UV-damaged DNA-binding Proteins Are Targets of CUL-4A-mediated Ubiquitination and Degradation*

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Cul-4A, which encodes a member of the cullin family subunit of ubiquitin-protein ligases, is expressed at abnormally high levels in many tumor cells. CUL-4A can physically associate with the damaged DNA-binding protein (DDB), which is composed of two subunits, p125 and p48. DDB binds specifically to UV-damaged DNA and is believed to play a role in DNA repair. We report here that CUL-4A stimulates degradation of p48 through the ubiquitin-proteasome pathway, resulting in an overall decrease in UV-damaged DNA binding activity. The R273H mutant of p48 identified from a xeroderma pigmentosum (group E) patient is not subjected to CUL-4A-mediated proteolysis, consistent with its inability to bind CUL-4A. p125 is also an unstable protein, and its ubiquitination is stimulated by CUL-4A. However, the abundance of p125 is not dramatically altered by CUL-4A overexpression. UV irradiation inhibits p125 degradation, which is temporally coupled to the UV-induced translocation of p125 from the cytoplasm into the nucleus. CUL-4A is localized primarily in the cytoplasm. These findings identify DDB subunits as the first substrates of the CUL-4A ubiquitination machinery and suggest that abnormal expression of Cul-4A results in reduced p48 levels, thus impairing the ability of DDB in lesion recognition and DNA repair in tumor cells.

Ubiquitin-dependent proteolysis plays an important role in controlling cell cycle, signal transduction, apoptosis, and a variety of other cellular processes. Through the action of a multi-enzyme system consisting of the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin-protein ligase, multiple ubiquitin moieties are delivered to the target protein to form a polyubiquitin chain through the isopeptide linkage between the ε-amino group of the lysine 48 residue of one ubiquitin and the carboxyl terminus of the adjacent ubiquitin. In turn, polyubiquitination serves as the signal for recognition and degradation by the 26 S proteasome (recently reviewed in Refs. 1 and 2).

The E3 component of the ubiquitin pathway is highly specialized in its ability to select specific cellular substrates for ubiquitination (reviewed in Ref. 3). The Rxbl-cullin subclass of RING E3s consists of multimeric protein complexes that are assembled around a core module composed of a cullin family member and the RING-H2 domain protein Rbx1/Roc1/Hrt1 (4–7, and reviewed in Refs. 1, 2, and 8). There are at least six identified mammalian cullins (9), and they share extensive sequence homology in a region of ~200 amino acid residues designated as the cullin homology domain (CH) (10, 11). Cullins interact with the RING-H2 domain protein Rxbl/Roc1/Hrt1 through their CH domains to form core ubiquitin-protein ligase modules that connect to the E2 ubiquitin-conjugating enzymes and other E3 components and facilitate ubiquitin transfer to substrates (4, 6, 7, 12, 13). Among members of the cullin family, Cul-1, Cul-2, and Cul-3 have been demonstrated to mediate the selective degradation of regulators of cell cycle and signaling pathways (reviewed in Refs. 1 and 2 and the references therein). Kamura et al. (12) recently demonstrated that the Cul-5/Rbx1 module associates with the elongin BC complex and a novel elongin BC-box/leucine-rich repeat-containing protein MUF1 to form a functional ubiquitin-protein ligase. Cul-4A and Cul-4B are still poorly characterized because of the lack of known proteolytic substrates and components of the putative Cul-4 complex. The Cul-4A gene is amplified or overexpressed in human breast cancer and many other tumor types (14, 15). Recent biochemical studies have identified a physical association between Cul-4A and the UV-damaged DNA-binding protein (DDB) (16),2 which is involved in the repair of damaged genomic DNA and the non-transcribed strand of expressed genes (17–19). However, the biochemical consequences of the interaction between Cul-4A and DDB have not been elucidated.

DBD is a heterodimeric protein complex consisting of a 127-kDa subunit (p125 or DDB1) and a 48-kDa subunit (p48 or DDB2) (20–23) that has high affinity for a variety of DNA lesions including UV-induced cyclobutane pyrimidine dimers and 6-4 photoproducts, as well as intrastrand cross-links by cisplatin and benzo[a]pyrene adducts. (24–26). For simplicity, the two DDB subunits are referred to as p125 and p48 from hereon. p125 is an abundant protein that is in excess of p48 (16, 21), whereas the p48 subunit is the limiting factor for UV-DDB activity and functions to activate p125 binding to damaged DNA (19, 27). UV irradiation induces p48 transcription in a

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1 The abbreviations used are: E3, ubiquitin-protein ligase; CH, cullin homology; DDB, damage-specific DNA-binding protein; p125, DDB complex consisting of a 125-kDa subunit; p48, DDB complex consisting of a 48-kDa subunit; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; F-p125, FLAG-tagged p125; F-p48, FLAG-tagged p48; DAPI, 4',6'-diamidino-2-phenylindole; HA-Ub, hemagglutinin-tagged ubiquitin; UV, ultraviolet light; XP-E, xeroderma pigmentosum complementation group E; EMSA, electrophoretic mobility shift assay; F-CUL-4A, FLAG-tagged human CUL-4A; f148, 148-bp DNA probe; LLnL, N-acetyl-Leu-Leu-Nle-CHO.

2 P. Zhou, unpublished results.
p53-dependent manner, resulting in the subsequent accumulation of p48 protein (18, 28). p48 is localized to the nucleus, whereas p125 is primarily cytoplasmic but translocates into the nucleus upon UV irradiation by a mechanism that is partially p48-dependent (28, 29). Mutations in the p48 subunit of DDB were identified in patients suffering from the autosomal recessive disease, xeroderma pigmentosum (complementation group E) (XP-E), which is characterized by defective nucleotide excision repair and predisposition to skin cancer (30–32). Recent studies (18, 27, 33) indicate that the p48 mutant cells are deficient in repair of UV-damaged DNA, consistent with their impaired DDB activity (DDB−). One such p48 mutant, 2RO (R273H), fails to associate with p125 and CUL-4A and is incapable of mediating nuclear accumulation of p125 (16, 29). Mutations of the p125 subunit have not been identified in XP-E patients, however, a 50% decrease in the steady-state levels of p125 has been reported in several DDB− XP-E cells (34). These results suggest a requirement for the precise control of the amount of p125 and p48 inside the cell.

Here we report that CUL-4A stimulates the ubiquitination and degradation of the p48 subunit of DDB, resulting in an overall decrease of UV-DDB activity. The 2RO mutant of p48, which is defective in binding to p125 and CUL-4A, is resistant to degradation induced by Cul-4A overexpression. Ectopic expression of Cul-4A also resulted in accelerated p125 ubiquitination. These studies identify DDB as the first target of the CUL-4A ubiquitination machinery and suggest a possible link between the abnormal Cul-4A expression in tumor cells and the inhibition of DDB-dependent repair of UV-damaged DNA.

MATERIALS AND METHODS

Cell Culture, Plasmids, and Antibodies—The hamster V79–4 lung fibroblast cells were purchased from ATCC (Manassas, VA). V79–4 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The expression plasmids carrying HA-tagged p125 (p125-HA), FLAG-tagged p125 (F-p125), FLAG-tagged p48 (F-p48), or FLAG-tagged p48 (R273H) were generous gifts of G. Chu (Stanford University, Palo Alto, CA) (27). MYC-tagged mouse Cul-4A (myc-Cul-4A) and human Cul-1 (myc-Cul-1) expression plasmids were generously gifted of T. Ohta (St. Marianna University, Kawasaki, Japan). FLAG-tagged human Cul-4A (F-Cul-4A) was amplified using total RNA prepared from HeLa cells (14). FLAG-tagged human Cul-4A (Δ), which carries an internal deletion of the CH domain (residues 346–532), was generated by PCR-directed mutagenesis. F-Cul-4A and F-Cul-4A (Δ) were cloned into pcDNA3 (Invitrogen) and sequenced. Transient transfection was carried out using plasmids as indicated in each figure (in µg) by calcium phosphate precipitation in HeLa cells or by FuGene 6 reagent (Promega). Cells were plated into 1% SDS lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1% SDS, 1 mM dithiothreitol) for 10 min and then diluted 10 times in Nonidet P-40 lysis buffer containing the protease inhibitor mixture (PharMingen). Equivalent amounts of extracts were separated by SDS-PAGE and immunoblotted to detect expression of F-p125, F-p48, MYC-CUL-1, and MYC-CUL-4A, respectively. V79–4 cells were also treated with 50 µg/ml pepstatin, leupeptin, and aprotinin (Roche Molecular Biochemicals) and rotated at 4°C for 30 min before harvesting and SDS-PAGE analysis.

Electrophoretic Mobility Shift Assay (EMSA)—V79–4 cells were transiently transacted with 10 µg of F-p125 plasmid, 8 µg of HA-Ub-expressing plasmid, and 4 or 8 µg of myc-Cul-4A, F-Cul-4A (Δ), or myc-Cul-1, respectively. Cells were washed with phosphate-buffered saline, and nuclei were isolated using the hypotonic buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1% SDS, 1 mM dithiothreitol) for 30 min followed by centrifugation at 13,000 rpm for 30 min at 4°C. 50 µg of each extract was first subjected to immunoblotting with the anti-CUL-4A antibody or anti-p125 polyclonal antibody or anti-FLAG monoclonal antibodies and resolved on 9% SDS-PAGE. The amount of 32P-labeled p125-HA or F-p48 at each time point was visualized using the anti-p125 polyclonal antibody or anti-FLAG (M2) monoclonal antibodies and resolved on 9% SDS-PAGE. The amount of 32P-labeled p125-HA or F-p48 at each time point was visualized using the anti-p125 polyclonal antibody or anti-FLAG monoclonal antibody, respectively. Immunohybridization was performed using either affinity-purified anti-CUL-4A polyclonal antibody or anti-CUL-4A monoclonal antibody resolved on 9% SDS-PAGE. The amount of 32P-labeled p125-HA or F-p48 at each time point was visualized using the anti-p125 polyclonal antibody or anti-FLAG monoclonal antibody, respectively. To evaluate the effect of UV irradiation on the stability of p125 or p48, cells were irradiated with UV at 10 J/m2 followed by recovery in DMEM for 8 h prior to pulse-chase analysis. In some instances, 100 µM MG132 proteasome inhibitor (Peptide International) was included in the chase medium, and the stability of p125-HA was subsequently addressed. The pulse-chase experiments were repeated three or more times, and representative results are shown in Figs. 3 and 4.

In Vivo Ubiquitination Assay—5 × 105 HeLa cells were transiently transfected with 10 µg of F-p125 plasmid, 8 µg of HA-Ub-expressing plasmid, and 4 or 8 µg of myc-Cul-4A, F-Cul-4A (Δ), or myc-Cul-1, respectively. Cells were washed with phosphate-buffered saline, and nuclei were isolated using the hypotonic buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1% SDS, 1 mM dithiothreitol) for 30 min followed by centrifugation at 13,000 rpm for 30 min at 4°C. 50 µg of each extract was first subjected to SDS-PAGE and immunoblotting to detect expression of F-p125, F-p48, MYC-CUL-4A, F-CUL-4A (Δ), and MYC-CUL-1, respectively.
isolated from the pSV2CAT plasmid by HindIII and PvuII digestion and was labeled with \( ^{32} \text{P}-\text{dCTP} \) by using the Klenow fragment of DNA polymerase I. \( ^{32} \text{P}-\text{Labeled f148 probe was purified by a } 4\% \text{ polyacryl-}

amide gel, and the eluted probe was damaged with UV at 5000 J/m \(^2\).

RESULTS

CUL-4A Interacts with p125 Independent of p48—To assess the function of CUL-4A, we searched for cellular proteins that could interact with human CUL-4A. Either F-CUL-4A or the pCDNA3 expression vector was transiently transfected into HeLa cells for immunoprecipitation by the anti-FLAG M2 monoclonal antibody, and the immunoprecipitates were analyzed by SDS-PAGE and silver staining. Two polypeptides with the molecular mass of \(-125\) and \(45\) kDa were specifically detected only from the CUL-4A immunoprecipitates (data not shown). While we were analyzing these polypeptides, Shiyanov et al. (16) reported the identification of CUL-4A as a T7-tagged p48-associated protein from the human osteosarcoma U2OS cell extracts. The two CUL-4A-interacting proteins we detected were subsequently confirmed to be p125 and p48.

To further examine how CUL-4A interacts with the individual subunits of DDB, we sought to analyze these interactions in the hamster V79–4 lung fibroblast cells that lack p48 expression (Fig. 1A) (19, 27). Plasmids carrying p125-HA were transfected into V79–4 cells along with a plasmid expressing F-Cul-4A or F-p48. The expression of each individual protein was verified by Western blotting (Fig. 1B). Coinmunoprecipitation was carried out using the anti-FLAG M2 antibody for F-p48 or F-CUL-4A and probed with anti-HA antibody to detect the presence of p125-HA in the immunoprecipitates. As shown in Fig. 1C, F-CUL-4A can form a complex with p125-HA in the absence of p48 in V79–4 cells, indicating that p48 is dispensable for this interaction (Fig. 1C, lane 4).

CUL-4A Is Localized Primarily in the Cytoplasm—p125 is localized primarily in the cytoplasm in a variety of cell lines examined (Fig. 2B) (28, 29). Given that UV irradiation stimulates p125 entry into the nucleus (28, 29), we sought to determine the subcellular localization of CUL-4A in the absence or presence of UV treatment. We first examined the cellular distribution of endogenous CUL-4A in V79–4 and HeLa cells using an affinity-purified anti-CUL-4A polyclonal antibody. In the absence of UV irradiation, CUL-4A, like p125, localized primarily in the cytoplasm in V79–4 cells and HeLa cells (Fig. 2C, left panel). At 8 h after UV irradiation (10 J/m \(^2\)), p125 was found predominantly in the nucleus in HeLa cells (Fig. 2C, upper right panel), whereas CUL-4A remained largely in the cytoplasm (Fig. 2C, bottom right panel). In contrast, p48 was always localized in the nucleus (28, 29) (data not shown). These results indicate that unlike p125, CUL-4A is retained in the cytoplasm following UV irradiation. We further analyzed the subcellular distribution of CUL-4A by immunofluorescence confocal microscopy. As shown in Fig. 2B, a subpopulation of CUL-4A (approximately 2–3%) was indeed detected in the nucleus (Fig. 2B). We, therefore, conclude that CUL-4A is predominantly localized in the cytoplasm, whereas a small fraction also resides in the nucleus.

CUL-4A Regulates p48 Levels through Ubiquitin-dependent Proteolysis—Because the nuclear-localized p48 is the limiting factor for UV-DDB activity, we sought to determine whether p48 is subjected to CUL-4A-mediated proteolysis. First, we assessed the steady-state levels of p48 in response to ectopic Cul-4A expression. V79–4 cells lacking endogenous p48 expression were transiently transfected with F-p48 along with the control vector pCDNA3, myc-tagged Cul-4A, or myc-tagged Cul-1. 1 \( \mu \)g of pCMV-CD19 plasmid was also cotransfected for immunomagnetic selection of transfected V79–4 cells, and the amounts of F-p48 were subsequently determined by immunoblotting using the anti-FLAG M2 monoclonal antibody. As shown in Fig. 3A, ectopic expression of myc-Cul-4A or but not myc-Cul-1 induced a dramatic decrease in the steady-state levels of p48 (Fig. 3A, lanes 1–3). Treatment of myc–Cul-4A and F-p48 coexpressing V79–4 cells with the proteasome inhibitor LLnL (Fig. 3A) or lactacystin (data not shown) dramatically inhibited the decrease of p48 levels, indicating that CUL-4A induced down-regulation of p48 through the proteasome (Fig. 3A, lane 4).

To assess whether CUL-4A reduces p48 levels through accelerating its ubiquitination, HeLa cells were transiently transfected with F-p48 alone (Fig. 3B, lane 1) or F-p48 and HA-tagged ubiquitin (Fig. 3B, lane 2) or F-p48, HA-Ub, and 3 or 6 \( \mu \)g of myc-Cul-4A (Fig. 3B, lanes 3 and 4). Extracts were immunoprecipitated with the anti-HA affinity matrix and probed with the anti-FLAG M2 antibody against F-p48. In the absence of exogenous CUL-4A, there was no obvious modification of F-p48 by HA-Ub (Fig. 3B, lane 2). When myc–Cul-4A was...
transfected into F-p48- and HA-Ub-expressing cells, a series of slower migrating F-p48 species was readily detectable in the anti-HA immunoprecipitates (Fig. 3B, lane 3), and the amounts of HA-Ub-modified F-p48 increased dramatically with the elevated Cul-4A expression (Fig. 3B, compare lane 3 with lane 4). These results indicate that p48 is specifically ubiquitinated by the CUL-4A ubiquitination machinery.

To evaluate whether CUL-4A-dependent down-regulation of p48 was the result of its accelerated turnover, we performed pulse-chase analysis to measure the half-life of p48 protein. Because of the unavailability of the anti-p48 antibody to detect endogenous p48, we first generated a stable V79–4/F-p48 cell line to monitor the turnover rate of chromosomally integrated FLAG-tagged p48 (Fig. 3C). In addition, we assessed the stability of transiently transfected F-p48 in HeLa cells, which exhibit high UV-damaged DNA binding activity (24). As shown by pulse-chase analysis in Fig. 3, p48 is turned over in both V79–4 and HeLa cells with a half-life of ~3.5 h. Ectopic expression of Cul-4A accelerated degradation of p48 (t½ of 1.2 h) (Fig. 3D, upper panel). The p48(R273H) mutant, which is defective for binding CUL-4A, was not subjected to CUL-4A-mediated p48 destruction (Fig. 3E). It is noted that the p48(R273H) mutant is still degraded, albeit independent of ectopic Cul-4A expression. The R273H mutation is within the WD40 domain of p48. One possible explanation is that the structural alteration resultant from the R273H mutation induces p48(R273H) degradation by an as yet unknown cellular proteolytic apparatus. Collectively, these results indicate that the CUL-4A machinery controls the stability of the p48 subunit of the UV-DDB complex through specifically enhancing the ubiquitination and degradation of p48.

CUL-4A Stimulates Ubiquitination of p125—The fact that CUL-4A also associates with the p125 subunit of the DDB complex independent of p48 (Fig. 1C) and that both CUL-4A and p125 are localized in the cytoplasm in non-irradiated cells prompted us to examine whether CUL-4A is involved in p125 ubiquitination. We first assessed the stability of endogenous p125 in HeLa cells that exhibit high UV-damaged DNA binding activity (24). As shown by pulse-chase analysis in Fig. 4A, p125 was also degraded in V79–4 cells with a half-life of ~2 h by pulse-chase analysis indicating that p48 is not required for p125 degradation (data not shown). Furthermore, ectopically expressed p125-HA was also rapidly turned over with a half-life of ~2 h by pulse-chase analysis indicating that p48 is not required for p125 degradation (data not shown). When the proteasome inhibitor MG132 (Fig. 4B) or lactacystin (data not shown) was included in the chase medium, degradation of p125-HA was dramatically inhibited with a half-life of 9.5 h indicating that the 26 S proteasome plays a major role in the regulation of p125 stability (Fig. 4B, middle panel).

We next investigated whether the stability of endogenous p125 could be altered in HeLa cells 8 h after UV treatment (10 J/m²) when the majority of p125 translocated from the cytoplasm to the nucleus (Fig. 2). As shown by pulse-chase, the
half-life of both endogenous and transfected p125 proteins were dramatically prolonged upon UV irradiation (Fig. 4, A, right panel, and B, lower panel). Because the majority of cytoplasmic p125 translocates into the nucleus in HeLa cells 8 h after UV irradiation and there is only a small fraction of CUL-4A in the nucleus (Fig. 2B), these experiments suggest that the CUL-4A might be limiting in the nucleus, and thus subcellular localization of p125 is a contributing factor that regulates p125 degradation within the cell.

Because the half-life of p125 was prolonged in the presence of the proteasome inhibitor, we further investigated whether p125 is directly modified by ubiquitin and whether CUL-4A plays a role in these processes. HeLa cells were transiently transfected with F-p125 alone (Fig. 5A, lane 1), F-p125 and HA-Ub (Fig. 5A, lane 2), or F-p125, HA-Ub, and increasing amounts of myc-CUL-4A (Fig. 5A, lanes 3 and 4). Expression of the transfected F-p125 was detected from 100 μg of extracts by immunoblotting using the anti-FLAG (M2) monoclonal antibody (Fig. 5A, top panel). In addition to the 127-kDa F-p125, a series of slower migrating species was also observed that was immunoreactive with F-p125 (Fig. 5A, lane 2) and whose intensity increased significantly with exogenous expression of myc-CUL-4A (Fig. 5A, lanes 3 and 4). This finding suggested that F-p125 might be modified by multiple ubiquitin molecules.

To determine whether these slower migrating species are indeed derived from ubiquitin conjugation to F-p125, the same lysates were immunoprecipitated with the anti-HA monoclonal antibody against HA-Ub and probed with anti-FLAG antibody against F-p125. The slower migrating species were readily observed, and an elevated level of these species was detected with increased CUL-4A expression, indicating that p125 is indeed polyubiquitinated in a CUL-4A dose-dependent manner (Fig. 5, A, lower panel, and B, lanes 2–4). Two relatively constant ubiquitin-modified F-p125 species at molecular masses 135 and 143 kDa were specifically detected under these experimental conditions (Fig. 5, B and C, marked with an asterisk), which were consistent with the size of mono- and di-ubiquitin-conjugated p125, and served as a sensitive readout of p125.
ubiquitination for the following experiment in Fig. 5C. When 4 or 8 µg of myc-Cul-4A plasmid was introduced into F-p125 and HA-Ub-expressing HeLa cells, we observed a CUL-4A dose-dependent increase in the level of these ubiquitinated p125 species (Fig. 5, B and C, lanes 3 and 4 marked by an asterisk on the right). However, the expression of 4 or 8 µg of either F-Cul-4A(Δ) or myc-Cul-1 plasmid DNA was incapable of stimulating p125 ubiquitination (Fig. 5C, lanes 5–8). The decrease of mono- and di-ubiquitinated F-p125 in the presence of 8 µg of myc-Cul-1 but not 8 µg of F-Cul-4A(Δ) (Fig. 5C, compare lane 8 with lane 6) is likely because of the competition of high levels of CUL-1 with the endogenous CUL-4A for the endogenous Rbx1 and therefore reduces the ubiquitin-protein ligase activity of the CUL-4A machinery. Taken together, these results indicate that p125 is specifically ubiquitinated by CUL-4A, and this process requires the CH domain of CUL-4A, which is responsible for connecting to the E1 ubiquitin-activating enzyme-E2 ubiquitin transfer machinery. Interestingly, the overexpression of CUL-4A did not significantly alter the steady-state levels of transfected p125 (Fig. 1B, data not shown). The endogenous p125 is known to be a highly abundant protein (16, 21). It is possible that CUL-4A is not the rate-limiting component of the p125 degradation machinery. Therefore, overexpression of Cul-4A alone was not capable of reducing the p125 levels. Alternatively, CUL-4A-stimulated p125 ubiquitination may serve other roles than proteolysis. Further studies should be conducted to address the functional role of CUL-4A-mediated ubiquitination of p125.

Overexpression of CUL-4A Down-regulates UV-DDB Activity—DDB exhibits high affinity to UV-damaged DNA. We investigated whether CUL-4A-mediated proteolysis of DDB plays a role in controlling the UV-damaged DNA binding activity by the established EMSA (36). V79–4 cells lack the UV-DDB activity because of the absence of p48 expression (Fig. 6, lane 2) (19, 27). The binding of DDB to UV-damaged t148 DNA was restored by transient transfection of F-p48 into V79–4 cells (Fig. 6, lane 3) or through stable F-p48 expression in V79–4/F-p48 cells (data not shown) (19). The DDB activity was characterized as two DNA protein complexes (Fig. 6, lane 3 marked as B1 and B2) as was previously demonstrated (27). Hwang et al. (27) showed that B2 consists of t148 DNA in the complex with both p125 and p48, whereas B1 is primarily p125/t148.

V79–4 cells were transiently transfected with F-p48 alone (Fig. 6, lane 3) or together with myc-Cul-4A and treated either in the absence or presence of proteasome inhibitor LLnL (Fig. 6, lanes 4 and 5) or transfected with F-p48 and myc-Cul-1 (Fig. 6, lane 6). A CD19 expression plasmid was included in the transfection for enrichment of transfected V79–4 cells by immunomagnetic selection prior to the preparation of cell extracts in EMSA analysis. In V79–4 cells transfected with myc-Cul-4A and F-p48, a dramatic decrease of the B2 DNA protein complex was readily observed (Fig. 6, compare lane 4 with lane 3), consistent with the accelerated p48 degradation by CUL-4A (Fig. 3A). Moderate down-regulation of the B1 complex was also detected (Fig. 6, lane 4). Treatment of Cul-4A and F-p48 coexpressing cells with the proteasome inhibitor LLnL stabilized F-p48 (Fig. 3A, lane 4) and restored the DDB activity (Fig. 6, lane 5). In contrast, the expression of myc-Cul-1 did not inhibit the UV-DDB activity (Fig. 6, lane 6). Furthermore, in the repair-competent 293T cells (19, 27), ectopic expression of CUL-4A also inhibited the UV-DDB activity (data not shown). These results provide functional evidence that CUL-4A-mediated proteolysis of p48 is involved in the regulation of UV-damage induced DNA binding activity.

**DISCUSSION**

Little is known about the proteolytic targets and the cellular processes controlled by the CUL-4A ubiquitination machinery. Recent biochemical studies indicate the association of CUL-4A with the DDB complex, however, the physiological role of this interaction is unknown. The results shown in this study provide compelling evidence that both subunits of the heterodimeric DDB complex are targets of CUL-4A-mediated ubiquitination. First, CUL-4A stimulated the accumulation of higher molecular weight species that were immunoreactive with both DDB subunits (p125 or p48) and HA-tagged ubiquitin (Fig. 3B and Fig. 5). Second, the CUL-4A(Δ) mutant defective for binding Rbx13 was also impaired in ubiquitinating p125 and p48 (Fig. 5, data not shown). This indicates that the association between Rbx1 and the conserved CH domain of CUL-4A is essential for CUL-4A-mediated ubiquitination, similar to what has been demonstrated for the Rbx1/CUL-1 ma-

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3. L. Douglas, unpublished result.
were subjected to immunoblotting to detect F-p125 or MYC-CUL-4A. The mono- or di-ubiquitin-modified F-p125 species are marked with an asterisk. Polyubiquitinated p125 species are also indicated on the right. The position of migration of unmodified F-p125 is indicated by an arrow on the right. C, an unknown protein in HeLa cell extracts that was precipitated by the anti-HA antibody and reactive with the anti-FLAG antibody. C, in vivo ubiquitination assays were carried out using HeLa cells transfected with F-p125 and HA-Ub along with increasing amounts of myc-Cul-4A, C, or myc-Cul-1 as in B (in μg). The mono- or di-ubiquitinated F-p125, marked with an asterisk, was detected and served as a sensitive readout to assess the levels of p125 ubiquitination in response to increased expression of myc-Cul-4A, F-Cul-4A(Δ), or myc-Cul-1 (data not shown). F-p125 levels were determined from 100 μg of extracts by immunoblotting using the anti-FLAG monoclonal antibody. Migration positions of molecular mass standards (in kilodaltons) are indicated on the left.

FIG. 5. CUL-4A stimulates p125 ubiquitination. A, HeLa cells were transiently transfected with plasmids as indicated (in μg). Lysates were subjected to immunoblotting to detect F-p125 or MYC-CUL-4A. B, 1 mg of lysate was immunoprecipitated with anti-HA monoclonal antibody and probed with anti-FLAG (M2) antibody to detect ubiquitinated F-p125. The mono- or di-ubiquitin-modified F-p125 species are marked with an asterisk. Polyubiquitinated p125 species are also indicated on the right. The position of migration of unmodified F-p125 is indicated by an arrow on the right. C, an unknown protein in HeLa cell extracts that was precipitated by the anti-HA antibody and reactive with the anti-FLAG antibody. C, in vivo ubiquitination assays were carried out using HeLa cells transfected with F-p125 and HA-Ub along with increasing amounts of myc-Cul-4A, C, or myc-Cul-1 as in B (in μg). The mono- or di-ubiquitinated F-p125, marked with an asterisk, was detected and served as a sensitive readout to assess the levels of p125 ubiquitination in response to increased expression of myc-Cul-4A, F-Cul-4A(Δ), or myc-Cul-1 (data not shown). F-p125 levels were determined from 100 μg of extracts by immunoblotting using the anti-FLAG monoclonal antibody. Migration positions of molecular mass standards (in kilodaltons) are indicated on the left.

Cherny (37). Third, ectopic expression of Cul-1 did not stimulate the ubiquitination and/or degradation of p125 or p48, suggesting that DDB proteins are specific targets of CUL-4A (Fig. 5, data not shown). Finally, the R276H (2RO) mutant of p48, which is defective for binding CUL-4A, is not subjected to CUL-4A-mediated degradation (Fig. 3E). These findings demonstrate that the CUL-4A ubiquitination machinery specifically targets the damaged DNA-binding proteins p125 and p48 for ubiquitination.

The CUL-4A-mediated degradation of DDB correlates with the cellular distribution of CUL-4A and the DDB subunits. Our immunofluorescence results indicate that CUL-4A is localized predominantly in the cytoplasm, and a small fraction (2–3%) of CUL-4A could be detected in the nucleus (Fig. 2). The presence of leucine-rich sequences within CUL-4A, which is similar to the human immunodeficiency virus rev-like nuclear export sequence (38), may account for its predominant cytoplasmic localization through nuclear export.4 Because nuclear translocation of p125 upon UV irradiation correlates with a reduction of its turn over rate (Fig. 4), it is likely that the ubiquitin-protein ligase activity of CUL-4A is limiting in the nuclear compartment. This result is consistent with the observation that p48, which resides exclusively in the nucleus (28, 29), was rapidly degraded when CUL-4A was overexpressed (Fig. 3). In accordance with p48 destabilization, we observed an overall reduction in damaged DNA binding activity by DDB (Fig. 6). Therefore, CUL-4A-induced degradation of p48 plays a critical role in restricting the UV-DDB activity.

p125 is a relatively abundant protein and is present in excess over p48 in unirradiated HeLa cells (16, 21). Our studies indicate that p125 is metabolically unstable, suggesting a requirement for the precise control of p125 levels inside the cell. Interestingly, a previous study (34) reported that in at least three DDB- XP-E cells, p125 levels were at least 50% lower than that in the DDB+ cells. We have shown that CUL-4A induced a robust p125 ubiquitination, but a significant decrease in the overall levels of cellular p125 has not been observed under these experimental conditions (Fig. 1C, data not shown). One possibility is that CUL-4A may not be rate-limiting for degradation of p125, therefore, the overexpression of Cul-4A could not further reduce the steady-state levels of p125.

4 L. Douglas and P. Zhou, unpublished result.
Alternatively, p125 has been shown to participate in transcriptional regulation as well as other cellular functions (39–43), and there might be only a fraction of cellular p125 that participates in DNA damage recognition and repair and is subjected to CUL-4A-mediated proteolysis. Because p125 is known to be a highly abundant protein inside the cell (10^5 molecules/cell) (21), it is likely that the endogenous p125 competes with the transfected p125-HA for the CUL-4A machinery. Alternatively, because proteolysis is not the only destination or immediate fate of an ubiquitinated protein (reviewed in Ref. 1), it is possible that ubiquitination of p125 might have additional roles besides degradation. Non-proteolytic functions have been possible that ubiquitination of p125 might have additional roles besides degradation. Non-proteolytic functions have been

Microinjection of purified DDB into DDB-/- XP-E cells restored the in vivo DNA repair synthesis to normal levels, establishing the function of DDB in DNA repair (17). However, in vitro reconstitution studies indicated that DDB is not required for nucleotide excision repair of either naked or nucleosomal DNA (48–50). Furthermore, Kazantsev et al. (49) observed an inhibitory rather than stimulatory effect of DDB on the excision repair of 6-4 photoproduct-containing DNA substrate in the in vitro nucleotide excision repair reactions. Thus, it has been suggested that DDB is involved in the initial DNA damage recognition step (28) and must subsequently be removed prior to the initiation of the nucleotide excision repair reactions. We propose that CUL-4A-stimulated ubiquitination and degradation of p48 may serve to restrict the steady-state levels of DDB inside the cell, therefore, ensuring the rapid dynamics of DDB at different stages of DNA damage recognition and repair.

The observation that abnormally high levels of CUL-4A are found in many human tumors suggests a potential role of CUL-4A in tumorigenesis. p48 has been shown to be the rate-limiting factor for UV-DDB activity and functions to suppress UV-induced mutations from genomic DNA and the non-transcribed DNA strand of expressed genes (19).

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