Cullin 4A-mediated Proteolysis of DDB2 Protein at DNA Damage Sites Regulates in Vivo Lesion Recognition by XPC*

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The genome of all living organisms is constantly subjected to damage by many kinds of physical and chemical agents. Prompt repair of DNA damage enables cells to overcome genotoxicity and retain normal cellular functions. Nucleotide excision repair (NER) is a versatile DNA repair pathway that eliminates a wide variety of helix-distorting DNA lesions, including UV-induced cyclobutane pyrimidine dimers (CPD) and 6–4 pyrimidine-pyrimidone photoproducts (6–4PP). Mammalian NER consists of two distinct subpathways: global genomic repair (GGR), which operates throughout the genome, and transcription-coupled repair (TCR), which works on damage within transcribed DNA strands of transcriptionally active genes (1, 2). Loss or impairment of NER is associated with several rare autosomal recessive genetic disorders, e.g. Xeroderma pigmentosum (XP) and Cockayne syndrome (3). XP syndrome is characterized by hypersensitivity to sunlight and a predisposition to skin cancer. Seven XP complementation groups have been identified, and all of the corresponding genes, from XPA to XPG, have been cloned (4).

Mutation in XPC results in the loss of GGR but not TCR, suggesting that the XPC gene encodes a damage recognition factor involved in GGR (5). The XPC protein binds in vivo to hHR23B, which is one of the two mammalian homologs of Saccharomyces cerevisiae Rad23 protein (6, 7). Biochemical studies have revealed that XPC-hHR23B is a structure-specific DNA binding factor (8) and it is one of the six core factors (Replication protein A, XPA, XPC-hHR23B, XPG, ERCC1-XPF, Transcription factor II H) essential for damage recognition and direct incision in vitro (9). Accumulating evidence indicates that the XPC protein plays an essential role in the damage recognition process of GGR (10–12). Like XPC cells, GGR is impaired in XP-E cells whereas TCR remains unaffected (13). XP-E cells lack the damaged DNA binding activity of the damaged DNA-binding protein (DDB), which is composed of the DDB1 (or p127) and the DDB2 (or p48) subunits (14). Mutation in the DDB2 gene is responsible for the phenotypic features of XP-E cells (15–20). DDB has a much higher affinity and specificity for damaged DNA than XPC, especially with regards to binding to 6–4PP (21–24). Despite its capability of binding to damaged DNA, DDB is not required for cell-free NER reconstituted in vitro (25–28). However, GGR of CPD is profoundly reduced in XP-E cells, whereas the repair of 6–4PP is only moderately impaired (16). Moreover, ectopic expression of human DDB2 in Chinese hamster cells enhances the removal of CPD from the genome and suppresses UV-induced mutagenesis (29). These findings suggest that DDB plays an important role in the recognition of CPD in vivo. DDB2, like XPC, accumulates at DNA damage sites immediately after UV irradiation (30, 31). Recent evidence further indicates that DDB activates the recruitment of XPC to CPD in vivo (32). Besides acting on CPD, DDB mediates efficient targeting of XPC to 6–4PP and accelerates its repair (33). Thus, DDB can be considered as the initial damage recognition factor for UV-induced photoproducts.

Although the precise role of DDB in NER remains unclear, it has been known that DDB2 is regulated at the transcriptional level by tumor suppressor p53 (13) and at the posttranslational level via the ubiquitin (Ub)-proteasome system (34–37). In eukaryotic cells, this pathway...
mediates a selective degradation of many cellular proteins (38). The target proteins are labeled by covalent attachment of multiple moieties of Ub, a highly conserved 76-residue eukaryotic protein. The poly(Ub)-conjugated substrates are recognized and destroyed by the proteasome. The attachment of Ub to a protein substrate is preceded by a cascade reaction of three enzymes, namely, the E1 Ub-activating enzyme, the E2 Ub-conjugating enzyme, and the E3 Ub-protein ligase. Cullin 4A (CUL-4A)-based Ub ligase appears to be responsible for UV-induced DDB2 degradation. CUL-4A belongs to a cullin family of proteins that assemble into multiple E3 ligase complexes (39, 40). It has been demonstrated that CUL-4A associates with DDB1 and overexpression of CUL-4A increases the ubiquitylation and the decay rate of DDB2 (35, 42). The DDB-CUL-4A complex can directly ubiquitylate DDB2 in vitro (37). These results, together with immediate recruitment of DDB2 to DNA damage and rapid turnover of DDB2 after UV irradiation (19, 36), suggest that the fate of DDB2 engaged in damage recognition is tightly controlled by Ub-mediated proteolysis.

Here, we have addressed the functionality (e.g. damage recognition and repair of UV-induced lesions) of DDB2 ubiquitylation and subsequent degradation mediated by CUL-4A-based E3 ligase. We present evidence that CUL-4A is an essential component for Ub-mediated proteolysis of DDB2 and is being physically recruited to DNA damage sites in chromatin. Degradation of DDB2 regulates the recruitment of XPC to DNA damage and subsequently the repair of UV-induced CPD. Our results support the idea that DDB2 degradation is integral to DNA damage handover from DDB to XPC during the initial steps of NER.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The normal human fibroblasts (OSU-2) were established in culture in our laboratory as described by Venkatachalam et al. (43). HeLa cells stably expressing N-terminal FLAG-HA epitope-tagged DDB2 (HeLa-DDB2) were kindly provided by Dr. Yoshihiro Nakatani (Dana-Farber Cancer Institute, Boston, MA). HeLa-DDB2 cells stably expressing c-Myc epitope-tagged CUL-4A (HeLa-DCH) and V5-His epitope-tagged XPC (HeLa-XPC) were established in our laboratory. For expression of CUL-4A in HeLa-DDB2 cells, the Myc3-tagged CUL-4A expression construct (a gift from Dr. Yue Xiong, University of North Carolina) was introduced into the HeLa-DDB2 cells and the transfectants were selected with G418. For expression of V5-His-tagged XPC, the XPC cDNA were inserted into pcDNA3.1/V5-His vector (Invitrogen). The expression constructs were transfected into the HeLa-DDB2 cells, and the stable transfectants were established through subcloning of the transfected cells. In both cases, the stably transfected cells were further subcloned by a single cell dilution and identified by Western blot analysis. The DNA transfection was performed using FuGENE 6 transfection reagents (Roche Applied Science) according to the manufacturer’s recommendation. All cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, antibiotics, and 500 μg/ml of G418 (for selection and maintenance of the transgenic cell lines) at 37 °C in a humidified atmosphere of 5% CO2. For experiments to assess DNA damage and repair, the monolayer cells were grown to confluence and then incubated with serum-free medium for 24 h.

**UV Irradiation**—The cells ready for UV-C irradiation were washed twice with phosphate-buffered saline (PBS). The UV-C light of 254 nm was delivered from a germicidal lamp at a dose rate of 0.5 J/m2/s as measured by a Kettering model 65 radiometer (Cole-Palmer Instrument Co., Vernon Hills, IL). For local UV irradiation, the cells were grown for 24–48 h to ~70% confluence on glass coverslips. The medium was aspirated, and the cells were washed with PBS. Prior to UV irradiation, an isopore polycarbonate filter (Millipore, Bedford, MA) with a pore size of 3, 5, or 8 μm diameter, was placed on top of the cell monolayer. The filter-covered coverslips were irradiated with the desired UV doses. The filter was then gently removed, and the cells were processed immediately or maintained in a suitable medium for the desired period and processed thereafter.

**Immunoprecipitation of the DDB2 Complex and Western Blotting**—The HeLa-DCH cells grown to confluence were UV irradiated at 25 J/m2 and incubated for the indicated periods in fresh medium. The cells were washed twice with PBS and fixed with 1% formaldehyde (final concentration in PBS) at room temperature for 10 min, followed by addition of glycine to a final concentration of 125 mM and incubation for 5 min to quench the cross-linking. After two additional washes with cold PBS, the cells were scraped from dishes and collected in radiolabeled precipitation assay buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing 10 μg/ml of leupeptin, 10 μg/ml of pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The cell extracts were made by sonication of the cell lysates on ice to break the DNA to a 100–500-bp fragment. The DDB2 complex in cell extracts was purified by immunoprecipitation with anti-HA affinity matrix (Roche Applied Science) followed by recovery with anti-FLAG gel (Sigma). Briefly, the cell extracts containing ~2 mg of protein were incubated with 25 μl of anti-HA affinity matrix in radiolabeled precipitation buffer at 4 °C overnight. The matrix beads were washed, and the bound proteins were eluted thrice each with 25 μl of elution buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA) containing 1 mg/ml of HA peptide at room temperature for 10 min. The eluted proteins were pooled and diluted in radiolabeled precipitation buffer and further immunoprecipitated with 25 μl of anti-FLAG gel. The bound proteins were recovered by dissolving the immunoprecipitates in IP elution buffer (1% SDS, 0.1 M NaHCO3). To reverse cross-link, the complex was incubated at 65 °C for 5 h after adding 0.2 M NaCl to the eluents. For Western blotting, the cell extracts were made from either transfected or non-transfected cells by boiling the cell lysates for 10 min in a sample buffer (2% sodium dodecyl sulfate, 10% glycerol, 100 mM dithiothreitol, and a protease inhibitor mixture in 62 mM Tris-HCl, pH 6.8). The proteins were quantified and separated by SDS-PAGE, and the immunoblot analysis was performed as described earlier (43). For detecting DDB2 complex, Novex 4% Tris-glycine gels (Invitrogen) were used for separating proteins under reduced or non-reduced conditions.

**Quantitation of CPD and 6–4PP Photolesions**—OSU-2 cells, either siRNA transfected or mock transfected or untransfected, were maintained in fresh serum-free medium for 12 h before exposure to 20 J/m2 dose of UV irradiation. At the indicated post-UV time, the cells were recovered for isolating genomic DNA. The initial formation of CPD and 6–4PP and that remaining in DNA after cellular repair for varying times were quantitated using a non-competitive immuno-slot blot assay as described earlier (44). The damage levels were calculated by comparing the band intensities of the samples with UV-irradiated DNA standards run in parallel with all the blots. The total amount of DNA loaded on the nitrocellulose membrane was kept constant for each sample blotted.

**Immunofluorescence**—Immunofluorescence staining of the cells was conducted essentially according to the method established in our laboratory (45). The cells were grown on coverslips in 60-mm dishes, washed twice with cold PBS, UV irradiated, and then fixed with 2% paraformaldehyde in 0.5% Triton X-100/PBS at 4 °C for 30 min, followed by three washes with PBS. For DNA denaturation, the cells were incubated in 2 N HCl for 5 min at 37 °C. The coverslips were rinsed three time with PBS and blocked with 20% normal goat serum in wash-
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Antibodies—Rabbit anti-DDB2 antibody (DDB2-A) and anti-XPC antibody (XPC-2) were generated by immunizing rabbits with synthetic peptides and were affinity purified with the corresponding peptide (BioSource, Hopkinton, MA). Peptide KRPTQKTSEIVLRPRNKR matches the N terminus of human DDB2 protein, whereas KTKREK-KAAASLFPPEKL matches to the C terminus of human XPC protein. Polyclonal anti-CUL-4A was a gift from Dr. Yue Xiong (University of North Carolina). Polyclonal anti-CPD and anti-6–4PP antibodies were generously provided by Dr. Tsukasa Matsumaga (Kanazawa University, Japan). Monoclonal antibodies recognizing c-Myc, FLAG, or HA epitopes were purchased from Sigma or Roche Diagnostics, respectively. Antibodies against Actin and XBP were from Neomarkers (Fremont, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Fluorescent-conjugated antibodies Alex Fluor® 488 goat anti-mouse IgG2a and IgG1 were from Molecular Probes (Eugene, OR); Texas Red goat anti-rabbit IgG and FITC goat anti-rabbit IgG were from Santa Cruz Biotechnology.

RNA interference—siRNA oligonucleotides were obtained from Dharmacon (Lafayette, CO) in a purified and annealed duplex form. The sequences targeting human CUL-4A gene are 5′-pUUGAUUACGAUCGCUGAC-GAUCGUAUCAAUU-3′ (sense) and 5′-pUUGAUUACGAUCGCU-GUUCUU-3′ (antisense). This set was chosen from four candidate siRNA through pilot experiments. siRNA transfection experiments were carried out using Lipofectamine transfection reagent according to the manufacturer’s instructions. Briefly, the Lipofectamine 2000 and OPTI-MEM medium (Invitrogen) were mixed for 5 min and then incubated with siRNA for 20 min at room temperature. After addition of the proper amount of 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium to the mixture, the siRNA-Lipofectamine mix was applied to the cell cultures. 48 h following siRNA transfection, the cells were used to analyze the expression of CUL-4A, DDB2, and other proteins. For DNA repair experiments, the transfected cells were maintained for 12 h in fresh serum-free medium after siRNA treatment. For immunofluorescent staining experiments, the siRNA transfection was performed with the cells grown on coverslips in 60-mm dishes.

RESULTS

CUL-4A Is an Essential Component for UV-induced DDB2 Degradation in Vivo—It has been reported that the DDB2 protein undergoes a Ub-mediated proteolysis shortly after cellular UV irradiation (19, 36). It has also been shown that DDB2 degradation is enhanced by overexpression of CUL-4A (35). We tested whether CUL-4A is an essential component for UV-induced degradation of DDB2 in vivo using RNA interference-based targeting technology. As shown in Fig. 1A, HeLa-DDB2, stably expressing FLAG-HA-tagged DDB2, produced DDB2 at significant levels in the absence of UV irradiation (lanes 1 and 2). However, upon UV irradiation these cells exhibited a sharp decrease in the DDB2 levels within 2–8 h of exposure (lanes 3, 5, and 7). Moreover, the levels of XBP, a component of transcription factor II H, were unaffected by UV irradiation at all post-irradiation times. Although both of these factors, DDB2 and XBP, are involved in NER, these data indicated that UV-induced loss of DDB2 protein is highly specific and presumably related to its function in initiating NER. Treatment of the cells with the proteasome inhibitor MG132 showed a clear inhibition of the UV-induced loss of DDB2 in HeLa-DDB2 cells (lanes 4, 6, and 8), suggesting the involvement of the Ub-proteasome system in this process. Controls, mock transfected with nonspecific siRNA, showed prompt degradation of DDB2 as early as 0.5 h after UV irradiation (Fig. 1B). The lowest level of DDB2 was seen at 4 h, and it started to recover at 8 h following irradiation. In contrast, no obvious loss of DDB2 could be seen after UV irradiation in cells that were transfected with CUL-4A siRNA. Western blot analysis showed that CUL-4A siRNA was specific and effective in diminishing the targeted CUL-4A protein at all the experimental time points used for assessing the fate of repair factors (Fig. 1C). Thus, silencing CUL-4A in cells prevented UV-induced DDB2 degradation, which is in agreement with the potential role of CUL-4A in DDB2 clearance.

CUL-4A Complexes with DDB and Is Recruited to DNA Damage Sites—Recent studies have identified a DDB-CUL-4A complex that possesses E3 Ub ligase activity (47). Biochemical analysis with purified DDB-CUL-4A complex also showed that the complex can directly ubiquitylate DDB2 in vitro (37). We hypothesized that by virtue of the DNA binding prowess of one of its associated components, CUL-4A would be physically recruited to DNA damage sites as a multimeric DDB-CUL-4A complex. This complex would there-
After micropore UV irradiation, the cells were fixed with 2% paraformaldehyde. The components within cross-linked complexes. In essence, no significant loss of DDB1 and DDB2 could be demonstrated upon cross-link reversal. It is possible that the antibody-reactive epitopes are buried within the complexes and the reactivity to individual component proteins is more efficient. The resulting increase of signal intensity from DDB1 and DDB2 precludes the visualization of UV-induced decrease that is clearly demonstrable in the complexes seen in Fig. 2A. Furthermore, it is well recognized that DDB in cells can itself exist within other complexes besides that with CUL-4A.

To determine whether CUL-4A actually translocates to DNA damage sites, we employed the micropore UV irradiation technique, which can deliver UV damage to a localized area of the cell nucleus (45). The HeLa-DCH cells, stably expressing Myc3-tagged CUL-4A, were irradiated through a 5-μm isopore filter and the cells were allowed to repair the damage for 15, 30, and 120 min. As shown in Fig. 2C, CUL-4A-specific signal (green) becomes intensified within subnuclear spots in UV-irradiated cells at 15 and 30 min after exposure. The same DNA damage-containing subnuclear spots were also visualized with anti-XPC antibodies (red), indicating the recruitment of repair factors for the assembly of productive NER machinery. Because subnuclear spots have been spatiotemporally characterized as localized damage spots (45), these results indicate that, like DDB2 and XPC, CUL-4A protein translocates to DNA-damaged sites immediately after UV irradiation. It may be noted that only some of the anti-XPC-visualized spots were also picked up by anti-Myc (CUL-4A) staining. In addition, at 2 h after micropore UV irradiation, some subnuclear spots were clearly detectable with anti-Myc staining, whereas none could be detected by anti-XPC (CUL-4A) staining. These results point to a temporal relationship according to which CUL-4A seems to get dislodged from the DNA damage and repair sites prior to releasing of XPC.
delivers a fraction of UV dose to cells. This low level irradiation would be insufficient to significantly deplete the DDB2 from cells to reveal appreciable intensity differences. Nevertheless, when the cells were treated with proteasome inhibitor MG132, DDB2 spots become clearly visible even at 4 h post-irradiation (Fig. 3B). MG132 treatment increased the DDB2/CPD ratio to more than three times at 4 h post-irradiation (Fig. 3D). The fact that DDB2 translocates to DNA damage sites very rapidly (48) and then promptly comes off points to the role of DDB2 proteolysis in the disappearance of DDB2 foci from DNA damage sites. Therefore, we further examined the effects of CUL-4A knock down on DDB2 elimination from the DNA damage sites. As illustrated in Fig. 3C, CUL-4A siRNA treatment did not affect the initial formation of DDB2 foci at damage sites (Fig. 3, C and D). The ratio of DDB2/CPD foci after CUL-4A silencing remained as high as 90% at 15 and 30 min post-irradiation. Nevertheless, the siRNA treatment clearly halted the normal disappearance of DDB2 foci at 2 and 4 h post-irradiation (Fig. 3, C and D). About 30% of DDB2 foci were still detectable at 4 h in CUL-4A siRNA-treated cells as compared with ~10% in mock-treated cells. The data strongly suggest that although CUL-4A is not required for the recruitment of DDB to DNA damage, it is clearly needed at the damage sites for the degradation of DDB2 and for the repair to move forward.

CUL-4A Knock Down Decreases XPC Recruitment and Affects GGR of CPD—Our previous studies, using a Ub-activating enzyme-defective thermosensitive cell line, have demonstrated that the Ub-proteasome system is required for efficient GGR and TCR (49). To investigate the functionality of DDB2 ubiquitylation and its subsequent degradation in NER, we next examined the effect of CUL-4A knock down on XPC recruitment. The spatiotemporal analysis of factor recruitment to damage sites was carried out after UV irradiation of cells at 40 J/m² through a 5-μm micropore filter. Considering that the nucleus is mostly shielded by the filter, this UV dose is comparatively lower and therefore the overall lesion load produced would be quantitatively repaired. As shown in Fig. 4A, both XPC and CPD foci were visualized within the nucleus of UV-irradiated cells. The XPC/CPD ratio at 30 min post-irradiation was determined to be ~66% in controls without a CUL-4A siRNA treatment. However, the initial factor recruitment of damage sites was significantly affected as the ratio decreased to ~32% upon CUL-4A siRNA-mediated knock down. The data suggest a clear involvement of CUL-4A-based E3 ligase in regulating the recruitment of XPC to UV-induced photolesions, presumably due to a lack of DDB2 clearance from damage sites.

We further examined the effect of CUL-4A knock down on GGR of UV-induced photolesions (Fig. 4, B–E). Both CPD and 6–4PP lesions were quantitated by immuno-slot blot assay of the genomic DNA isolated at varying post-irradiation times from the CUL-4A siRNA-transfected or mock-transfected repair-proficient human fibroblasts (Fig. 4, B and D). The mock-transfected cells exhibited the typical kinetics of CPD repair, i.e., the unrepaired CPD remaining in the genome were ~80, 65, and 46% of initial damage at 4, 8, and 24 h, respectively. In contrast, in CUL-4A siRNA-transfected cells, the unrepaired CPD was ~88, 78, and 63% at 4, 8, and 24 h, respectively (Fig. 4C). Obviously, the CUL-4A knock down decreased the efficiency of GGR at all the time points
tested. In contrast to CPD repair, the kinetics of 6–4PP repair, which is known to occur at a faster rate than CPD, was identical in both siRNA- and mock-transfected cells (Fig. 4E). Therefore, the GGR of 6–4PP was not impacted by impairing the action of CUL-4A.

**Availability of DDB2 and XPC Proteins Is Differentially Affected by UV**—Once DDB2 binds to DNA damage and impinges on the initial repair event by being consumed through degradation, it cannot be available for the removal of any residual or newly induced lesions. To test that this actually happens, we designed a "two-hit experiment" to ascertain the participation of DDB in lesion repair. First, the entire nuclear DNA was subjected to UV-induced damage by global irradiation of cell monolayers. The cells were allowed to repair the damage for indicated times. The CPD (A and C) or 6–4PP (D and E) in genomic DNA were detected by immuno-slot blot assay using cognate damage-specific antibodies. The intensity of lesion-specific signals was determined by laser densitometric scanning, and the amount of damage was calculated upon comparing the band intensities with a standard reference run in parallel. The repair was expressed as the percentage of initial damage remaining in isolated cellular DNA.

**FIGURE 4. Knock down of CUL-4A decreased XPC recruitment and affected GGR of CPD.** A, normal human fibroblasts on coverslips were transfected or mocked-transfected with CUL-4A siRNA for 48 h and irradiated with 40 J/m² UV-C through a 5-μm isopore filter. The cells were incubated for another 30 min, fixed with 2% paraformaldehyde, and immunostained with both anti-XPC and anti-CPD antibodies. B–E, the transfected or mock-transfected fibroblasts were maintained overnight in serum-free medium and irradiated with UV-C at 20 J/m². The cells were allowed to repair the damage for indicated times. The CPD (B and C) or 6–4PP (D and E) in genomic DNA were detected by immuno-slot blot assay using cognate damage-specific antibodies. The intensity of lesion-specific signals was determined by laser densitometric scanning, and the amount of damage was calculated upon comparing the band intensities with a standard reference run in parallel. The repair was expressed as the percentage of initial damage remaining in isolated cellular DNA.
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FIGURE 5. Two-hit experiments show differential effects of damage load on the recruitment of DDB2 and XPC to freshly induced DNA damage sites. HeLa-DDB2 cells on coverslips were globally irradiated with UV-C at indicated UV doses and maintained for 4 h in fresh medium to allow the first phase of repair. A second cycle of 100 J/m² UV was delivered to the cells through a 5-μm micropore filter. The cells were incubated for another 30 min, fixed, and double immunostained with anti-HA (DDB2) and anti-XPC antibodies. The relative recruitment of DDB2 and XPC was calculated based on the numbers of DDB2 or XPC foci in 200 cells from at least three microscopic fields.

Unlike XPC, prevents its recycling for the repair of additional photolesions.

DISCUSSION

Cellular exposure to UV irradiation is known to cause several changes of the DDB complex, e.g. prompt translocation from cytoplasm into nucleus, tight association with chromatin, and rapid degradation of its DDB2 protein component (19, 51, 52). These findings, together with recent identification of the DDB-CUL-4A E3 ligase complex, suggest that the complex could be functioning at the very early step of DNA damage recognition and repair. Here, we have provided evidence about how the two damage recognition factors, DDB2 and XPC, cooperate in early steps of GGR. Based on our accumulated data, it is posited that the clearance of DDB2 by Ub-mediated degradation facilitates the access of XPC and other NER factors to DNA lesions. It should be emphasized that knock down of CUL-4A delayed the dispersal of DDB2 from the damage sites while it decreased the recruitment of XPC to DNA damage and subsequently reduced the removal of CPD from the genome (Figs. 3 and 4). These results are consistent with previous published observations that knock down of CSN5, a critical component of COP9, leads to defect of GGR and TCR (47). While COP9 is a negative regulator of associated E3 ligase in vitro, it would be required for the same E3 activity in vivo (41). Therefore, interpretation of the requirement of CSN5 for GGR suggests the importance of the ligase activity of DDB-CUL-4A E3 complex in GGR. Our siRNA experiments specifically targeted CUL-4A-based E3 ligase and therefore provided direct evidence that the ligase activity of DDB-CUL-4A E3 complex is involved in GGR. In these experiments, the decrease in XPC recruitment by CUL-4A knock down further suggested that the ligase activity of DDB-CUL-4A E3 complex is required for an efficient recruitment of XPC to DNA damage. Sugawara et al. (50) recently reported that the ubiquitylation of DDB2 alters the DNA binding property of DDB in vitro, suggesting that the ubiquitylation regulates the departure of DDB from damage DNA. Our results, however, favor the suggestion that both ubiquitylation and subsequent proteasomal degradation of DDB2 are responsible for the clearance of DDB, allowing XPC to load at damage sites. In support of this view, we have previously shown that treatment of cells with proteasome inhibitors compromises the repair of UV-induced CPD and also inhibits the UV-induced XPC recruitment to DNA damage sites (49).

Interestingly, the fates of DDB2 and XPC are quite different, although both of them appear to be the substrates of DDB-CUL-4A E3 complex (50, 53). In our two-hit experiments (Fig. 5), 40 J/m² global UV irradiation (first hit) diminished the DDB2 recruitment ~90% to damage that was freshly produced by micropore UV irradiation (second hit). This indicates that the damage loading following the first exposure depletes most of the available DDB activity. Nevertheless, at the same damage load, 50% XPC recruitment was observed at the freshly induced damaged sites. The difference between the availability of XPC and that of the DDB2 strongly suggests that XPC is re-used for the repair of persistent damage. Because DDB2 is required for in vivo XPC recruitment and it is
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degraded after the global UV irradiation, it is reasonable to assume that 6–4PP presents a major stimulus for the recruitment of XPC to newly formed damage sites.

In summary, we have examined the CUL-4A-mediated proteolysis of DDB2 at the DNA damage sites and explored the functional relevance of such DDB2 degradation to GGR with regard to XPC recruitment and removal of UV-induced photolesions. It should be recognized, however, that DDB-CUL-4A E3 complex could regulate NER through substrates other than DDB2. For example, XPC has been recently identified as one of the key DNA repair-related substrates of the complex (50). Clearly, regulation of NER by the Ub-proteasome system remains an exciting research area for further exploration.

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