeLa cells (obtained from American Type Culture Collection), were grown in DMEM supplemented with 10% FCS, essential and nonessential amino acids, and glutamax. XP-E primary fibroblasts (XP23PV; ref. 6) and normal human lymphoblastoid (GM01953) and XP-E (GM01646) lymphoblastoid cells (purchased from Coriell Cell Repository) were grown as previously described (6, 32).

UV irradiation and indirect immunofluorescence. Total or micropore UV irradiation and indirect immunofluorescence have been previously described (32, 35). The digital images were captured with a cooled CCD camera, processed and superimposed using SPOT software (Diagnostic Instruments, Inc.).

Plasmids and transfections. The plasmids expressing human DDB2 and CUL4A have been previously described (32). The CUL4A deletion construct *ΔCUL4A* was made by PCR using the full-length human *CUL4A* clone as a template. Restriction sites at the 5′ and 3′ ends and a V5 tag at the COOH terminus were also introduced by PCR, and cloning was performed as described for CUL4A. The human *CUL4B* clone was obtained from Open Biosystems. Full-length *CUL4B* cDNA was PCR-amplified with primers designed to introduce restriction sites at the 5′ and 3′ ends and an HA-tag at the COOH terminus. The PCR product was cloned into the pcDNA 3.1 vector (Invitrogen). *DDB1* cDNA was cloned into pEGFP-N1 (Clontech). WI38-VA13 cells were transiently transfected with the above constructs using Fugene 6 (Roche) according to the manufacturer's instructions, and exposed to ±UV-irradiation 40 h after transfection. XP-E cells were prepared for electroporation using the basic nucleofactor kit for primary fibroblasts (Amaxa Inc.). Cells were then electroporated with several combinations of the above-described plasmids using the program V-13 of the Nucleofactor device (Amaxa) and then were plated onto coverslips and fixed after 40 h.

RNA interference. CUL4 small interfering RNA (siRNA) oligonucleotides and nonspecific siRNA oligonucleotides were synthesized by Qiagen in a purified and annealed duplex form. The sequences targeting the *CUL4A* gene and the *CUL4B* gene were 5′ GAACAGCGAUCGUAAUCAAUU 3′ (sense) and 5′ GAACGUACCUGGUCUUCAUUU 3′ (sense), respectively. The nonspecific sequence used was 5′ UUCUCCGAACGUGUCACGUUU 3′ (sense). siRNA transfections were done with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. HeLa cells were transiently transfected with nonspecific oligonucleotides or with CUL4A and CUL4B oligonucleotides. Cells were collected 4 days after transfection and cellular fractionations were performed. The nuclear fractions were immunoprecipitated with chicken DDB1 antibody.

Antibodies. The production of rabbit DDB2, rabbit DDB1, and chicken DDB1 (DDB1-IgY) antibodies have been previously described (6, 32). Mouse anti-HA (Covance) and mouse anti-chicken IgY (Sigma) were obtained commercially. All other antibodies used were described in a previous publication (32).

Cellular fractionations. Cells were collected and washed several times in cold PBS. Cytosolic, nuclear, and solubilized chromatin fractions were prepared as previously described (32, 36). The solubilized chromatin fraction was obtained by a complete micrococcal nuclease (MNase) treatment of the insoluble chromatin which contains all of the tightly bound chromatin-associated proteins.

Immunoprecipitations and immunoblotting. The methods used for the coimmunoprecipitation of free or chromatin-bound E3 ligase subunits and immunoblotting were described previously (32). In the case of the immunoprecipitations performed with DDB1-IgY antibody, the chicken antibody was immunoprecipitated with mouse anti-chicken IgY for 1 h and then protein G–conjugated agarose beads (Life Technologies) were added. For the purification of DDB1-CUL4A^{DDB2} and DDB1-CUL4B^{DDB2} complexes, human WI38-VA13 cells were cotransfected with *CUL4A-V5* and *Flag-DDB2* cDNAs or *CUL4B-HA* and *Flag-DDB2* cDNAs. The amount of cells plated was 1.5×10^6 and 2.4×10^6, respectively. Forty hours after transfection, cells were collected and lysed in NP40 buffer. The DDB1-CUL4A^{DDB2} or DDB1-CUL4B^{DDB2} complexes were purified through anti-Flag and anti-V5 or anti-HA immunoprecipitations. The lysates were incubated with equilibrated Flag-M2 agarose beads (Sigma) for 2.5 h at 4°C on a rotating platform. Agarose beads were washed with NP40 buffer and then eluted with 100 μg/mL of Flag peptide (Sigma) for 1 h at 4°C on a rotating platform. Each Flag elution was transferred to a new tube and doubled in volume with an NP40 buffer. Anti-V5 or anti-HA antibody was added overnight to immunoprecipitate the CUL4A and CUL4B complexes, respectively. The complexes were further incubated with protein G–conjugated agarose beads for 80 min at 4°C on a rotating platform. The agarose beads were washed with NP40 buffer and the coimmunoprecipitates were separated on 8% SDS gels and transferred to polyvinylidene difluoride membranes which were probed with DDB1, CUL4, and DDB2 antibodies. In parallel, the same coimmunoprecipitates coupled to protein G were used for the *in vitro* ubiquitination reactions.

***In vitro* ubiquitination reactions.** Reactions were performed in 50 mmol/L of Tris (pH 7.4), 1 mmol/L of DTT, 10 mmol/L of $MgCl_2$, 0.2 mmol/L of $CaCl_2$, 4 mmol/L of ATP, 0.1 μg/μL of bovine serum albumin, 2 μmol/L of ubiquitin aldehyde (BostonBiochem), 100 ng of E1 (BostonBiochem), 300 ng of E2 (UbcH5a, BostonBiochem), and 1 μg of FLAG-ubiquitin (BostonBiochem). Human recombinant H2A (Upstate Biotechnology) was used as a substrate. The reactions were incubated for 2 h on a shaking platform at 32°C and were terminated by the addition of a protein-loading buffer. Samples were boiled and analyzed on a 12% SDS-PAGE gel.

Results

Ectopically expressed CUL4A and CUL4B form independent E3 ligases with UV-DDB. It was reported by Wang and colleagues that an E3 ligase containing CUL4A and CUL4B (CUL4-DDB-RBX1) mediated histone ubiquitination (34). Because of the method by which the above E3 ligase was purified, it remained undefined as to whether the two cullins form a single or two separate ligase complexes. To test if each CUL4 could form a complex with UV-DDB *in vivo*, the *CUL4A* and *CUL4B* genes were cloned in a pcDNA 3.1 expression vector and the constructs were transiently transfected into fibroblasts. The cell lysates were immunoprecipitated with either V5 or HA-tag antibodies to pull down CUL4A-V5

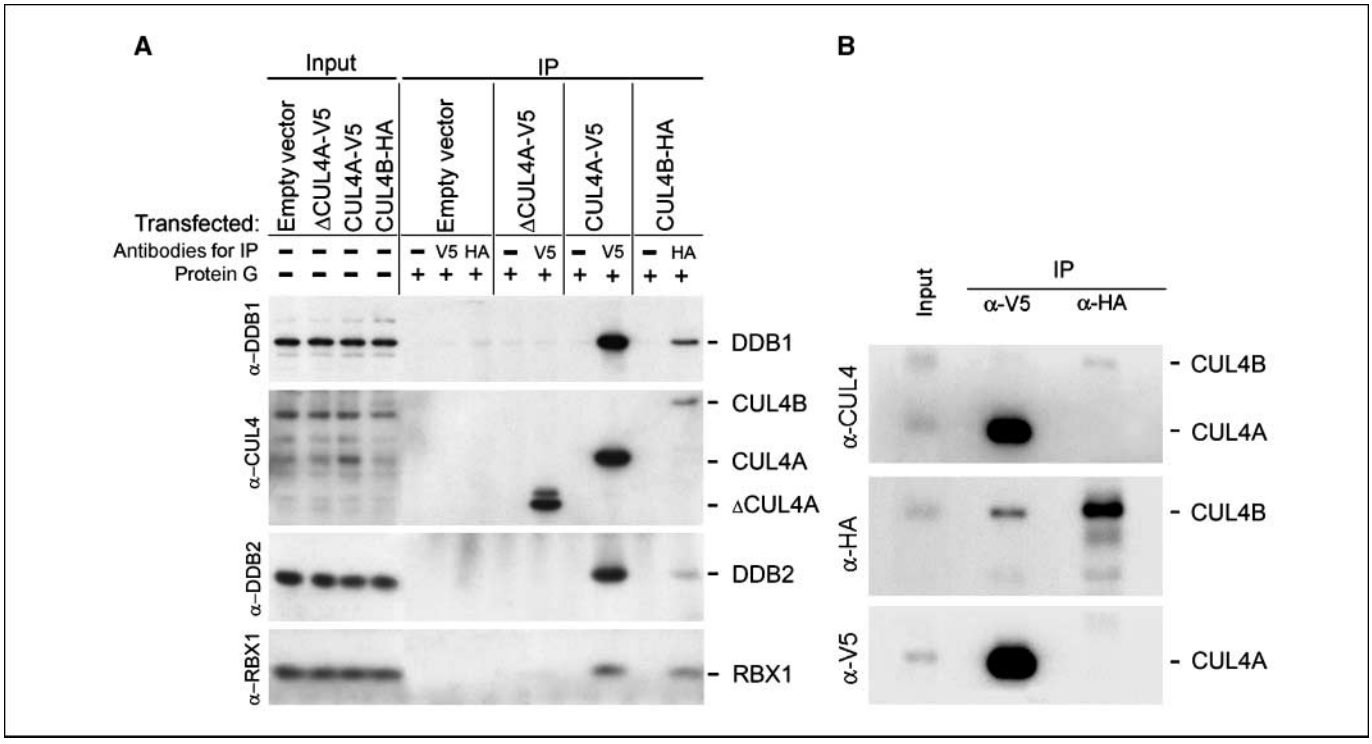

Figure 1. CUL4A and CUL4B interact with UV-DDB to form independent E3 ligases. *A,* human fibroblasts WI38-VA13 were transiently transfected with either empty vector or Δ*CUL4A-V5*, *CUL4A-V5*, or *CUL4B-HA* cDNAs. Cell lysates from the cells transfected with empty vector or Δ*CUL4A-V5* or *CUL4A-V5* were immunoprecipitated with V5 antibody whereas cell lysates from the cells transfected with empty vector or *CUL4B-HA* were immunoprecipitated with HA antibody. Mock immunoprecipitations with protein G alone were also included. The coimmunoprecipitates were analyzed by Western blot with DDB1, CUL4, DDB2, and RBX1 antibodies. *B,* WI38-VA13 cells were transiently cotransfected with both *CUL4A-V5* and *CUL4B-HA* cDNAs. Equal amounts of cell lysates were used to perform immunoprecipitations with V5 or HA antibodies. The coimmunoprecipitates were analyzed by Western blot with CUL4, HA, and V5 antibodies. Two percent of the cell lysates used for immunoprecipitation (*A* and *B*) was loaded as input.

and CUL4B-HA–containing complexes, respectively. Western blot analysis showed that each cullin coimmunoprecipitates with endogenous DDB2, DDB1, and RBX1, and only one cullin form is present in each immunoprecipitate (Fig. 1*A*). Judging by the intensity of the CUL4A and CUL4B signals from the input lanes, obtained with the CUL4 antibody, the ectopically expressed cullins do not seem to contribute substantially to the overall levels of expression (Fig. 1*A*). However, the ectopically expressed CUL4s can be detected with the V5 and HA-tag antibodies (Fig. 1*B*). The *ΔCUL4A* mutant which is missing the first 100 amino acids of the full-length CUL4A did not coimmunoprecipitate DDB1 (Fig. 1*A*). This result is supported by the structural analysis of the DDB1-CUL4A-RBX complex, revealing that the CUL4A amino-terminal domain interacts with DDB1 (13, 37).

To further investigate how the complex would be affected by the coexpression of both forms of CUL4, WI38-VA13 cells were cotransfected with *CUL4A-V5* and *CUL4B-HA* cDNAs, and experiments were performed as described in Fig. 1*B*. Small amounts

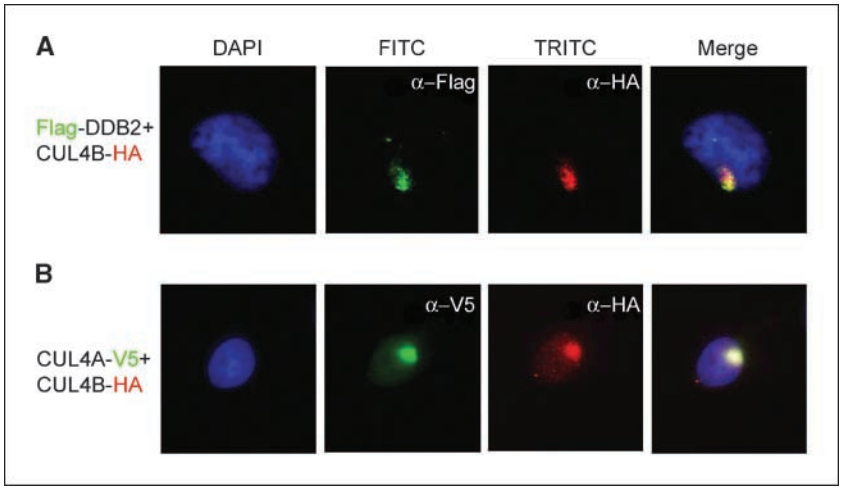

Figure 2. CUL4B colocalizes with DDB2 and CUL4A at UV-damaged DNA sites. *A,* WI38-VA13 cells were transiently transfected with *Flag-DDB2* and *CUL4B-HA* cDNAs. Forty hours after transfection, cells were irradiated through an 8-μm pore filter with a dose of 60 J/m^2. Immediately after treatment, the cells were washed with CSK buffer [100 mmol/L NaCl, 300 mmol/L sucrose, 10 mmol/L PIPES (pH 7.0), 3 mmol/L $MgCl_2$, and protease inhibitors], incubated 5 min in CSK plus 0.2% Triton X-100, and then fixed. Cells were counterstained with 4′,6-diamidino-2-phenylindole (*blue*). DDB2 and CUL4B were visualized with the antibodies against the FLAG (*green*) or HA (*red*) epitope present on the proteins, respectively. Merge shows that the proteins colocalize within the irradiated area. *B,* the experiment was performed as described in *A*, except that the plasmids encoding *CUL4A-V5* and *CUL4B-HA* were cotransfected into WI38-VA13 cells. Merge shows that CUL4A-V5 (*green*) and CUL4B-HA (*red*) colocalize in the UV-irradiated subnuclear spot.

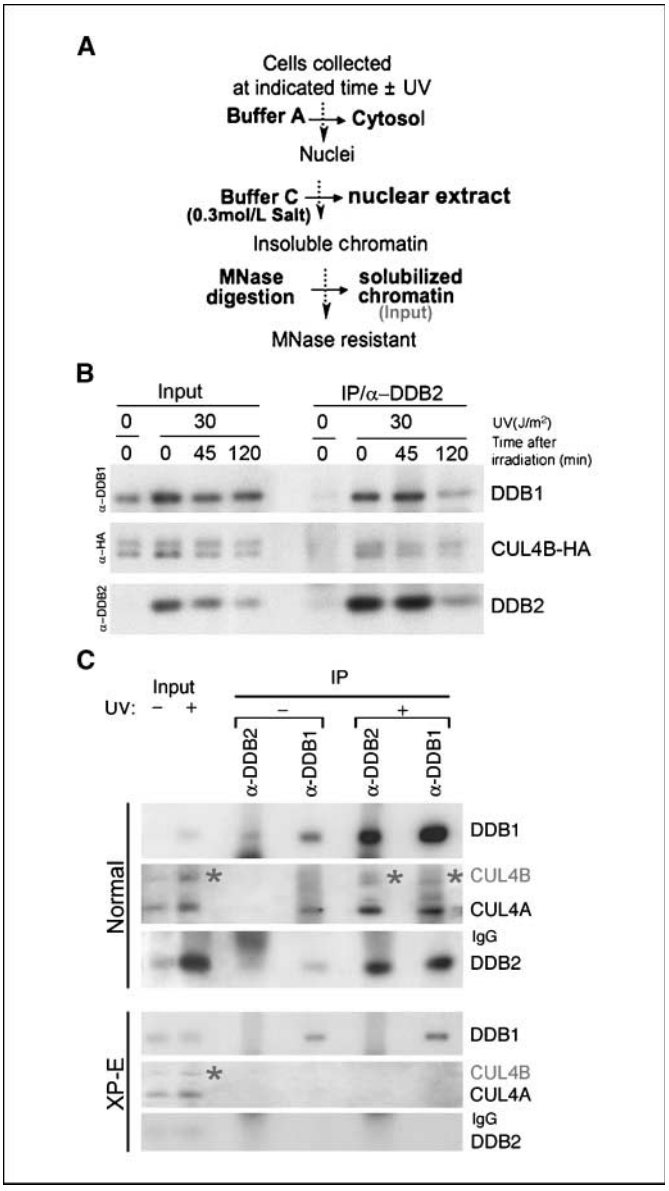

Figure 3. UV irradiation results in elevated levels of DDB1-CUL4B^{DDB2} E3 ligase components in chromatin-bound fractions. *A,* the scheme of the cell fractionation. *B,* ectopically expressed CUL4B, as DDB1-CUL4B^{DDB2} E3 ligase, binds to the chromatin immediately after UV irradiation and is depleted 120 min after the treatment. WI38-VA13 cells were transiently transfected with *CUL4B-HA* cDNA. Forty hours after transfection, cells were irradiated (30 J/m^2) and allowed to recover for the indicated time before being collected. Immunoprecipitation with DDB2 antibody was performed on solubilized chromatin fractions. Coimmunoprecipitates were separated on an 8% SDS gel and proteins were transferred on a polyvinylidene difluoride membrane which was probed with DDB1, DDB2, and HA antibodies. *C,* in XP-E cells, the endogenous CUL4B as a part of DDB1-CUL4B^{DDB2} E3 ligase fails to bind UV lesions. Cellular fractionations were performed on normal and XP-E lymphoblastoid cells that had not been irradiated or had been allowed to recover for 45 min after irradiation (30 J/m^2). The solubilized chromatin-bound fractions were immunoprecipitated with rabbit DDB2- and DDB1 IgY antibodies. The coimmunoprecipitates were analyzed by immunoblotting with DDB1, CUL4, and DDB2 antibodies. One aspect of this figure (the strips with immunoblotting with DDB1 and DDB2 antibodies) was published in our previous article (32) and was included here as a control.

of CUL4B-HA were detected by the anti-HA antibody in the CUL4A-V5 immunoprecipitation, suggesting that the two cullins may occasionally coexist as a complex. However, the immunoprecipitation of CUL4B-HA failed to bring down CUL4A-V5, as verified by CUL4 and V5-tag antibodies (Fig. 1*B*). Taken together, our data suggest that there are two individual UV-DDB–based ubiquitin E3 ligases: DDB1-CUL4A^{DDB2} and DDB1-CUL4B^{DDB2}.

CUL4B colocalizes with UV-damaged DNA. We recently showed that CUL4A, as a part of the DDB1-CUL4A^{DDB2} ligase, colocalizes with DDB2 on UV-damaged DNA (32). To test whether CUL4B colocalizes with DDB2, we applied local UV-irradiation and indirect immunofluorescence. The coexpressed Flag-DDB2 and CUL4B-HA colocalized and produced very strong signals at the damaged DNA subnuclear spot, with very little background in unirradiated areas of the cell (Fig. 2*A*). Furthermore, when WI38-VA13 cells were transfected with cDNAs expressing CUL4A-V5 and CUL4B-HA and treated as above, both cullins colocalized to UV-irradiated areas (Fig. 2*B*). Under our experimental conditions, no cross-reactivity between anti-FLAG and anti-HA antibodies was observed (Supplementary Fig. S1).

The DDB1-CUL4B^{DDB2} complex binds to chromatin after UV irradiation. The DDB1-CUL4A^{DDB2} E3 ligase binds to UV-damaged DNA (32, 38), and subsequently, DDB2, as a substrate receptor

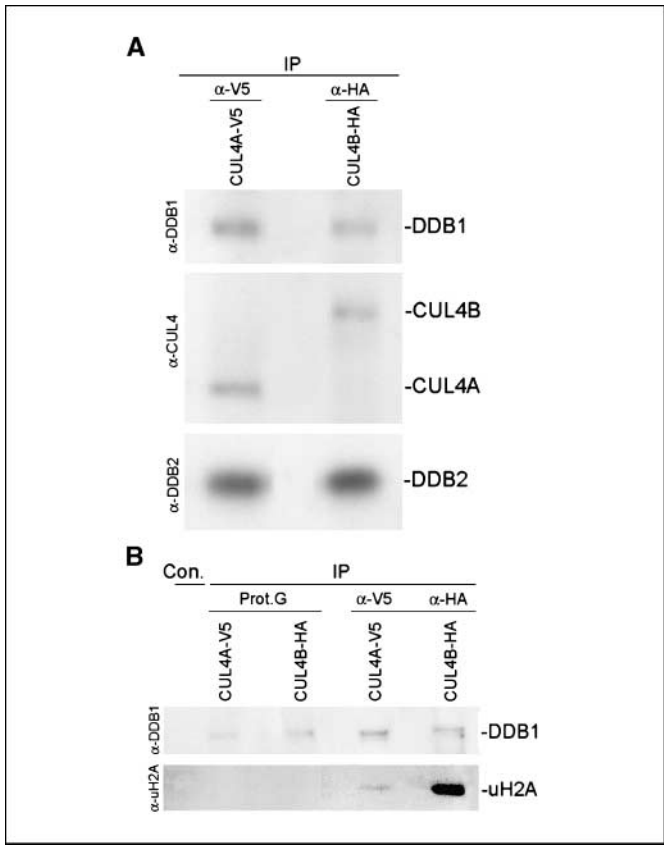

Figure 4. The DDB1-CUL4B^{DDB2} E3 ligase is more efficient than DDB1-CUL4A^{DDB2} E3 ligase in ubiquitinating histone H2A. *A,* WI38-VA13 cells were cotransfected with *CUL4A-V5* and *Flag-DDB2* cDNAs or *CUL4B-HA* and *Flag-DDB2* cDNAs. Forty hours after transfection, the cells were collected and lysed in NP40 buffer. The DDB1-CUL4A^{DDB2} and DDB1-CUL4B^{DDB2} complexes were purified through double-tag immunoprecipitations; first with FLAG and then with either V5 or HA antibodies. Coimmunoprecipitates were separated on 8% SDS gels and polyvinylidene difluoride membranes were probed for the components of the DDB1-CUL4A^{DDB2} or DDB1-CUL4B^{DDB2} complexes (with DDB1, CUL4, and DDB2 antibodies). In parallel, the purified E3 ligases, bound to the protein G beads and mock immunoprecipitated material (without anti-V5 or anti-HA), were used for the *in vitro* ubiquitination reactions in *B*. A ubiquitination reaction without an immunoprecipitated source of E3 was a control for these assays. Reactions were analyzed by Western blot with DDB1 and uH2A antibodies.

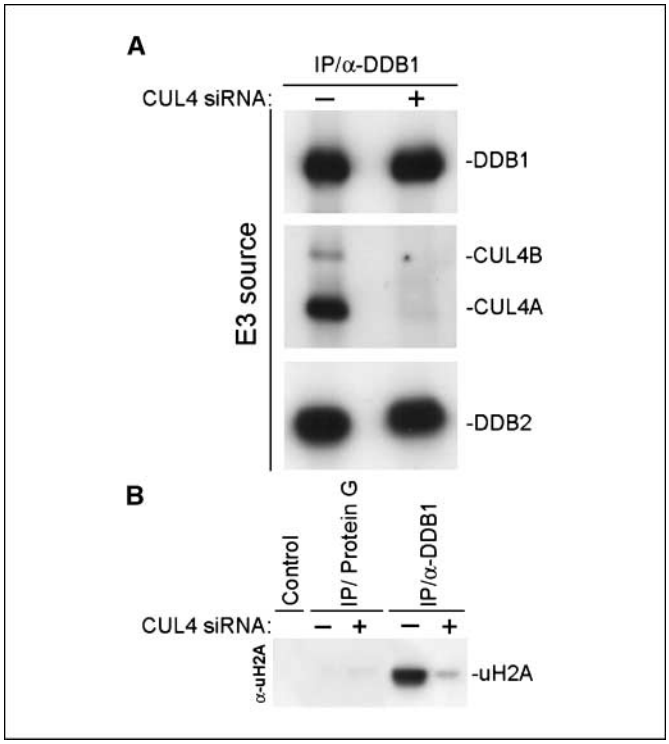

Figure 5. The DDB1-CUL4^{DDB2} targets histone H2A for monoubiquitylation. *A,* HeLa cells were transfected with either nonsilencing siRNA or a combination of siRNAs against CUL4A and CUL4B. Nuclear extracts were prepared and immunoprecipitations were performed with DDB1-IgY antibody. The coimmunoprecipitates were analyzed by Western blot with DDB1, CUL4, and DDB2 antibodies. *B,* immunoprecipitated material from nonsilenced and CUL4-silenced cells was used as a source of E3 ligase in the *in vitro* ubiquitylation reaction with histone H2A as a substrate. Mock immunoprecipitations (without DDB1-IgY and anti-IgY antibodies) were included in the assays. The control for the assays was a ubiquitination reaction without an immunoprecipitated source of E3. Reactions were analyzed by Western blot with uH2A antibody.

protein, undergoes autoubiquitylation and degradation (29, 35, 39), which leads to disengagement of this E3 from the UV-irradiated chromatin (9). To test whether ectopically expressed CUL4B-HA, as the DDB1-CUL4B^{DDB2} E3 ligase, has a similar UV response, we set up a time course experiment. The solubilized chromatin fractions (Fig. 3*A*), collected immediately, 45 min, and 120 min after UV irradiation were subjected to immunoprecipitation using anti-DDB2 (Fig. 3*B*). Western blot analysis of the immunoprecipitated proteins using anti-DDB1, anti-DDB2, and anti-HA confirmed that (*a*) a complex formed by UV-DDB and CUL4B-HA bound tightly to chromatin after UV-irradiation, and (*b*) as DDB2 is degraded following UV treatment, less DDB1 and CUL4B-HA are associated with chromatin as components of the DDB1-CUL4B^{DDB2} E3 ligase. The latter observation is important because it shows that DDB2, not only as a part of the DDB1-CUL4A^{DDB2} E3 ligase but also as a part of the DDB1-CUL4B^{DDB2} E3 ligase, undergoes degradation. The signals for the levels of DDB2, DDB1, and CUL4B-HA in the inputs reflect the response of the DDB1-CUL4B^{DDB2} E3 ligase to UV irradiation. In nonirradiated cells, DDB2 is not detected in the solubilized chromatin, whereas detectable amounts of DDB1 and CUL4B are bound to the chromatin independent of the UV-irradiation (input for 0 min, 0 J/m^2), presumably apart from the complex which they form with DDB2 (Fig. 3*B*).

We further asked if endogenous CUL4B binds to the chromatin in a DDB2-dependent manner. In the XP-E cells the interaction between DDB1 and mutated DDB2 is impaired and consequently, DDB1-CUL4A^{DDB2} E3 ligase fails to bind to UV-damaged chromatin and ubiquitinate histone H2A (32). If DDB2, as a substrate receptor for DDB1-CUL4B^{DDB2} E3 ligase, is responsible for targeting a substrate for ubiquitination at the site of a photolesion, the accumulation of this E3 ligase would be impaired in the solubilized chromatin fraction of XP-E cells. We probed the membrane which was used in the past for the detection of CUL4A (32) to investigate the chromatin binding of the DDB1-CUL4B^{DDB2}E3 ligase in normal and XP-E cells before and 45 min after UV irradiation. CUL4 antibody was used to detect both forms of CUL4 in the chromatin-bound fraction (*inputs*, Fig. 3*C*) but the signal corresponding to CUL4A was stronger. This suggests that either CUL4A is more abundant than CUL4B, or the CUL4 antibody has higher affinity towards CUL4A. In addition to CUL4A, CUL4B was coimmunoprecipitated from the UV-damaged chromatin of normal cells with DDB1 and DDB2 antibodies (Fig. 3*C*). UV-treated XP-E lymphoblastoid cells failed to show any enrichment for CUL4B, CUL4A, or DDB2 proteins in chromatin (Fig. 3*C*), demonstrating a deficiency in both CUL4-based UV-DDB E3 ligases. The presence of CUL4s in the input lanes of the XP-E fractions is in agreement with the formation of multiple CUL4-based complexes interacting with chromatin (17, 18). Taken together, the above data (Figs. 2 and 3) suggest that DDB1-CUL4B^{DDB2} E3 ligase is targeted to UV-damaged chromatin by DDB2 and presumably plays a role in the initiation of NER.

The CUL4-based UV-DDB E3 ligases ubiquitinate histone H2A with a different efficiency. Finding DDB1-CUL4B^{DDB2} as well as DDB1-CUL4A^{DDB2} associated with chromatin after UV irradiation has raised the question as to which E3 ligase is responsible for the previously observed ubiquitination of histone H2A. For the purpose of testing these two E3 ligases, WI38-VA13 cells were transfected to express either CUL4A-V5 or CUL4B-HA along with Flag-DDB2. After two sequential immunoprecipitations, first with anti-FLAG and then either with anti-V5 or anti-HA antibodies, the composition of the precipitated cullin complexes was verified by a Western blot. The amounts of both DDB1 and DDB2 were comparable between the two complexes but importantly, only one form of CUL4 was detected in each (Fig. 4*A*). In parallel, the same complex preparations and the appropriate mock controls (Fig. 4*B*) were tested in an *in vitro* ubiquitination assay using histone H2A as a substrate. By probing for DDB1, we were able to normalize for the amounts of the two immunoprecipitated cullin complexes and compare their ubiquitination activities. As shown in Fig. 4*B*, the DDB1-CUL4B^{DDB2} can ubiquitinate histone H2A much more efficiently than DDB1-CUL4A^{DDB2}.

To further investigate the role of CUL4-based UV-DDB E3 ligases in histone H2A ubiquitination and to eliminate the possibility that the immunoprecipitations of UV-DDB (Figs. 3*C* and 4*A*) somehow pulled down an undetected non–CUL4-based E3 ligase (e.g., Ring2 E3 ligase that has also been implicated in monoubiquitination of histone H2A; ref. 33), we performed *in vitro* ubiquitination assays with immunoprecipitated UV-DDB E3 complexes from cells in which the levels of CUL4A and CUL4B were decreased by silencing. For that purpose, HeLa cells were transiently transfected with siRNAs against CUL4A and CUL4B or control nonsilencing siRNAs. The endogenous UV-DDB E3 complexes were affinity-purified from the 0.3 mol/L nuclear fractions. Although DDB1-IgY antibody pulled down similar amounts of UV-DDB complexes from both the control and silenced samples, there was an obvious decrease of CUL4A

and CUL4B in the cells which were treated with CUL4s-siRNA (Fig. 5*A*). It is apparent from these data that the UV-DDB complex can form in the absence of CUL4A and CUL4B.

The immunoprecipitated complexes from nonsilenced and CUL4s-silenced cells were tested in an *in vitro* ubiquitination assay with histone H2A as a substrate. Appropriate mock and control reactions were also included. As shown in Fig. 5*B*, the treatment of cells with siRNAs against CUL4A and CUL4B severely reduced the ability of the immunoprecipitated complexes to monoubiquitinate histone H2A. Based on the above observations, we conclude that the attenuation of CUL4 expression by specific siRNA treatment results in the loss of ligase activity, verifying that it is indeed CUL4-based ligases which perform the ubiquitination of histone H2A.

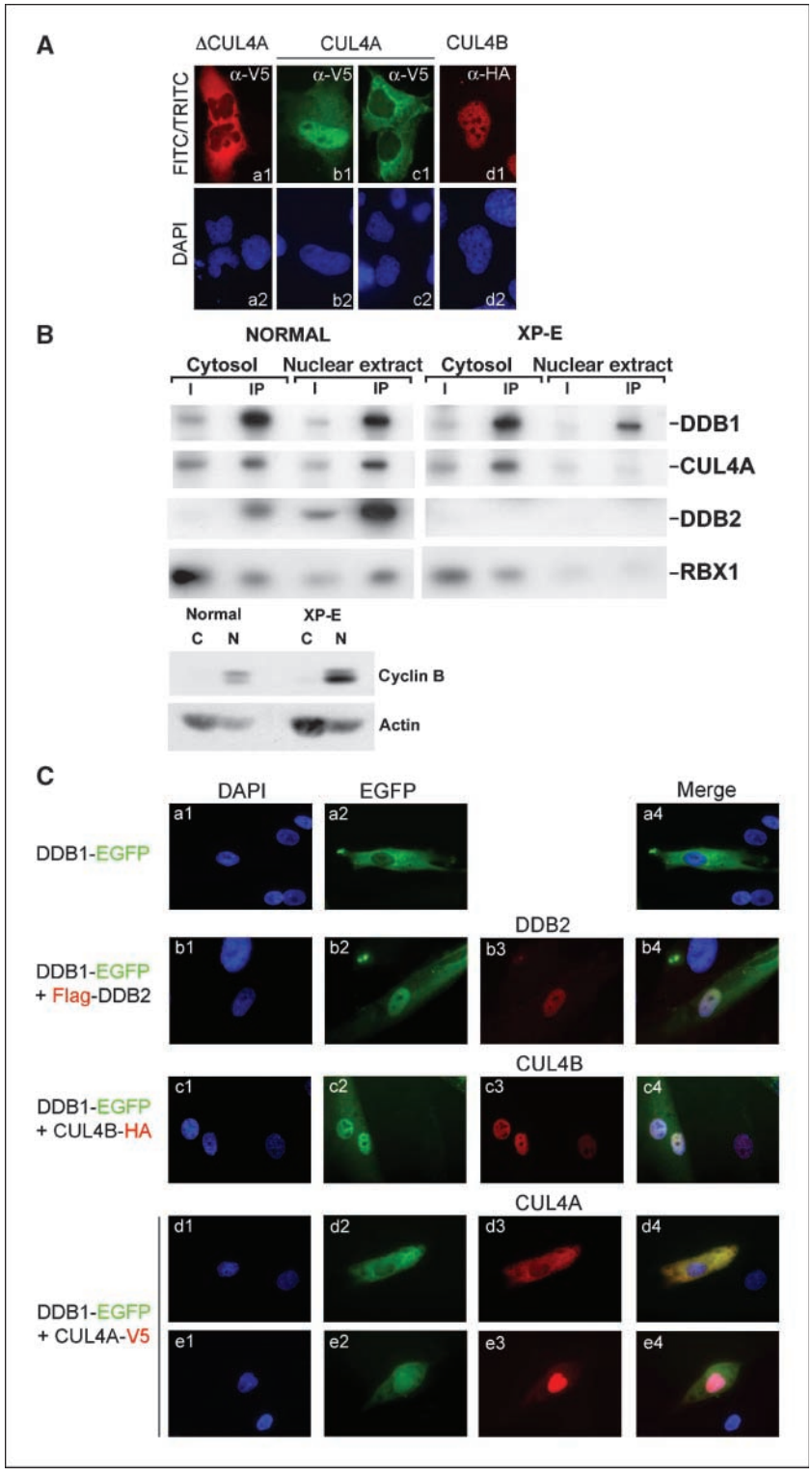

Figure 6. CUL4B and DDB2 facilitate the transfer of DDB1-CUL4-RBX1 E3 ligases into the nucleus. *A,* subcellular localization of ΔCUL4A, CUL4A, and CUL4B. WI38-VA13 cells were transiently transfected with *ΔCUL4A-V5*, *CUL4A-V5*, or *CUL4B-HA* cDNAs. Cells were fixed 40 h after transfection and counterstained with 4′,6-diamidino-2-phenylindole (*blue*). Indirect fluorescent images of CUL4A were visualized by the antibody to the V5 epitope present on CUL4A (*green*) and ΔCUL4A, or the HA epitope present on CUL4B (*red*). *B,* mutations in DDB2 affect the composition and the subcellular localization of DDB1-CUL4A^{DDB2} E3 ligase in XP-E cells. Cytosol and nuclear extract were prepared from 1×10^7 cells from a normal (GM01953) and an XP-E cell line (GM01646), as depicted on Fig. 3*A*, and immunoprecipitated (*IP*) with DDB1-IgY antibody. The coimmunoprecipitates were analyzed by Western blotting with DDB1, CUL4, DDB2, and RBX1 antibodies. As a control for cell fractionation, the inputs (*I: C,* cytosol; *N,* nuclear extract) used for coimmunoprecipitations were additionally probed with actin and cyclin B antibodies. *C,* CUL4B assists the translocation of DDB1 into the nucleus in XP-E cells. XP-E primary fibroblasts (XP23PV) were electroporated with EGFP-DDB1 alone or in combination with each of the following expressing cDNAs: *Flag-DDB2, CUL4B-HA*, and *CUL4A-V5*. The cells were then plated on coverslips and fixed 40 h later. DDB1 was visualized directly by the EGFP-tag on it. DDB2, CUL4A, and CUL4B were visualized as in Fig. 2.

Subcellular localization of CUL4A and CUL4B. Using the program for prediction of subcellular localization PSORTII,[1] only one putative nuclear localization signal (NLS) was identified in CUL4A, which resided between the 729 and 735 amino acid residues. In addition to this signal, three putative NLSs were identified within the first 114 residues of CUL4B (a unique sequence for CUL4B). According to calculations of NLS scores by the same program, CUL4A has a low nuclear localization potential (−0.22), whereas CUL4B shows a value of 0.62. The abundance of NLSs in CUL4B and the higher NLS score suggests a predominantly nuclear localization for this molecule whereas CUL4A's nuclear localization is less certain.

For the purpose of determining the cellular localization of the two forms of CUL4, the WI38-VA13 cells, in which CUL4A-V5 or CUL4B-HA were expressed, were stained with the appropriate antibodies against the V5 and HA-tags. As predicted, CUL4B was exclusively localized in the nucleus (Fig. 6*A, d1*). However, CUL4A was found in both the cytosol and the nucleus (Fig. 6*A, b1* and *c1*). Approximately 30% of the cells have cytoplasmic staining, 30% have nuclear staining, and the rest are stained in both cellular compartments. Upon deletion of the first 100 amino acids of CUL4A (ΔCUL4A), the protein's detection was restricted to the cytosol (Fig. 6*A, a1*).

CUL4B mediates the nuclear entry of DDB1 independently of DDB2. DDB2 regulates the nuclear import of the UV-DDB complex, which is in agreement with the observation of several putative NLS identified only in DDB2 (6, 40). To address the question of whether DDB2 is the main factor for delivering the DDB1-CUL4A-RBX complex into the nucleus, we used a DDB1 antibody to perform immunoprecipitations with the cytosol and nuclear fractions extracted from normal and XP-E cells, and assessed the levels of the E3 components. The DDB1-CUL4A-RBX complex is precipitated in the cytosol fraction independently of the DDB2 status and a decreased amount of DDB1 is localized in the nuclear extract of XP-E cells (Fig. 6*B*). Compared with normal controls, the levels of CUL4A and RBX were also significantly diminished in the nuclei of XP-E cells (Fig. 6*B*), supporting the role of DDB2 in the nuclear import of the DDB1-CUL4A-RBX complex. Nevertheless, the presence of some DDB1 in the nuclear fraction of XP-E cells prompted us to speculate that CUL4B, having four putative NLSs, could contribute to the translocation of DDB1 into the nucleus in the absence of functional DDB2.

To investigate this hypothesis, we used the primary fibroblasts from an XP-E patient, XP23PV, in which we previously confirmed that mutations in the *DDB2* gene result in an undetectable level of the protein and a low level of UV-induced DNA-repair synthesis (6). We electroporated the fibroblasts with different combinations of cDNAs and stained with the appropriate antibodies. When EGFP-DDB1 alone was expressed, it localized primarily in the cytosol (Fig. 6*C, a2*). As expected, the coexpression of EGFP-DDB1 and Flag-DDB2 resulted in the translocation of DDB1 into the nucleus (Fig. 6*C, b2*), presumably as a UV-DDB complex. Importantly, when EGFP-DDB1 was coexpressed with CUL4B-HA, similar to CUL4B, the EGFP-DDB1 was exclusively localized in the nucleus (Fig. 6*C, c2*). However, the coexpression of EGFP-DDB1 with CUL4A-V5 resulted in a DDB1 distribution pattern similar to the localization of CUL4A: predominantly cytosolic or predominantly nuclear (Fig. 6*C, d2* and *e2*).

Discussion

The work presented in this article focuses on the properties and the role of a novel CUL4B-based UV-DDB E3 ligase in NER. We have previously shown the association of CUL4A and DDB2 with UV-damaged chromatin after micropore UV irradiation (32), and here, we provide evidence for the accumulation of CUL4B at the same sites, suggesting that both cullins play a role during NER. Moreover, the association of both CUL4A and CUL4B with damaged DNA and the coimmunoprecipitation of each one with DDB1, DDB2, and RBX1 argues for an early recruitment during the initiation of NER.

Our current data supports the formation of two separate CUL4 complexes, although the proposed model by Wang and colleagues portrays the two CUL4s as parts of the same E3 ligase (34). Under our experimental conditions, either CUL4A or CUL4B associates with UV-DDB and RBX1 to form an E3 ligase. This result corroborates the currently accepted model of cullin-based E3 ligases in which E3 ligase contains one cullin molecule (14). The crystal structure of CUL1- and CUL4A-based E3 ligases further supports the above model (13, 41). The notion that CUL4B-based ligases exist on their own and have distinct roles is supported by the recent finding that the dioxin receptor (AhR) is a CUL4B-based E3 ubiquitin ligase and no CUL4A was detected in the complex (42). Nevertheless, we cannot exclude the possibility that DDB1-CUL4A^{DDB2} and DDB1-CUL4B^{DDB2} could dimerize *in vivo*, as recently shown for SCF^{Fbw7} (43).

The identification of two separate complexes has inevitably provoked questions regarding their subcellular localization as well as their role in NER. The fact that ectopically expressed CUL4B is primarily nuclear supports the notion that CUL4B can be transported into the nucleus independently of other factors, probably due to its strong NLS, as shown for mouse Cul4B (44). More importantly, CUL4B as a part of a DDB1-CUL4-RBX1–based E3 ligase could contribute to the complex's transport into the nucleus. This concept is reinforced by the translocation of ectopically expressed cytoplasmic DDB1 into the nucleus of XP-E cells upon the coexpression of CUL4B. Contrary to CUL4B, CUL4A appeared in both the nucleus and the cytosol, suggesting a more complex mechanism for entering the nucleus. The fact that ΔCUL4A, which is missing the DDB1 interaction domain, is exclusively cytoplasmic, suggests that this cullin depends on DDB1 to enter the nucleus. It is known that DDB1 does not translocate into the nucleus on its own (40, 45), but it can act as a bridge for the recruitment of proteins with a strong nuclear localization ability such as DDB2. As shown in this article, in XP-E cells, some of the ectopically expressed CUL4A will still enter the nucleus, presumably by means of DDB1's interaction with other substrate receptor proteins (13, 15–17). Overall, the difference in the subcellular localization of CUL4A and CUL4B suggests an independent regulation of the nuclear import of the two CUL4-based E3 ligases.

Similar to other factors which are involved in the recognition step of NER, the newly identified E3 ligase, DDB1-CUL4B^{DDB2}, was tightly bound to chromatin after UV irradiation, posing a question about its substrate for ubiquitination at the site of UV lesions. After we reported that the DDB1-CUL4A^{DDB2} E3 ligase is involved in UV-induced histone H2A ubiquitination, as a response to UV

[1] http://psort.nibb.ac.jp/

treatment, another group identified the Ring2 E3 ligase as the main ligase responsible for the monoubiquitination of H2A in response to UV irradiation (33). Furthermore, as mentioned above, a third group identified histones H3 and H4 as targets of the CUL4-DDB-RBX1 E3 ligase (34). In an attempt to combine all reports, it was proposed that both DDB1-CUL4^{DDB2} and Ring2 ligases are recruited to UV-induced lesions to modify histones, and thus, facilitate different steps of the NER pathway (18). With this new information, it was necessary to exclude the possibility that another non–CUL4 ligase was coimmunoprecipitated in our previous (32) and current preparations, and that this undetected ligase was responsible for the observed ubiquitination of H2A. For that purpose, we silenced both CUL4s through siRNA and we tested the efficiency of the immunoprecipitated complex in ubiquitinating histone H2A, with the assumption that the missing CUL4 component would not affect the formation and immunoprecipitation of the undetected non–cullin ligase. The dramatic decrease of uH2A levels as a result of CUL4 silencing confirms that it is indeed CUL4-based E3 ligases which target histone H2A for ubiquitination. The selective silencing of either CUL4A or CUL4B was also attempted in an effort to dissect the role of each CUL4. However, our endeavors to lower the expression of each of the CUL4s individually, using published sequences of siRNA (26, 29, 46), resulted in CUL4B silencing by siRNA against CUL4A and vice versa. In the case in which the reported silencing of CUL4 was achieved by a cotransfection of siRNAs against CUL4A and CUL4B (26, 46), the effect of each individual siRNA was unknown. In another study in which siRNA against CUL4A was used, the effect on CUL4B was not assessed (29).

We reported that histone H2A is monoubiquitinated by DDB1-CUL4A^{DDB2} E3 ligase at UV-induced DNA lesions (32), but based on the data presented here, we speculate that the CUL4B-based E3 ligase was also present in our previous preparations. Our current findings created a certain ambiguity as to which cullin complex was responsible for the observed ubiquitination of histone H2A, which needed to be addressed. In lieu of a highly specific antibody against each of the CUL4 forms, we engaged in the ectopic expression of tagged CUL4A and CUL4B molecules. The tagged cullin molecules were used to pull down the E3 ligase complexes which were used to ubiquitinate histone H2A in *in vitro* assays. The immunoprecipitated DDB1-CUL4B^{DDB2} E3 ligase proved to be a more potent ligase for the *in vitro* ubiquitination of histone H2A compared with DDB1-CUL4A^{DDB2}. Based on this result, we conclude that it is the DDB1-CUL4B^{DDB2} E3 ligase which is mainly responsible for H2A ubiquitination.

In this study, we show that the CUL4B-based UV-DDB E3 ligase is involved in the initiation step of NER and targets histone H2A for monoubiquitination. This finding, together with our previous publication (32), reinforces the notion that UV-DDB assembled on the CUL4A or CUL4B-RING platforms of ubiquitin ligases, is a link between DNA repair, chromatin, and ubiquitination. More studies are necessary to define the target specificity of each CUL4 ligase, but it is possible that each complex targets a different substrate during global genomic NER. It is not inconceivable that under extreme circumstances, the intrinsic ability of CUL4B to localize to the nucleus would ensure the presence of at least one active E3 ligase to support the cellular UV response. The availability of cell lines from patients carrying CUL4B mutations would allow further studies to determine in detail the role of CUL4B in NER and in other pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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