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Mammalian DET1 Regulates Cul4A Activity and Forms Stable Complexes with E2 Ubiquitin-Conjugating Enzymes▽

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DET1 (de-etiolated 1) is an essential negative regulator of plant light responses, and it is a component of the *Arabidopsis thaliana* CDD complex containing DDB1 and COP10 ubiquitin E2 variant. Human DET1 has recently been isolated as one of the DDB1- and Cul4A-associated factors, along with an array of WD40-containing substrate receptors of the Cul4A-DDB1 ubiquitin ligase. However, DET1 differs from conventional substrate receptors of cullin E3 ligases in both biochemical behavior and activity. Here we report that mammalian DET1 forms stable DDD-E2 complexes, consisting of DDB1, DDA1 (DET1, DDB1 associated 1), and a member of the UBE2E group of canonical ubiquitin-conjugating enzymes. DDD-E2 complexes interact with multiple ubiquitin E3 ligases. We show that the E2 component cannot maintain the ubiquitin thioester linkage once bound to the DDD core, rendering mammalian DDD-E2 equivalent to the *Arabidopsis* CDD complex. While free UBE2E-3 is active and able to enhance UbcH5/Cul4A activity, the DDD core specifically inhibits Cul4A-dependent polyubiquitin chain assembly in vitro. Overexpression of DET1 inhibits UV-induced CDT1 degradation in cultured cells. These findings demonstrate that the conserved DET1 complex modulates Cul4A functions by a novel mechanism.

The ubiquitin conjugation system is used as a common regulatory strategy in all eukaryotic organisms (12). Ubiquitin is activated by the E1 ubiquitin-activating enzyme. It is then transferred to the catalytic site cysteine residue in a ubiquitin-conjugating enzyme (E2), forming an E2~ubiquitin thioester intermediate. Finally, the ubiquitin-charged E2 cooperates with an E3 ligase to attach ubiquitin to a substrate protein. For those substrates targeted to the 26S proteasome, the initial ubiquitin attachment is followed by chain elongation (14).

DET1 (*d*e-*et*iolated 1) was originally isolated as a key regulator of light-activated development in *Arabidopsis thaliana* (6) and in tomato (known as *HP2* for *h*igh *p*igment 2) (26). *Det1* mutants have an altered gene expression pattern and are unable to undergo the etiolation developmental path in darkness (6, 24, 28, 33). In addition, the weak mutants are hypersensitive to light signals and exhibit an abnormal circadian rhythm. DET1 has been shown to negatively regulate ubiquitin-proteasome-mediated turnover of an important circadian clock regulator, LHY (late elongated hypocotyl) (34). DET1 forms a protein complex known as the CDD complex with the *Arabidopsis* homolog of DDB1 (damaged DNA binding protein 1) (33) and COP10 (41). COP10 is a ubiquitin E2 variant (UEV) that has the conserved catalytic core domain (UBC) but lacks the cysteine residue that forms the thioester linkage with ubiquitin and is therefore catalytically inactive (36). COP10 is distinct from other known UEVs, such as MMS2, UEV1, and TSG101, as it displays highest homology to the *Saccharomyces cerevisiae* Ubc4/5 family of canonical E2s (20, 36). The *Arabidopsis COP10* belongs to the *COP*/*DET*/*FUS* class of loci that also includes conserved genes such as *DET1*, *COP1*, and most of the COP9 signalosome (CSN) genes. This group is defined by the characteristic photomorphogenic phenotype of the mutant plants (38, 42). Typically, *cop*/*det*/*fus* mutants, now including tomato *hp1*(*ddb1*), display a constitutive or hypersensitive light response (6, 22, 24). Recent studies have demonstrated that members of this group mediate their functions via the ubiquitin system (38, 42). Unlike DET1 and DDB1, the human counterpart of COP10, has not been identified to date by conventional bioinformatic approaches. Likewise, it remains unclear whether a homologous CDD complex exists in animals.

DDB1 functions as an adaptor for the Cul4A ubiquitin ligase, a member of the cullin-RING ubiquitin E3 ligase family (CRL). DDB1 forms a complex with WD domain-containing substrate receptors and assembles with Cul4A-Rbx1 (Roc1/Hrt1) into specific E3 ligase complexes (1, 10, 11, 17). The Cul4A-DDB1 E3 ligases regulate cell cycle progression, replication, and DNA damage responses (4, 13, 17, 31, 35). One of the Cul4A-DDB1 substrates is the DNA replication licensing factor CDT1, which is degraded in S phase and after DNA damage (13, 17).

Human DET1 has been shown to interact with human COP1, DDB1, and Cul4A and together mediate c-Jun ubiquitination and degradation (39). In addition, SCF^{FWD7} and Itch, which are CRL family E3 and HECT-domain E3, respectively, have also been demonstrated to mediate signaling-dependent ubiquitination of c-Jun in various biological contexts (9, 27). In this study, we report that DET1, which does not

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contain a WD40 domain, exhibits unique characteristics distinct from conventional Cul4-DDB1 substrate receptors, such as DDB2, CSA, and Cdt2. Our data show that DET1 interacts with multiple E3 ubiquitin ligases and negatively regulates Cul4A-mediated polyubiquitination in vitro. In addition, mammalian DET1 forms a stable DDD core complex with DDB1 and DDA (DET1, DDB1-associated 1). The DDD complex recruits a member of the UBE2E group of canonical E2s, forming a DDD-E2 complex. The UBE2E group shows highest sequence homology to COP10 in the *Arabidopsis* genome. Moreover, the DDD-associated UBE2E-3 is not charged with ubiquitin, while the free UBE2E-3 is. We suggest that the DDD-E2 complex is the mammalian counterpart of the *Arabidopsis* CDD complex.

MATERIALS AND METHODS

Cell culture and siRNA experiment. HeLa cells, HEK293 cells, and Flag-DET1 stable cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a CO_2 (5%) incubator at 37°C. The small interfering RNA (siRNA) oligos against DDA1 were made using SMARTpool reagent (Dharmacon). The siRNA sequences (sense) against other genes were as follows: UBE2E-3, 5′-GCAUAGCCAC UCAGUAUUU; UBE2E-1, 5′-GCGA UAACAU CUAUGAAUG; UBE2E-2, 5′-UCACCAGACU AUCCGUUUA. Transfections were mediated by Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Cells were collected 48 h posttransfection for the siRNA experiment and at 24 h for regular plasmid transfection.

Purification of Flag-DET1 complex. To generate Flag-DET1 stable cell lines in HEK293 cells, pFlag-DET1 was cotransfected with pBABE, which confers puromycin resistance. One day later, cells were replated in selection medium containing 3 μg/ml puromycin (Sigma). A cell line with moderate expression of Flag-DET1 was chosen for purification of the complex.

Flag-DET1 cells from 40 15-cm dishes were collected 48 h after seeding. Cells were extracted by sonication in hypotonic buffer (20 mM HEPES pH 7.2, 5 mM KCl, 2.5 mM EDTA) supplemented with Complete protease inhibitors (Roche) and 0.4% NP-40. The extract was mixed with high-salt buffer (20 mM Tris pH 7.4, 1 M NaCl, 0.4% NP-40, 10% glycerol) to a final volume of 40% (vol/vol), incubated on ice for 10 min, and clarified by centrifugation (14,000 × *g* for 15 min at 4°C). M2 beads (Sigma) were incubated with the supernatant in a ratio of 80 μl slurry to 6 mg of total protein for 6 to 12 h on a roller at 4°C. The beads were washed in 50% high-salt buffer twice for 10 min at 4°C and then twice in elution buffer (25 mM Tris-HCL, pH 7.4, 150 mM NaCl, 10% glycerol). Finally, Flag peptide (1 mg/ml; Sigma) dissolved in elution buffer was incubated with the sample for 15 min at room temperature. To estimate the quantity of the complex, first the amount of associated E2 was determined based on silver staining intensity, and then the amount of the complex was calculated assuming that the E2s represented 10% of the total mass of the complex.

Gel filtration chromatography and immunoprecipitation. Cells were washed twice in phosphate-buffered saline (PBS) and collected in cold hypotonic buffer supplemented with 1 mM dithiothreitol (DTT), 0.1% NP-40, and protease inhibitor cocktail (Roche), with or without 10 mM ATP and 20 mM $MgCl_2$. Cells were lysed by freeze-thaw in liquid nitrogen and a 37°C water bath or sonication. The samples were cleared by microcentrifugation for 10 min at 4°C. Supernatant was filtered through a 0.2-μm filter and applied to a prepacked Superose-6 gel filtration column (Amersham-Pharmacia). The equilibration and elution buffers contained 20 mM Tris-HCl pH 7.2, 200 mM NaCl, and 10% glycerol. Fractions of 0.5 ml each were collected starting from 7 ml and were mixed with sample buffer for immunoblot analysis.

For immunoprecipitation, cell extract was prepared in hypotonic buffer and sonicated as described above. The extract was then mixed 1:1 with PBS buffer containing 0.4% Triton X-100 and incubated on ice for 10 min. The sample was clarified by centrifugation (14,000 × *g* for 15 min at 4°C) twice, and the supernatant was incubated with antibody-linked agarose beads for 4 to 12 h at 4°C. The beads were washed four times with PBS buffer containing 0.1% Tween 20. For T7-Cul4A and Flag-DET1 complexes used for in vitro E3 assays, two additional washes with the ubiquitin reaction buffer were performed.

UV treatment. UV treatment of HeLa cells in 12-well or 6-well dishes was carried out 24 hours after transfection. Culture medium was removed, and the cells were washed once in PBS. The dishes were placed under UV-C lamps (254 nm) in the chamber of a UV Stratalinker 2400 model (Stratagene). After irradiation (40 J/m^2), culture medium was added back and the cells were returned to the incubator for the specified times. To collect the samples, cells were washed with PBS and directly lysed with 3× sodium dodecyl sulfate (SDS) sample buffer, with about 80 μl per well for 12-well dishes and 160 μl for 6-well dishes. The samples were boiled for 6 to 10 min and cleared by centrifugation before SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Antibodies and plasmids. To generate antibodies against UBE2-E1 and UBE2E2, N-terminal amino acid (aa) residues 1 to 44 of UBE2E-1 or aa residues 1 to 52 of the UBE2E-2 fragment were expressed as His-S-tagged fusion proteins in BL21 cells. Proteins were purified by Ni-nitrilotriacetic acid (NTA) affinity resin and introduced into rabbits for antibody production. Each antibody was then demonstrated to recognize only itself but not other homologous E2s. Antibodies specific to UBE2E-3 or UbcM2 were made against the specific N-terminal extension as described elsewhere (7, 30).

Anti-CDT1 (13), anti-COP1 (42), and anti-COP10 (36) have been described previously. Anti-Flag (M2; Sigma), anti-His (penta-His monoclonal; QIAGEN), antiubiquitin (UG9510; BioMol/Affinity), anti-UbcH5 and anti-UbcH12 (Boston Biochem), anti-elongin B (Santa Cruz), anti-T7 (Novagen), antihemagglutinin (anti-HA; sc-805; Santa Cruz), and anti-glutathione *S*-transferase (anti-GST; Amersham Bioscience) were purchased. The rabbit antibody against Cul4A was raised against the peptide ERDKDNPNQY HYVA (ProteinTech Group, Inc.). The rabbit antibody against human DDA was raised against the C-terminal peptide CAPPRKVARTDSPDMHEDT (ProteinTech Group, Inc.). Rabbit anti-DDB1 was raised against the human DDB1 C-terminal 384-aa fragment (aa 756 to 1140), which was produced in *Escherichia coli* from pET-DDB1C384 and purified by using Ni-NTA. Similarly, anti-DET1 antibody was raised against a C-terminal region of mouse DET1 expressed from pRSET-DET1C in *E. coli*.

Information for construction of plasmids used in this study is available upon request.

Purification of recombinant proteins and complexes. Recombinant proteins were expressed in the *E. coli* BL21(DE3) strain carrying the specified plasmids. The DDA1/DDB1 complex was obtained by expressing His-DDA1 and untagged DDB1 from the pET-Duet-HisDDA1-DDB1 vector. The DDA1/DET1 complex was obtained by expressing His-DDA1 and a T7-tagged DET1 from the pET-Duet-HisDDA1-T7DET1 vector (see Fig. 3A, below). The DDA1/DDB1/DET1 (DDD) complex was obtained by coexpressing pET-Duet-HisDDA1-DDB1 (Amp^r) and pET28-HisT7DET1 (Kan^r). All three complexes were purified via His tag (see Fig. 3A) as described below.

The bacteria were cultured in LB at 37°C to an optical density at 600 nm of 0.6, at which time protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside for 3 h or overnight at 25°C. His-tagged proteins were purified with Ni-NTA–agarose beads (QIAGEN or Amersham Biosciences) and eluted with 250 mM imidazole. GST-E2 fusion proteins were purified with glutathione-Sepharose beads (Amersham Biosciences) and eluted with 10 mM reduced glutathione. Samples were dialyzed against a buffer containing 20 mM Tris-HCl pH 7.5 and 20 mM NaCl and aliquoted for later use in the activity assay. Protein amounts were estimated by comparing the Coomassie blue staining intensity with a bovine serum albumin standard.

E2~ubiquitin thioester assay. To detect the E2~ubiquitin thioester linkage in vitro, 100 ng of recombinant GST–UBE2E-3 purified from *E. coli* was incubated with 50 ng E1 (Boston Biochem), 50 μM ubiquitin (Sigma) in the reaction buffer (25 mM Tris-HCl pH 7.4, 3 mM ATP, 5 mM $MgCl_2$, 0.5 mM DTT) for 10 min at 30°C. The sample was incubated with an equal volume of nonreducing SDS sample buffer (8 M urea, 4% SDS, 15% glycerol, 50 mM Tris-HCl pH 6.8, and 0.001% bromophenol blue) for 30 min at 30°C. For reduced samples, a half volume of each of the nonreduced samples was mixed with DTT to a final concentration of 50 mM and boiled for 5 min before loading onto the SDS-PAGE, followed by immunoblotting with anti-GST or anti-UbcM2 antibodies. To detect the endogenous ubiquitin-charged form of UBE2E-3, cells were extracted in an ATP-containing buffer as described in "Gel filtration chromatography and immunoprecipitation," above. Samples were mixed in nonreducing or reducing sample buffer for immunoblotting.

In vitro E3 ligase activity assay. For the T7-Cul4A immunocomplex activity assay, various components as indicated in the figures were incubated at 30°C for 60 min in 40 μl of assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM DTT, and 3 mM ATP. Additional ingredients included 50 ng E1 (Boston Biochem), UbcH5c (Boston Biochem) in amounts specified in the figure legends, and 5 μg ubiquitin in a 1:5 ratio of N-terminal biotinylated ubiquitin (Boston Biochem) to unlabeled ubiquitin (Sigma). T7-Cul4A immunocomplex was obtained by immunoprecipitation from transfected 293 cells using anti-T7-conjugated resin (Novagen).

For the recombinant Cul4 and Cul1 complex experiment, reactions were

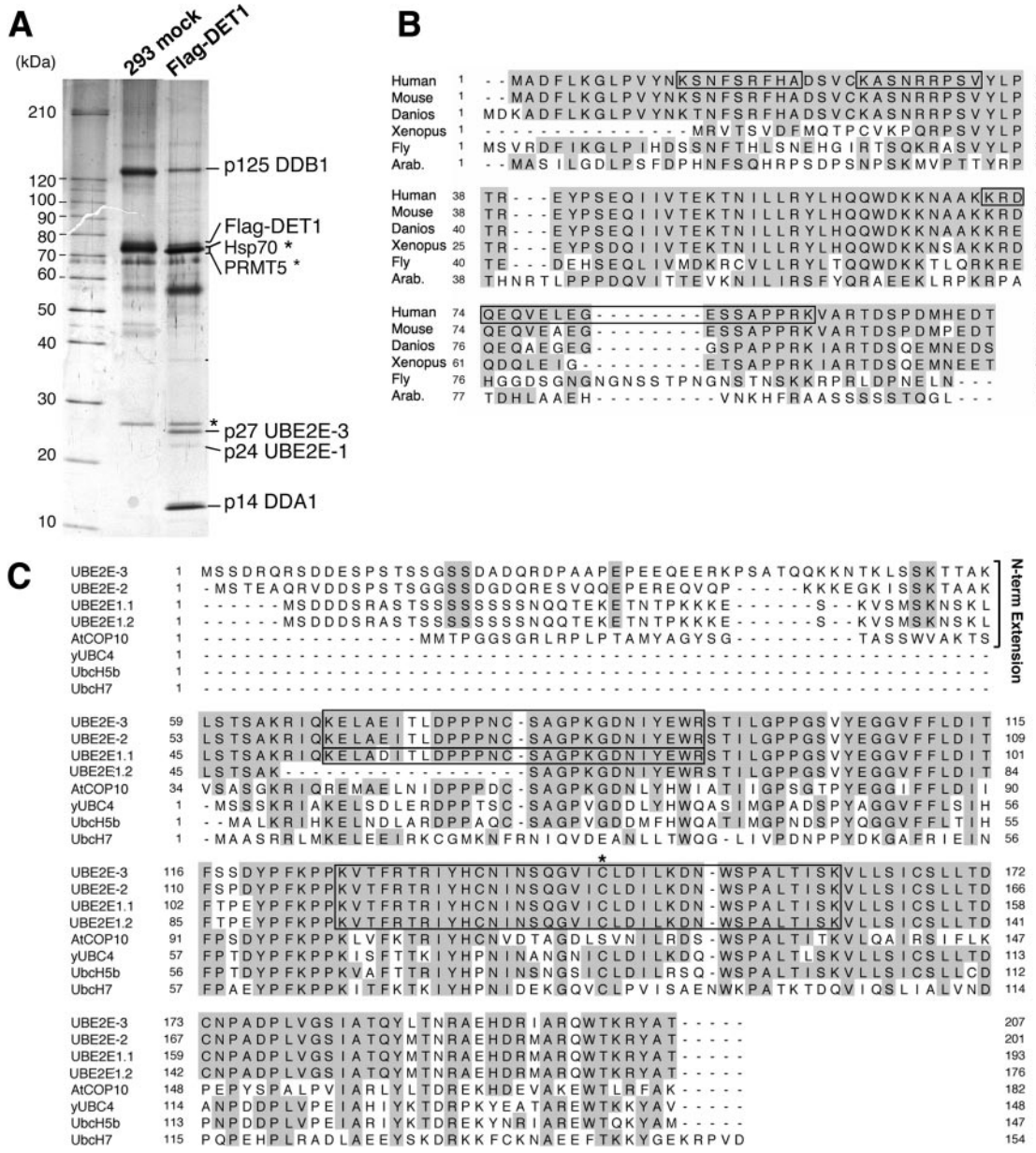

FIG. 1. Isolation of human DET1 complex. (A) Silver staining of Flag-DET1 complex components isolated from HEK293 cells (mock) or Flag-DET1 stable cells. Asterisks indicate nonspecific bands. (B) Sequence alignment of DDA1 orthologs from various organisms. Homologous residues are shaded. (C) Sequence alignment of human UBE2E family E2s, including UBE2E-1 isoforms along with yeast UBC4 (yUBC4), *Arabidopsis* COP10, and UbcH5b and UbcH7. Identical residues are shaded. The conserved catalytic cysteine is indicated by an asterisk. Boxed sequences indicate the peptides identified by mass spectrometry and peptide sequencing.

performed in a total volume of 15 μl that contained 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM ATP, 50 ng yeast E1 (Boston Biochem), 200 ng UbcH5b (Boston Biochem), and 5 μg ubiquitin (Boston Biochem), together with either 300 ng GST-Roc1-Cul1(324–776) (a gift from Zhen-Qiang Pan, Mount Sinai School of Medicine) or 2 μg of DDB1/CUL4A/Rbx1 (a gift from Ning Zheng, University of Washington). Varying amounts of bacterially purified His-DDA1/DDB1, His-DDA1/T7-DET1, or His-DDA1/T7His-DET1/DDB1 (recombinant DDD complex [rDDD]) complexes and bovine serum albumin (Sigma) were added to the reaction mixtures as specified in the figure legends. Reaction mixtures were incubated for 1 h at 37°C before being terminated with DTT-containing SDS sample buffer.

Nucleotide sequence accession number. The sequence for DDA1 was submitted to GenBank (accession number DQ090952).

RESULTS

The human DET1 complex is composed of DET1, DDB1, DDA1, and the UBE2E group of E2s. To isolate human DET1 complex, a cell line stably expressing moderate levels of Flag-tagged mouse DET1 (98% identical to human DET1) was established in HEK293 cells. Flag-DET1 was immunopurified via the Flag tag, and the associated proteins were visualized by silver or Coomassie blue staining (Fig. 1A). Protein bands specific to the Flag-DET1 samples were excised and analyzed by mass spectrometry and direct peptide sequencing. Peptides

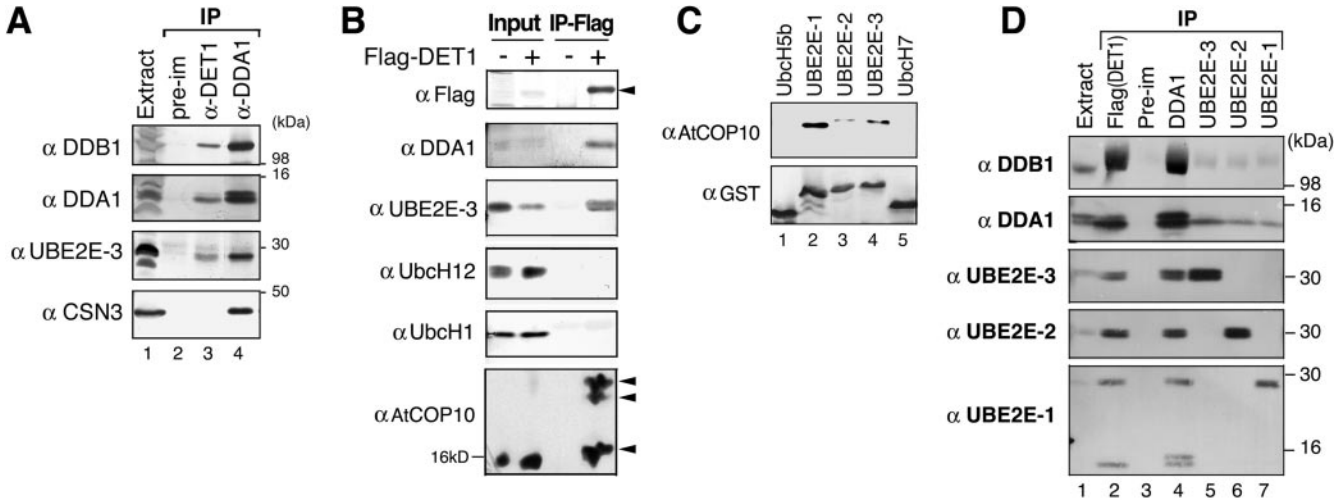

FIG. 2. Endogenous DET1 complex and specificity of DET1-associated E2s. (A) Endogenous DET1 and DDA1 were immunoprecipitated from HeLa cell extracts using their respective antisera or preimmune serum. The samples were immunoblotted using the indicated antibodies. (B) Whole-cell extract (input) and Flag-DET1 immunocomplex (IP-Flag) were analyzed by Western blotting using the indicated antibodies. (C) Recombinant GST-tagged UbcH5, UbE2E-1, UbE2E-2, UbE2E-3, and UbcH7 (50 ng per sample) were loaded on the SDS-PAGE gel. Anti-COP10 or anti-GST blots are shown. (D) Immunoprecipitations from Flag-DET1 cell extracts were performed using specific antibodies to UBE2E enzymes along with preimmune serum (pre-im) or other antibodies as indicated. The samples were analyzed by immunoblotting.

corresponding to Flag-DET1 and DDB1 (p125) were identified. Note that the 124-kDa band in the mock purification was not DDB1 and did not react with the anti-DDB1 antibody (see Fig. 6A). The 72- to 75-kDa samples included nonspecifically bound heat shock proteins and PRMT5.

We consistently found a p14 doublet in stoichiometric amounts to DET1 and DDB1. Peptides from both bands matched MGC2594 or PCIA1 (cross-immune reaction antigen). We named this protein DDA1, for *D*ET1, *D*DB1 *a*ssociated 1 (GenBank accession number DQ090952). DDA1 is a conserved protein of 102 aa residues whose orthologs can be found in vertebrates and invertebrates as well as in *Arabidopsis* (Fig. 1B), but it contains no recognizable motifs or domains.

A group of specific bands around 24 to 28 kDa were extensively analyzed. A peptide (UBE2E-3 aa 126 to 161) encompassing a conserved region of the UBC domain, identical among the UBE2E group of E2s, was repeatedly recovered (Fig. 1C). In addition, peptide sequences matching UBE2E-3 aa 68 to 94 and UBE2E-1 (or UbcH6) aa 54 to 80 were obtained, indicating that multiple E2s from the UBE2E family were in the complex. Each member of this family has a unique N-terminal extension, an identifying feature of class III E2s (25), and is highly homologous to yeast Ubc4/5 and *Arabidopsis* COP10 at the UBC domain (Fig. 1C). Importantly, because the peptide sequence data covered the region containing the active site cysteine-145 (UBE2E-3), we can definitively conclude that these proteins are canonical ubiquitin-conjugating enzymes rather than E2 variants.

Our data showed that stably expressed Flag-DET1 forms a stable protein complex consisting of DDB1, DDA1, and UBE2E-3 E2 enzyme. To verify that this complex exists in normal cells, we generated antibodies against DET1 and DDA1. Immunoprecipitation of endogenous DET1 or DDA1 from HEK293 cells readily pulled down DDB1, DDA1, and UBE2E-3 (Fig. 2A). In addition, DDA1, but not DET1, also coimmunoprecipitated CSN3 (Fig. 2A).

Each DET1 complex contains a specific member of the UBE2E family of enzymes. We next investigated the specificity of the E2s associated with DET1. Antibodies recognizing the unique N-terminal extensions from UBE2E-1 (UbcH6), UBE2E-2, and UBE2E-3 confirmed that all three enzymes were in the DET1 complex (Fig. 2D). In contrast, UbcH12, a Nedd8-specific E2 that also contains an N-terminal extension, and UbcH1, an E2 with a C-terminal extension, were not detected (Fig. 2B). Similarly, UbcH5 and UbcH7 were not detected in the complex (not shown). Interestingly, a polyclonal antibody against *Arabidopsis* COP10 can cross-react with recombinant human UBE2E-1, UBE2E-2, and UBE2E-3, but not UbcH5 and UbcH7 (Fig. 2C). Anti-COP10 strongly reacted with several proteins in the human DET1 complex, which were probably the UBE2E group of E2s (Fig. 2B, bottom panel). Together, the data show that only the UBE2E groups of E2s are specifically associated with DET1 and that the complexes contain multiple E2 enzymes from this group.

Two different scenarios can be envisioned with regard to how multiple E2s are held in the complex. In one model, two or more E2 molecules are bound simultaneously as a dimer or through multiple E2-docking sites in one complex. Alternatively, each complex contains a single E2 molecule and different E2s are assembled into different DET1 complexes. These two models can be distinguished by asking whether the E2s can associate with each other. Each of the UBE2E group of E2s was immunoprecipitated from Flag-DET1 stable cells using specific antibodies for each enzyme (Fig. 2D). Each specific E2 antibody only precipitated itself and DDA1, but not the other E2 enzymes (lanes 5 to 7), while Flag-DET1 and DDA1 pulled down all of the E2s (lanes 2 and 4). These data conclusively show that UBE2E-1, UBE2E-2, and UBE2E-3 do not form

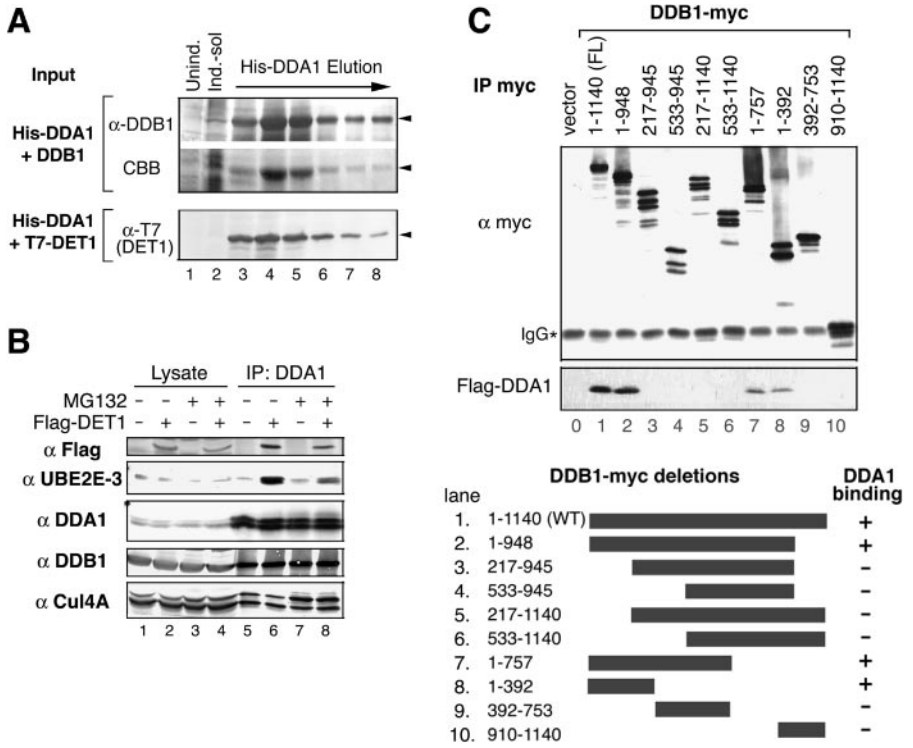

FIG. 3. Characterization of DDA1. (A) His-DDA1 was coexpressed with untagged DDB1 (upper panel) or with T7-DET1 (lower panel) in *E. coli*. The elution from the Ni-NTA resin was examined by Coomassie brilliant blue staining (CBB) and anti-DDB1 or anti-T7 blot assays. (B) HeLa cells were transfected with Flag-DET1 or vector and were incubated with MG132 (10 μM) for 6 h in the indicated samples. Endogenous DDA1 was immunoprecipitated and analyzed by Western blotting. (C) DDA1 binds to the N-terminal domain of DDB1. Myc-tagged DDB1 and the truncation mutants were coexpressed with Flag-DDA1 in HEK293 cells. DDB1 proteins were pulled down by anti-myc resin, and association of Flag-DDA1 was detected by anti-Flag blotting. The data are summarized along with a diagram of the corresponding DDB1 derivatives in the bottom panel.

stable heterodimers under these experimental conditions, nor are they present in a single DET1 complex. Therefore, each DET1 complex carries a single E2 enzyme, while different E2s are held in different complexes.

To distinguish different UBE2E-containing complexes, we hereafter refer to the complex composed of DET1, DDB1, and DDA1 as the DDD core complex. The DDD core recruits a specific UBE2E enzyme, for example, UBE2E-3, to assemble a specific DDD-UBE2E-3 holo-complex. Our data show that different UBE2E enzymes assemble with distinct DDD cores to form a heterogeneous population of DDD-E2 complexes in the cell.

DDA1 directly binds DET1 and DDB1 and is an integral subunit of the DDD core complex. We further characterized DDA1 with respect to its interactions with DDB1, DET1, and E2s. Although recombinant DDB1 and T7-DET1 were insoluble when expressed alone in *E. coli*, each formed a soluble complex when coexpressed with His-DDA1 and coeluted with it from the Ni-NTA resin (Fig. 3A). This result indicates that DDA1 is able to bind DDB1 and DET1 independently. In mammalian cells, anti-DDA1 antibody efficiently coprecipitated DDB1, DDA1, and UBE2Es (Fig. 2A and D), as well as Cul4A (Fig. 3B). Notably, overexpression of DET1 significantly increased the association of DDA1 with the E2, while its association with DDB1 or Cul4A remained unchanged (Fig. 3B, lanes 6 and 8). This result indicates that DET1 is the limiting factor for E2 binding.

DDB1 folds into a three-propeller structure (21). To understand how DDB1 binds DDA1, we expressed a series of DDB1 truncation mutants in cultured cells and tested their ability to bind DDA1. As shown in Fig. 3C, DDB1 N terminal aa 1 to 392, which form propeller A (BPA), is necessary and sufficient to bind DDA1. Thus, the DDA1 binding site resides in the BPA propeller of DDB1.

UBE2E-3 dynamic association with DET1 and role of its N-terminal extension. Gel filtration fractionation of cell extract showed that Flag-DET1 and DDA1 predominantly coeluted in the 250-kDa fractions as DDD-E2 complexes (Fig. 4A). A low-abundance shoulder of approximately 650 kDa was also detected. In addition, DDB1 displayed a wide elution profile, while the CSN1 blot served as an internal marker indicating the CSN peak position of approximately 500 kDa. Notably, UBE2E-3 was separated into two populations; about 10% or less cofractionated with DET1 and DDA1, while the majority eluted as "free UBE2E-3" in fractions corresponding to its monomer size (Fig. 4A). Other UBE2Es behave similarly to UBE2E-3 according to an anti-COP10 blot on these fractions (not shown). In addition, we found that association of UBE2E-3 with DET1 appeared to be more sensitive to ATP concentrations above 15 mM compared to DDA1 (Fig. 4B)

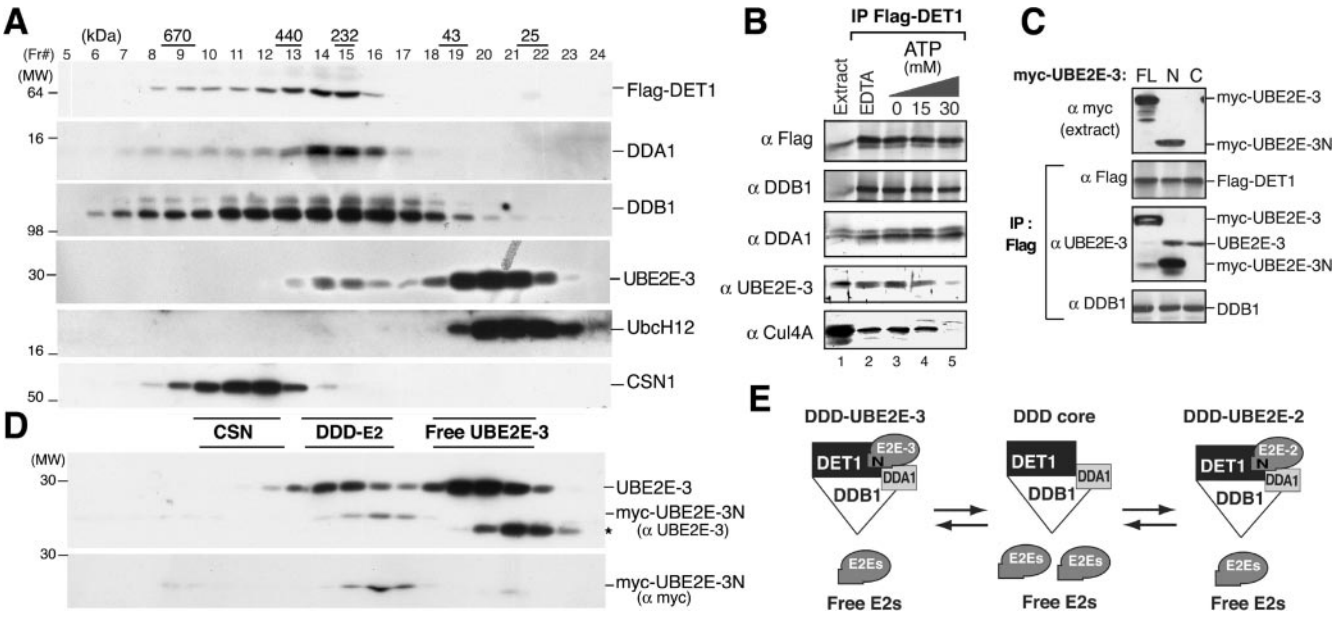

FIG. 4. Characterization of the UBE2Es in DET1 complexes. (A and D) Superose-6 gel filtration analyses of total cell extracts from a Flag-DET1 stable line (A) or Flag-DET1 stable cell lines expressing myc-UBE2E-3N (D). Fractions were probed with the indicated antibodies. Fractions corresponding to CSN, DDD-E2, and free UBE2E-3 are labeled at the bottom. The myc-UBE2E-3N-containing DDD complex appeared slightly smaller than the endogenous DDD-UBE2E-3 complex. The Asterisk indicates the presumed partial breakdown product recognized by anti-UBE2E-3 but not by anti-myc antibody. (B) Flag-DET1 cell extracts were incubated with EDTA (5 mM) or increasing concentrations of ATP as indicated prior to Flag immunoprecipitation (IP). Decreased associations with UBE2E-3 and Cul4A were observed, while steady-state levels of UBE2E-3 and Cul4A did not change (not shown). (C) Myc-tagged UBE2E-3 full length (FL), UBE2E3-N(1–66) (N), or UBE2E3-C(60–207) (C) was transfected to Flag-DET1 cells. Total extract or Flag immunocomplex samples were blotted as indicated. (E) Proposed model describing the dynamic interactions of the UBE2Es and the DDD core complex.

and that knockdown of the UBE2E family of E2s does not detectably affect association of DET1, DDB1, and DDA1 (data not shown). We suggest that E2 association with DDD is a dynamic event, as illustrated in Fig. 4E.

Besides the overall sequence homology, the UBE2E group of E2s shares with *Arabidopsis* COP10 an N-terminal extension (Fig. 1C). The function of this extension is unknown, because all of the reported interactions between UBE2E enzymes and other proteins, such as importin-11 and the E3s, are mediated through the UBC domain (7, 16, 29, 30). To evaluate the role of this domain in DDD binding, we transiently expressed full-length UBE2E-3 (myc-UBE2E-3), the N-terminal aa 1 to 66 domain (myc-UBE2E-3N), or the C-terminal aa 60 to 207 fragment (myc-UBE2E-3C) in Flag-DET1 cells (Fig. 4C). Unfortunately UBE2E-3C failed to express or accumulate in the cells, precluding further analysis. Still, Flag-DET1 effectively pulled down myc-UBE2E-3 and endogenous UBE2E-3, as well as myc-UBE2E-3N, indicating that the N-terminal extension of UBE2E-3 is sufficient to bind DET1. In agreement, gel filtration analysis showed that myc-UBE2E-3N was in the complex-associated fractions (Fig. 4D). The UBC domain also plays a role, because the full-length myc-UBE2E-3, but not the N-terminal fragment, could effectively compete against endogenous UBE2E-3 for Flag-DET1 binding (Fig. 4C).

Association of uncharged UBE2E-3 with the DDD core complex. The function of E2s involves the formation of an unstable E2~Ub thioester intermediate via the catalytic site cysteine residue (ubiquitin loading or charging). We set out to determine whether the DDD core complex has a binding preference for the catalytic state of the E2. Toward this end, a recombinant complex comprised of HisT7-DET1, His-DDA1, and substoichiometric amounts of untagged DDB1 was isolated from *E. coli* (rDDD) (Fig. 5A). This complex could bind to GST–UBE2E-3 in vitro without ubiquitin charging (Fig. 5B, lane 6), indicating that ubiquitin charging is not required for the E2s to associate with DDD.

To extend this observation in vivo, immunoprecipitation of Flag-DET1 was performed in an extraction buffer supplemented with 10 mM ATP to better maintain the ubiquitin-charged state of the E2s. Under nonreducing conditions, UBE2E-3~ubiquitin thioester conjugates could be detected in total cell extracts and the unbound fraction, but not in DET1-bound samples (Fig. 5C). This result indicates that DET1 preferentially associates with the uncharged form of UBE2E-3. Further, we examined the distribution of ubiquitin-charged and uncharged UBE2E-3 in the gel filtration fractions. Remarkably, all of the ubiquitin-charged UBE2E-3 was found in the free E2 fractions, while the complex-bound UBE2E-3 was exclusively in the uncharged state (Fig. 5D). The relative ratio of the complex-associated and free UBE2E-3 did not significantly change with or without the 10 mM ATP supplement (Fig. 4A and 5D).

An in vitro thioester assay was carried out to compare the ubiquitin-charging efficiency of recombinant GST–UBE2E-3 and DDD–UBE2E-3, the latter isolated as Flag-DET1 immunocomplex. Recombinant GST–UBE2E-3 could be detectably charged with ubiquitin by using as little as 20 ng of the protein (Fig. 5E, lanes 4 to 6). In contrast, the Flag-DET1 complex,

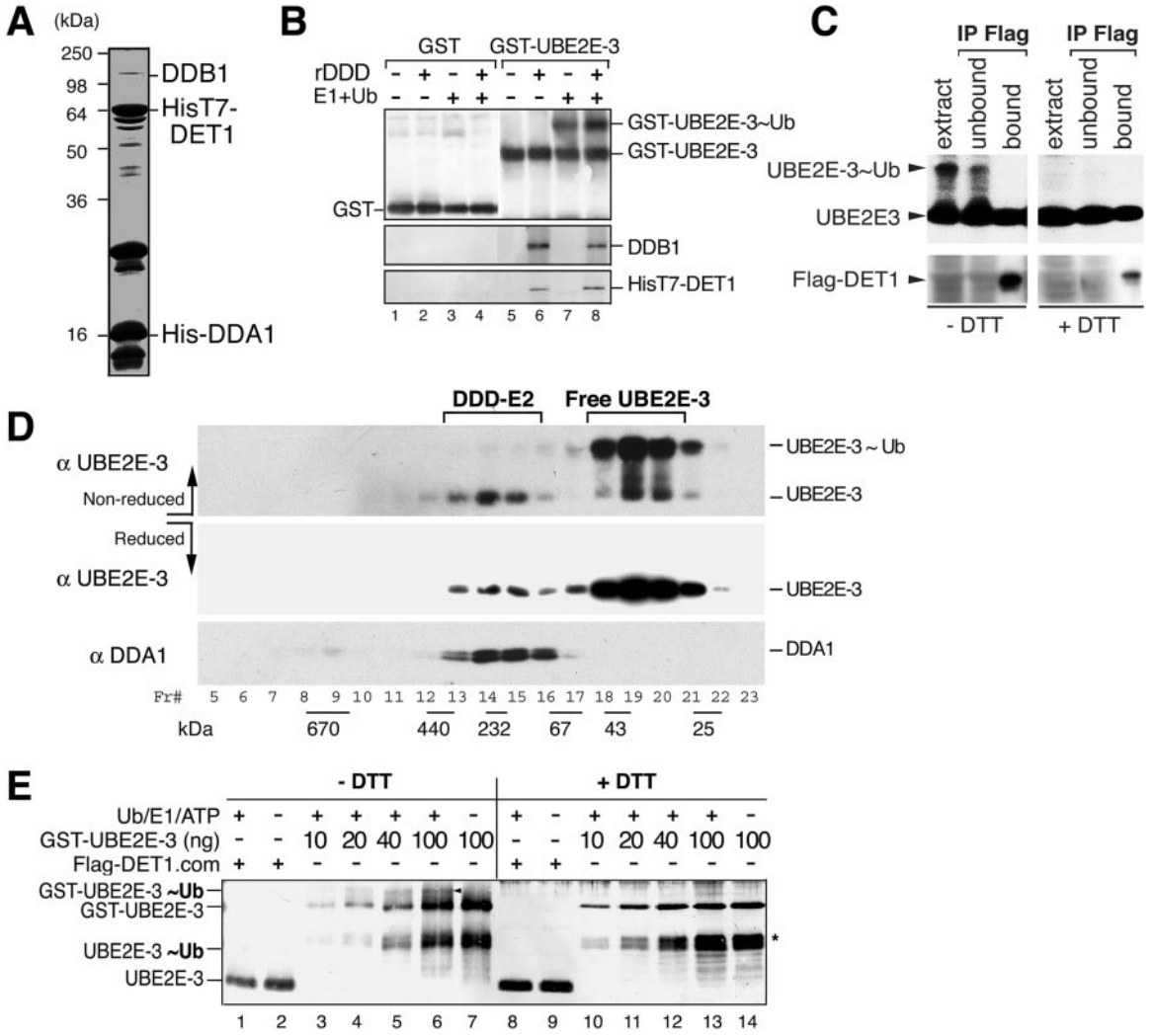

FIG. 5. (A) Coomassie blue gel of the rDDD complex consisting of HisT7-DET1, His-DDA1, and untagged DDB1. The complex was purified from *E. coli* by Ni-NTA resin. Identity of each component labeled on the right was verified by immunoblotting. (B) GST or GSTUBE2E-3 was preincubated under ubiquitin-loading conditions (E1+Ub + lanes) or not (- lanes) and then mixed with rDDD. Following GST pull-down, association of rDDD components was examined by immunoblotting. (C) Flag-DET1 cells were extracted in an ATP-containing buffer, followed by Flag immunoprecipitation (IP). Samples were denatured in a nonreducing (-DTT) or standard reducing (+DTT) sample buffer and probed with anti-UbcM2/UBE2E-3 (top) or anti-Flag (bottom). (D) Flag-DET1 cell extract containing 10 mM ATP was fractionated on a Superose-6 column. Fractions were mixed with nonreducing (top panel) or reducing (lower panels) sample buffer before immunoblotting. The ubiquitin-charged UBE2E-3 (UBE2E-3~Ub) was found only in the "free" UBE2E-3 fractions. (E) In vitro ubiquitin thioester assay. Increasing amounts of recombinant GST–UBE2E-3 or Flag-DET1 immunocomplex were incubated with ubiquitin, E1, in an ATP-containing buffer in a 40-μl volume. The samples were probed with anti-UBE2E-3 antibody. The arrowhead indicates the ubiquitin thioester conjugate to UBE2E-3. The Flag-DET1 complex in lane 2 contained about 70 ng of UBE2E-3 as estimated by silver staining.

which contained more than 40 ng of UBE2E-3 as judged by immunoblotting and silver staining, failed to produce a detectable level of ubiquitin thioester conjugates (Fig. 5E, lanes 1 and 2). Because the E2s do not carry thioester-linked ubiquitin in the DDD-E2 form, mammalian DDD-E2 complexes essentially resemble the *Arabidopsis* CDD complex, which is incapable of forming a ubiquitin thioester.

Association of DET1 with multiple E3 ubiquitin ligases. Substrate receptors for Cul4A-DDB1 E3s, such as DDB2, CSA, and Cdt2 WD40 proteins, can pull down approximately stoichiometric amounts of Cul4A and Rbx1, as well as abundant amounts of CSN components (10, 13, 23). Though DET1 resembles these WD40 proteins in binding to DDB1, we did not recover any peptides matching Cul4A or CSNs in the Flag-DET1 immunocomplex. Nonetheless, we detected COP1 and Cul4A in the Flag-DET1 complexes by immunoblotting (Fig. 6A) as previously reported (39). DET1 also coprecipitated a 75-kDa form of Mdm2. In addition, a transiently expressed ARA54, a RING E3 previously shown to bind the UBE2E family of E2s (16), could coprecipitate with DET1 (Fig. 6B). However, unlike CDT2, CSA1, and DDB2, the DET1 complexes did not contain detectable amounts of CSNs even by immunoblotting. In addition, DET1 showed no interaction with elongin B, a component of the Cul2 E3 complex (Fig. 6A), or Cul1 (not shown). Our data are consistent with the notion that DDD-E2 accumulates as a distinct protein

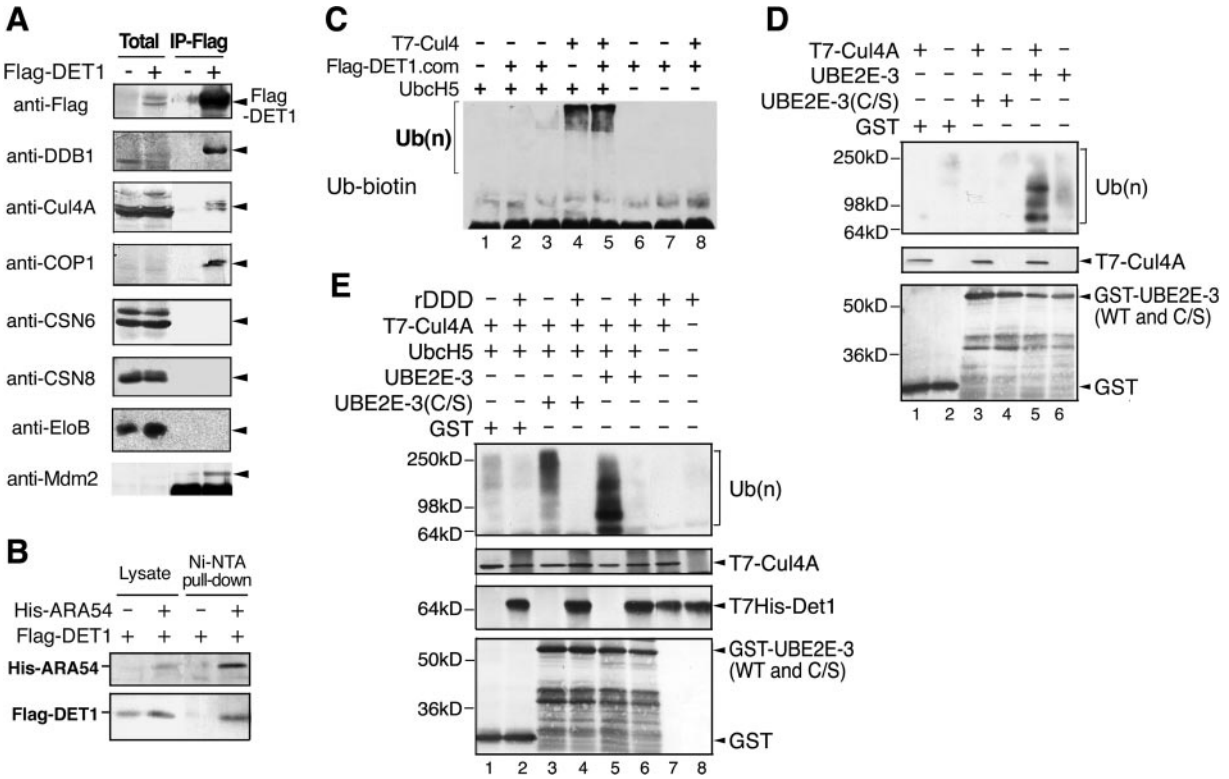

FIG. 6. DDD-E2 complex association with E3 ligases and its effect on Cul4A E3 activity. (A) Whole-cell extract (total) or Flag-DET1 immunocomplex (IP-Flag) was analyzed by Western blotting using the indicated antibodies. (B) His-ARA54 was transiently expressed in Flag-DET1 cells. Proteins in the Ni-NTA pull-down were blotted for anti-His and anti-Flag. (C) All reaction mixtures contained ubiquitin, biotinylated ubiquitin, and E1. UbcH5c (50 ng) or Flag-DET1 complexes containing approximately (50 ng) of E2s were used as E2 sources. (D) UBE2E-3 is an active E2 for Cul4A. GST, GST–UBE2E-3, or its catalytic site C/S mutant (2.5 ng/μl) was used as E2. Ubiquitin chains were detected by antiubiquitin blotting. Equal amounts of Cul4A and GST fusion proteins were confirmed by immunoblotting (lower two panels). (E) A similar experiment was set up as for the experiment in panel D, except that the E2s were UbcH5 (2.5 ng/μl) in combination with GST (5 ng/μl; lanes 1 and 2), UBE2E-3 (5 ng/μl; lanes 5 and 6), or UBE2E-3(C/S) mutant (5 ng/μl; lanes 3 and 4). UBE2E-3(C/S) enhanced polyubiquitination when combined with UbcH5 and Cul4A, while addition of rDDD complex (50 ng/μl) strongly inhibited polyubiquitination detected by antiubiquitin blotting (upper panel). Amounts of UBE2E-3, Cul4A, and DET1 were confirmed by anti-GST and anti-T7 blotting.

complex that transiently interacts with Cul4A and other E3 ligases.

Free UBE2E-3, but not DDD-UBE2E-3, can act as an active E2 or an E2 enhancer for the Cul4A E3 ligase. Toward understanding the function of DDD-E2, we first tested whether this E2-containing complex can act as an E2 to support Cul4A E3 activity in vitro. T7-Cul4A immunocomplex was isolated from transfected cells, and Flag-DET1 immunocomplexes or UbcH5 were used as the sources of E2 (Fig. 6C). As widely used in combination with CRL E3s in vitro, UbcH5 was active in supporting T7-Cul4A immunocomplex, as indicated by the high-molecular-weight polyubiquitin products (lanes 4 and 5). These polyubiquitin products could be unanchored or could be attached to heterogeneous proteins in the T7-Cul4A immunocomplex. Since these polyubiquitin products were generated in a T7-Cul4A- and UbcH5-dependent fashion (Fig. 6C), we used it as a measure of Cul4A E3 activity. We found that the Cul4A complexes were active when supplied with UbcH5 (lane 4), but not with the DET1 complexes (lane 8). These data indicate that DDD-E2 complexes do not have classical E2 activities for Cul4A.

In the following series of experiments, we have dissected DDD-E2 complexes and addressed the activities of DDD and the associated E2s separately. With the same experimental setup as in Fig. 6C, we found that UBE2E-3 was an active E2 for Cul4A in vitro (Fig. 6D, lane 5). Curiously, the ubiquitin chains assembled by UBE2E-3 differed from those assembled by UbcH5, as the former generated shorter and more discrete ubiquitin conjugates in a Cul4A-dependent manner (Fig. 6D, lane 5, compared to E, lanes 1 and 3). The UBE2E-3(C/S) mutant, in which the catalytic cysteine residue was mutated to serine (30), was inactive on its own (Fig. 6D); however, it significantly enhanced UbcH5/Cul4A activity (Fig. 6E, lane 3, compared to lane 1). This observation echoes the E2-enhancing activity described for COP10 UEV (41). While UBE2E-3(C/S) enhanced the amount of polyubiquitin products, it did not change the characteristics of UbcH5-generated chains (Fig. 6E, lanes 1 and 3). Taken together, UBE2E-3 can act as an independent E2 or an E2 enhancer to support Cul4A ubiquitin ligase activity.

DET1/DDD inhibits Cul4A-mediated polyubiquitination in vitro. To investigate the role of DDD on Cul4A E3 activity, we first tested rDDD (Fig. 5A) in the Cul4A immunocomplex assay described above. Remarkably, addition of the rDDD complex at roughly double the molar amount relative to UBE2E-3 drastically inhibited all Cul4A-mediated polyubiq-

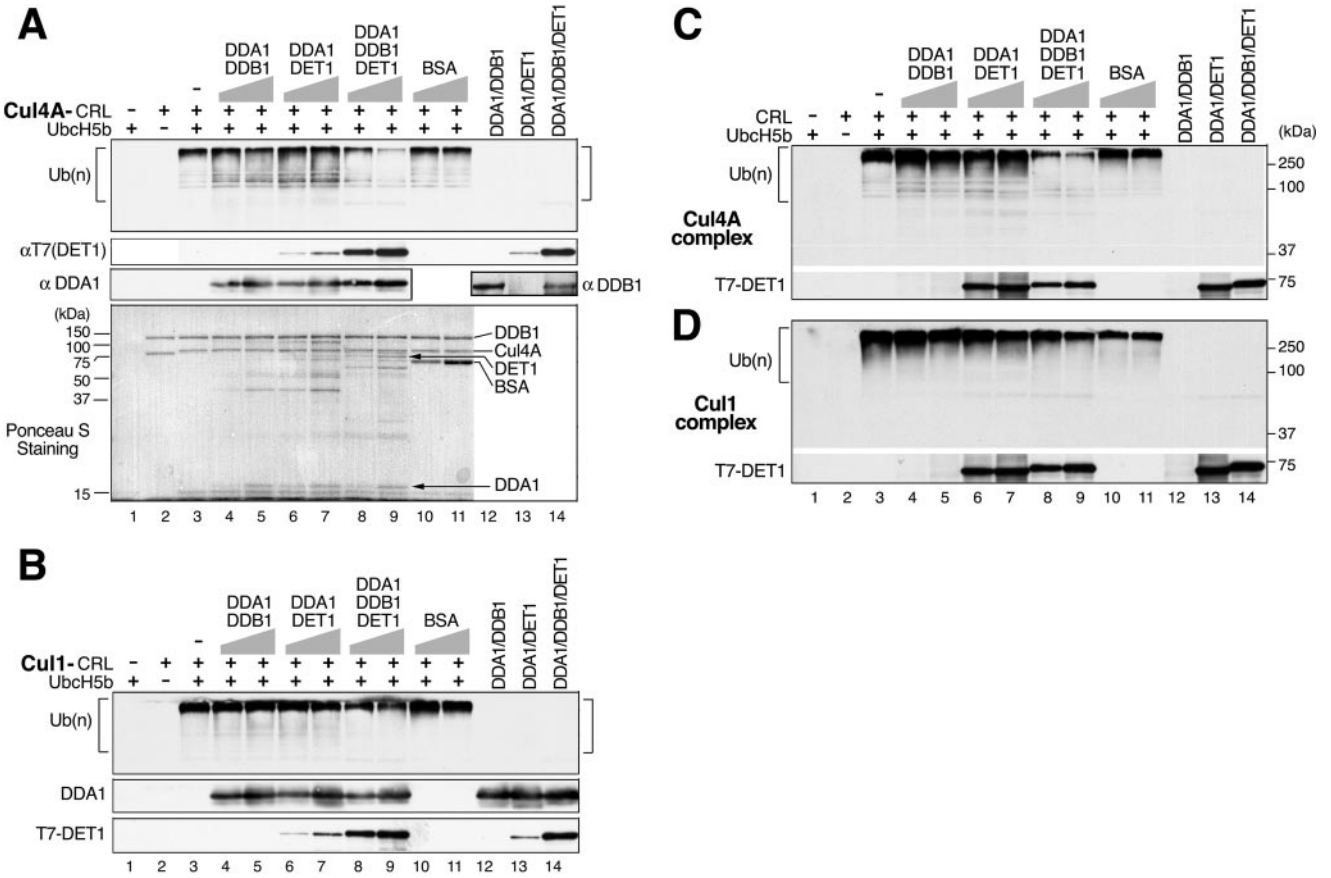

FIG. 7. In vitro E3 activity assay using reconstituted CRL E3. (A) Recombinant Cul4A-DDB1-Rbx1 complex (2 μg) or (B) recombinant Cul1(324–776)-Roc1 complex (0.3 μg) was incubated in a 15-μl reaction mixture containing ubiquitin, ATP, E1, and UbcH5b (0.2 μg). As indicated, bovine serum albumin (BSA; 1.25 and 2.5 μg) or the following recombinant proteins that had equal amounts of DDA1 were added to the reaction mixture: His-DDA1, DDB1 (0.6 and 1.2 μg); His-DDA1, T7-DET1 (0.75 and 1.5 μg); His-DDA1, HisT7-DET1, and DDB1 (rDDD, 0.75 and 1.5 μg). Ubiquitin chain was detected by antiubiquitin blotting. Total protein amounts in the reaction mixtures were examined by Ponceau S staining of the membrane. Identity of labeled protein bands was confirmed by immunoblotting. (C and D) Experiments similar to those in panels A and B, except that the recombinant complexes were normalized for equal amounts of DET1 in the reaction: His-DDA1, DDB1 (3 and 6 μg); His-DDA1, T7-DET1 (5.5 and 11 μg); His-DDA1, HisT7-DET1, DDB1 (rDDD, 0.75 and 1.5 μg). Amounts of DET1 were confirmed by anti-T7 blotting. Ubiquitin chain was detected by antiubiquitin blotting.

uitination without affecting the level of Cul4A in the reaction mixtures (Fig. 6E, lanes 2, 4, and 6). The inhibition was observed in reaction mixtures containing UbcH5 (lanes 1 to 4) or combinations of UbcH5 and UBE2E-3 (lanes 5 and 6), suggesting that DDD-mediated inhibition of polyubiquitination is probably not specific for particular E2s.

We next addressed whether DDD exhibits any specificity at the level of E3s. Since DET1 interacts with Cul4A but not Cul1, we utilized a reconstituted Cul4A-DDB1-Rbx1 E3 complex (1) and a reconstituted Cul1-Rbx1 system comprised of the Cul1 C-terminal fragment (aa 324 to 776) in complex with Roc1 (Rbx1) (40). Both complexes were isolated from bacteria and were able to assemble polyubiquitin chains independent of substrate or substrate receptors (Fig. 7, lanes 3). In addition to rDDD complex, recombinant complexes containing DDA1/DDB1 and DDA1/DET1 were also tested. In Fig. 7A and B, recombinant complexes containing equal amounts of DDA1 were added to the reaction mixture, whereas in Fig. 7C and D, the complexes were normalized according to DET1. Addition of rDDD, but not partial complexes, robustly inhibited Cul4A-mediated polyubiquitination in both cases (Fig. 7A and C, lanes 8 and 9), while only slightly affecting Cul1 activity (Fig. 7B and D). The DDA1/DDB1 and DDA1/DET1 complexes were inactive, despite their containing equal or more DDA1, DDB1 (Fig. 7A), or DET1 (Fig. 7C and D) than DDD. This indicated that all three components were necessary for the inhibition. In addition, these results demonstrate that rDDD specifically inhibits Cul4A-dependent polyubiquitination, supporting the idea that direct interaction with the E3 is necessary for efficient inhibition. On the other hand, the identity of E2, which was UbcH5 in the above assays, appears not to be critical.

Stabilization of CDT1 by DET1. DNA replication licensing factor CDT1 is a Cul4A substrate. Ubiquitination and degradation of CDT1 can be triggered by UV irradiation (13, 17). We examined the role of DET1 and other components of the complex in UV-induced CDT1 degradation. The levels of DET1, DDA1, and UBE2E were knocked down in HeLa cells by siRNA transfection. Fifteen minutes after UV irradiation (40 J/m^2), CDT1 was effectively degraded in negative control

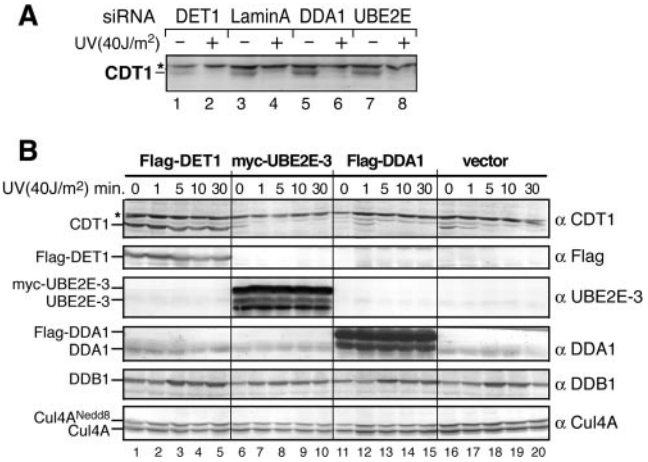

FIG. 8. DET1 inhibits UV-stimulated degradation of CDT1. (A) HeLa cells in 12-well plates were transfected with siRNA oligos corresponding to Lamin A (control), DET1, DDA1, and a combination of UBE2E-1, -2, and -3. After 48 h, cells were irradiated with UV-C light (40 J/m^2). Samples were collected 15 min after the UV treatment by direct lysis. (B) HeLa cells in 12-well plates were transfected with 4 μg of the plasmids expressing different DDD-E2 subunits. After 24 h, cells were exposed to UV-C light (40 J/m^2) and collected by direct lysis in SDS sample buffer at the time points indicated. Levels of CDT1 and overexpressed proteins were detected by immunoblotting. An asterisk denotes a nonspecific band cross-reacting with the anti-CDT1 antibody.

siRNA (lane 4) as well as in siDET1, siDDA1, and siUBE2E cells (Fig. 8A). This indicates that DET1 and the DDD-E2 complexes are not required for UV-induced CDT1 degradation. On the other hand, transient overexpression of Flag-DET1, but not DDA1 or UBE2E-3, abolished UV-stimulated degradation of CDT1 (Fig. 8B). CDT1 stabilization was not caused by alterations in Cul4A ligase level, as the levels of Cul4A and its neddylation state remained largely unchanged (Fig. 8B, bottom panel). These results are consistent with the in vitro data showing that DET1 inhibits Cul4A-mediated polyubiquitination. However, we cannot rule out that CDT1 stabilization resulted indirectly from DET1 overexpression. We also noticed that DET1 overexpression caused an elevation of steady-state CDT1 levels before UV treatment (lane 1 compared to lane 16), suggesting that DET1 may also inhibit cell cycle-dependent degradation of CDT1, which is the major route of CDT1 degradation in undamaged normal cells.

DISCUSSION

Function of DET1 in Cul4A-DDB1 ubiquitin ligases. A large number of DDB1-associated proteins have recently been isolated, most of which are WD40 domain proteins, including DDB2, CSA, and Cdt2, which recruit substrates to the Cul4A E3 ligase for ubiquitination (1, 11, 17). DET1 was also isolated as one of the DDB1-associated proteins. In this study, we demonstrate that DET1 is distinct from the reported Cul4A-DDB1 substrate receptors in both biochemical behavior and activity. First, DET1 does not contain a WD40 domain, a characteristic of Cul4A-DDB1 substrate receptors. Second, the conventional substrate receptors, such as DDB2, CSA, and Cdt2, can each form a stable complex with stoichiometric amounts of Cul4 and Rbx1 such that Cul4 can be visualized by silver or Coomassie blue staining in the immunocomplexes (10, 17, 23). Other CRL substrate receptors, such as VHL of Cul2 complex, SOCS-box proteins of Cul5 complex (18), and Skp2 of Cul1 complex (32), share this feature. This indicates that substrate receptors tend to be associated with their respective E3s in its functional state. In contrast, DET1 complexes contain very small amounts of Cul4 that are detectable only by immunoblotting, suggesting that the majority of DDD-E2 complexes are not associated with Cul4. Instead, DDD-E2 represents a separate structural entity that only transiently interacts with a variety of E3s. Third, DDB2, CSA, and Cdt2 avidly recruit CSN complex such that CSN components can be detected by silver staining (10, 23). In the case of DDB2, CSN could be separated from the DDB2-DDB1-Cul4A-Rbx1 E3 complex by an additional chromatography step, indicating that the association of CSN is secondary to the E3 complex (10). In contrast, purified DET1 complexes do not contain detectable amounts of CSN, even by immunoblotting (Fig. 2). Instead, DET1 uniquely forms a stable complex with DDA1, DDB1, and a specific group of E2 enzymes that are absent from all reported Cul4A substrate receptor complexes. Fourth, the DDD complex inhibits Cul4A-mediated polyubiquitin chain assembly in vitro. Forced overexpression of DET1, but not DDB2 (not shown), abolishes CDT1 degradation in vivo. Consistent with our finding on mammalian DET1, a genetic study in *Arabidopsis* also indicated a negative role of DET1 in ubiquitin-proteasome-dependent degradation of the LHY circadian regulator (34).

Nevertheless, our data on DET1 are mutually compatible with a role of DET1 as an unconventional substrate receptor for Cul4A. This may apply to the case of c-Jun ubiquitination, in which DET1 works with COP1 (39). It remains unclear whether DET1 exerts a different effect on Cul4A in a substrate-dependent manner. Given that *Arabidopsis* DET1 has been shown to bind histones (2), it is an appealing possibility that DET1 may function in specific histone ubiquitination. This idea is further encouraged by recent reports that Cul4A-DDB1 ubiquitin ligases can mediate histone ubiquitination (19, 37) and that a member of the UBE2E family, UBE2E-1 (or UbcH6), participates in histone H2B monoubiquitination by RNF20/RNF40 (43).

DDD-E2 is the mammalian counterpart of *Arabidopsis* CDD despite distinctions between UBE2E enzymes and COP10. *Arabidopsis* COP10 is not homologous to any of the human UEVs but instead displays highest homology to the UBE2E family of E2s in humans (20). The obvious distinction of UBE2Es from COP10 is the fact that UBE2Es are canonical E2 enzymes that have important functions outside of the DDD-E2 complexes. For example, UBE2E-3 is an active E2 for RNF8 and ARA54 RING E3s (16). It also participates in HECT-domain E3 Nedd4-mediated ubiquitination of the Na^+ channel protein EnaC (7). UBE2E-1 works with the RNF20/40 complex to mediate monoubiquitination of histone H2B (43). These differences may account for the observation that the majority of the UBE2E-3 population exists as a monomer, whereas the predominant population of COP10 is in the CDD complex (36). In addition, the mechanisms of nuclear translocation appear to differ. The UBE2E enzymes require ubiquitin loading to interact with importin-11 and to translocate into the

nucleus (30), whereas COP10 localizes to the nucleus by piggybacking on the CDD complex (36).

Importantly, both UBE2Es and COP10 form stable complexes with DET1 and DDB1. Moreover, despite UBE2Es being functional E2s by themselves, once integrated into the DDD-E2 complex, they can no longer maintain ubiquitin~thioesters. As a consequence, the DDD-E2 complex is equivalent to a UEV-containing CDD complex with respect to their biochemical capabilities. Based on these analogies, we suggest that the DDD-E2 complex is the mammalian counterpart of the *Arabidopsis* CDD complex.

Function of DDD-E2 complexes. Among the DDD components, DDA1 strongly binds DDB1 and DET1, and DDB1 provides a platform for interacting with Cul4A and WD40 proteins, while DET1 is the determinant factor in E2 binding and the key component in inhibition of Cul4A. We sometimes found weak polyubiquitination activities in the DET1 immunocomplexes, but the activities were highly variable, depending on the isolation conditions (not shown). Interpretation of these activities is further complicated by the tertiary association of various E3s with DET1.

All three UBE2E group E2s can independently assemble with DDD and together constitute a heterogeneous population of DDD-E2 complexes in the cell. We have shown that the DDD core can inhibit polyubiquitin chain assembly by Cul4A, while UBE2E-3 can facilitate Cul4A reactions in a capacity as either an E2 or an E2 enhancer. In a recent study of plant CDD (5), it was shown that a complex composed of COP10-DDB1-DET1 (without DDA1) enhanced Cul4A activity similar to COP10 alone (41). Still, the function of various DDD-E2 complexes remains elusive at present. It is conceivable that the DDD-E2 complexes may work with other E2s and promote unconventional ubiquitin chain assembly, similar to UbcH13/MMS2 (or UEV1) complexes (8, 15). Alternatively, it may facilitate specialized ubiquitination reactions, such as monoubiquitination.

The *Arabidopsis COP/DET/FUS* loci and the tomato *HP (DDB1/DET1*) loci (22, 26) define a class of regulators critical in plant light responses. This group includes CDD components, the RING-WD protein COP1, and the CSN components (38). Recently, *Arabidopsis* Cul4A has been shown to participate in light responses (3, 5). In this study, we have identified the mammalian DDD-E2 complex as a CDD homolog. It will be interesting to see whether the *Arabidopsis* DDA1 homolog is a component of CDD and whether a genetic mutant of DDA1 bears any resemblance to the phenotype for *cop/det/fus*. The mammalian counterparts of the *COP/DET/FUS/DDB1* genes, along with Cul4A, are involved in cellular responses to UV irradiation (10, 38, 42). CSN is an established regulator of the Cul4A E3 complex. UV activation of Cul4A-DDB1-DDB2 ligase is believed to involve the dissociation of CSN from the E3 complex (10, 35). In this study, we found that DET1 also regulates Cul4A, revealing another level of complexity in the control of the Cul4A E3 ligase system.

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