Ubiquitin-specific Protease 7 Is a Regulator of Ubiquitin-conjugating Enzyme UbE2E1*

Ubiquitin-specific protease 7 (USP7) is a deubiquitinating enzyme found in all eukaryotes that catalyzes the removal of ubiquitin from specific target proteins. Here, we report that UbE2E1, an E2 ubiquitin conjugation enzyme with a unique N-terminal extension, is a novel USP7-interacting protein. USP7 forms a complex with UbE2E1 N-terminal extension, is a novel USP7-interacting protein. UbE2E1, an E2 ubiquitin conjugation enzyme with a unique ubiquitin from specific target proteins. Here, we report that dynamic balance of the ubiquitin-proteasome system. A deubiquitinating enzyme to maintain and modulate the protein turnover of target proteins in the cell. This study reveals a new cellular mechanism that couples the opposing activities of the ubiquitination machinery and a deubiquitinating enzyme to maintain and modulate the dynamic balance of the ubiquitin-proteasome system.

Ubiquitination is a post-translational modification that regulates protein turnover, function, and localization. The attachment of ubiquitin takes place in several steps and involves at least three distinct types of enzymes. First, ubiquitin is activated through adenylation at its C terminus by the E1 activating enzyme. The activated ubiquitin is then transferred to an E2 conjugating enzyme. Finally, an E3 ligase transfers the ubiquitin from the E2 to the e-amino group of a lysine residue on a target protein in a substrate-specific manner (1, 2). There are two ubiquitin E1 activating enzymes, approximately 40 E2 conjugating enzymes, and over 600 E3 ligases in the human genome, allowing precise spatial and temporal regulation of ubiquitination (3–5).

All E2s have a conserved ubiquitin-conjugating (UBC)5 domain, consisting of about 150 residues with a central cysteine essential in formation of the ubiquitin thioester bond. E2s can be further divided into four classes: Class I E2s consist of only the UBC domain; Class II E2s have C-terminal extensions to the UBC core; Class III E2s have N-terminal extensions in addition to the UBC core; and Class IV contains both N-terminal and C-terminal extensions to the core domain. Emerging evidence suggests that the additional N- or C-terminal extensions are essential for the functions of the E2s, contributing to substrate specificity and/or regulation (6, 7). UbE2E (UbE2E1, UbE2E2, and UbE2E3) is a subfamily of class III E2 enzymes, homologs of yeast UBC4 and -5, that mediate degradation of misfolded and damaged proteins (8). They interact with a broad range of ubiquitin E3 ligases and are involved in ubiquitination of a large number of substrates (9). UbE2E1 was shown to play a role in histone ubiquitination, and UbE2E3 was implicated in the redox homeostasis following oxidative stress, required for retinal pigment epithelial cell proliferation (10, 11). All three UbE2E proteins contain the core UBC domain with ~94% sequence similarity and a less conserved N-terminal extension of about 50 amino acids. The function of the N-terminal extensions of the UbE2E proteins is not clear. However, these extensions are naturally disordered and rich in serine and lysine residues, suggesting that they are subject to protein-protein interaction and post-translational modifications, which may regulate UbE2E family members.

Ubiquitination, like other post-translational modifications, is reversible. Reversal of ubiquitination, or deubiquitination, is carried out by deubiquitinating enzymes (DUBs), which belong...
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to the metallo and cysteine families of proteases (12). By reversing the actions of ubiquitin ligases, DUBs offer a way to fine-tune the effects of ubiquitination as a post-translational modification. This is perhaps best illustrated by the regulation of the p53 tumor suppressor by the DUB, USP7. USP7 not only deubiquinates and stabilizes p53, but also Hdm2, the primary E3 ubiquitin ligase of p53 (13, 14). The effect of USP7 on Hdm2 is through direct interaction and independently of p53. Coexistence of both the DUB and the E3 ligase activity in the same protein complex offers an elegant way to tightly regulate p53 levels, which are key in determining the fate of the cell by governing cell growth arrest and apoptosis. Interestingly, the coupling of opposing activities of ubiquitination and deubiquitination is not unique to the USP7-Hdm2 complex. Indeed, USP7 was first discovered as a protein in complex with the herpes simplex virus protein ICP0, a viral E3 ligase (15), and was termed HAUSP for herpes-associated ubiquitin-specific protease. Several other E3 ligases have been identified that are also coupled to USP7 including CHFR, HLTF, ARF-BP1, and MARCH7 (16–19) USP7 has emerged as a key regulator of a range of cellular processes as exemplified by the large number of USP7 substrate proteins, which include p53, Hdm2, HdmX, clasin, histone H2B, PTEN, and FOXO4 (13, 16, 20–22).

Although USP7 generally regulates the levels, function, and localization of its interacting proteins through its deubiquitinase activity, it has also been shown to negatively regulate the PML domain of USP7 (USP7-NTD) recognizes a sequence motif ((P/A)XX) found in its interaction partners such as p53, Hdm2, and HdmX (24, 25). Interestingly, the class III E2 conjugating enzymes, UbE2Es, contain a conserved (P/A)XX motif within their N-terminal extensions. In this study, UbE2E1 was identified as novel USP7 interaction partner. Further structural and functional analysis revealed that USP7 deubiquinates UbE2E1 and is important for UbE2E1 stability in vivo. This study provides a new facet of USP7 in modulating and sustaining the basic ubiquitination machinery through the regulation of yet another component of the ubiquitination machinery: the E2 conjugation enzymes.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies—HCT116 wild type and USP7−/− cells were kindly provided by Bert Vogelstein (Johns Hopkins Medical Institutions). Human U2OS cells were grown in McCoy’s medium. HCT116, 293T, and HeLa cells were grown in DMEM supplemented with 10% FBS. The antibodies used for immunoblotting and immunostaining experiments include the mouse antibody to ubiquitin (Covance MMS-258R), actin (Calbiochem, CP01), the Myc epitope tag (Millipore 05-724) and the FLAG epitope tag (Sigma F3165), the mouse, rabbit, and goat antibodies to UbE2E1 (BD Biosciences, 611218; Boston Biochem, A-630; and Santa Cruz Biotechnology, sc-47547), and the rabbit antibody to USP7 (Bethyl Laboratories A300-033A).

Expression and Purification of USP7-NTD and UbE2E1 Proteins—USP7-NTD, UbE2E1, and ΔN-UbE2E1 were expressed from pET15b plasmids in Escherichia coli BL21(DE3) cells and purified using affinity chromatography. Briefly, the cells were harvested and lysed using sonication in 50 mM Tris, pH 7.5, 500 mM NaCl, 10 mM imidazole, 1 mM benzamidine, and 0.5 mM PMSF. The lysates were cleared and poured onto nickel-nitrilotriacetic acid beads (Qiagen). After extensive washing with 50 mM Tris, pH 7.5, 500 mM NaCl, and 30 mM imidazole, the proteins were eluted with the addition of 50 mM Tris, pH 7.5, 500 mM NaCl, and 250 mM imidazole. USP7-NTD was incubated with thrombin to remove the His tag and further purified using size-exclusion chromatography (Sephacryl S200 16/60) on an ÄKTA purifier 10 UPC (GE Healthcare) prior to crystallization trials.

Peptide Synthesis—The UbE2E1 peptides were synthesized by CanPeptide Inc. (Montreal, Canada) with both N-terminal acetylation and C-terminal amidation to mimic the native peptides. All of the peptides were greater than 95% pure and were dissolved in either the crystallization or the fluorescence binding assay buffers prior to use.

Crystallography, Data Collection, and Structure Determination of the USP7-UbE2E1 Complex—USP7-NTD (100 mg/ml) was co-crystallized with UbE2E1 (6DSRASTSSSS14) peptide at ~5-fold molar excess of peptide. Several rounds of micro-seeding using USP7-NTD-HdmXASTS crystals as seeds were performed. The seeded crystals grew after 1 week at 4 °C in the dark in conditions containing 30% PEG 4000, 0.1 M Tris, pH 8.5, and 0.2 M lithium. X-ray data from a frozen crystal of the USP7-NTD-UbE2E1ASTS complex was tested at 100 K on a Rigaku MicroMax007 rotating anode diffractometer with Saturn 944+ CCD detector and collected at 100 K at the 19ID Structural Biology Center beamline (Advanced Photon Source, Argonne National Laboratory, Argonne, IL). The crystal belongs to space group P4₁ with approximate unit cell dimensions a = b = 70.0 Å and c = 45.8 Å³. Data were integrated and scaled using HKL2000. A summary of data collection statistics is presented in Table 1. The

### TABLE 1

| X-ray data                          | USP7-UbE2E1ASTS |
|------------------------------------|-----------------|
| Space group                        | P4              |
| Resolution (Å)                     | 50.0–1.95       |
| Unit cell axes (Å³)                 | 69.9 × 69.9 × 45.7 |
| Molecules/asymmetric unit          | 1               |
| Total observations (No.)           | 189292          |
| Unique reflections (No.)           | 16086           |
| Intensity (I/σ(I))                 | 29.0 (5.2)      |
| Completeness (%)                   | 98.9 (95.9)     |
| Rmerge (%)                         | 0.133 (0.789)   |

* Rmerge = Σ[I−⟨I⟩]/ΣI where I is the observed intensity and ⟨I⟩ is the average intensity of multiple observations of symmetry-related reflections.
structure was determined by using the molecular replacement component of CNS (version 1.2) with USP7-NTD (Protein Data Bank (PDB) ID 1YY6) as the search model without any peptide (26). Electron density visualization and model building were done with O (27). Rigid body and simulated annealing torsion angle refinement were normally followed by individual B-factor refinement and performed using CNS 1.2. Several rounds of refinement were combined with model rebuilding in O after inspection of both $2F_{o} - F_{c}$ and $F_{o} - F_{c}$ maps. A summary of refinement statistics is presented in Table 1. PyMOL was used for the preparation of the figure.

**GST Pulldown Assays**—The GST-USP7-NTD and GST-USP7-NTD<sup>DW</sup> fusion proteins were generated by PCR amplification and insertion between the BamHI and XhoI sites of pGEX-4T-2 plasmid (GE Healthcare) as described previously (24). Proteins were expressed in *E. coli* BL21 pLYsS cells with 3 h of 1 mM isopropyl-1-thio-β-D-galactopyranoside induction at 37 °C, purified on glutathione-Sepharose resin (GE Healthcare) using standard methods, and dialyzed against assay buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, 5% glycerol, 1 mM benzamidine, and 0.5 mM PMSF). Purified UbE2E1 and ΔN-Ube2E1 were incubated with glutathione S-transferase (GST) alone and GST-USP7-NTD and GST-USP7-NTD<sup>DW</sup> fusion proteins in a 1:1 molar ratio at 4 °C for 1 h in the presence of 50 μl of glutathione-Sepharose beads (GE Healthcare). The mixture was then transferred to a micro column, and after extensive washing with assay buffer, bound proteins were eluted with 20 mM reduced glutathione and detected by Coomassie Blue staining following SDS-PAGE.

**Intrinsic Tryptophan Fluorescence Assays**—UbE2E1 peptides were titrated (0–150 μM) with wild type USP7-NTD (1 μM) in 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT. The change in tryptophan fluorescence was monitored using a Cary Eclipse fluorescence spectrophotometer (Varian Inc.), and $K_{d}$ values for USP7-NTD binding were calculated using GraphPad Prism as described previously (25). The $K_{d}$ values were calculated based on three individual experiments.

**In Vitro Ubiquitination and Deubiquitination Assays**—The *in vitro* ubiquitination and deubiquitination assays were performed in a volume of 20 μl in 50 mM Tris, pH 7.6, 5 mM MgCl<sub>2</sub>, 2 mM ATP, and 2 mM DTT. The reaction mixture typically contained E1 (100 ng), E2 (200 ng), ubiquitin (5 μg), and E3 (the catalytic domains of Nedd-4L or Itch), 0.5 μM photometer (Varian Inc.), and the change in tryptophan fluorescence was monitored using standard methods, and dialyzed against assay buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl, 5% glycerol, 1 mM benzamidine, and 0.5 mM PMSF). Purified UbE2E1 and ΔN-Ube2E1 were incubated with glutathione S-transferase (GST) alone and GST-USP7-NTD and GST-USP7-NTD<sup>DW</sup> fusion proteins in a 1:1 molar ratio at 4 °C for 1 h in the presence of 50 μl of glutathione-Sepharose beads (GE Healthcare). The mixture was then transferred to a micro column, and after extensive washing with assay buffer, bound proteins were eluted with 20 mM reduced glutathione and detected by Coomassie Blue staining following SDS-PAGE.

**In Vitro Ubiquitin Loading Assays**—The *in vitro* ubiquitin loading assays were performed as described previously (28). The reactions were carried out in a volume of 10 μl in 10 mM HEPES, pH 7.5, 100 mM NaCl, 40 μM ATP, and 2 mM MgCl<sub>2</sub> containing E1 (1 μg), E2 (1 μg), ubiquitin (5 μg), and increasing amounts of USP7 (0–2 μg). After incubation at 30 °C for 10 min, the reactions were stopped by the addition of SDS-PAGE sample buffer without DTT and resolved on 15% SDS-polyacrylamide gels. Loaded E2 protein were visualized and evaluated by Western blotting using antibodies against ubiquitin (Covance) and UbE2E1 (Boston Biochem).

**Immunoprecipitations**—HeLa cells transfected with FLAG-tagged UbE2E1 and Myc-tagged USP7 were used for immunoprecipitations. Cells were harvested 48 h after transfection and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate and protease inhibitor mixture (Roche Applied Science)). Protein A-Sepharose bead precleared lysates were incubated with antibodies against the FLAG tag for FLAG-tagged UbE2E1 (Sigma) or against the Myc tag for Myc-tagged USP7 overnight at 4 °C followed by the addition of protein A beads for another 60 min. Immunoprecipitates were washed three times using radioimmunoprecipitation assay buffer. The immunoprecipitated complexes were released by boiling the beads for 5 min in SDS sample buffer and resolved on 12% SDS-polyacrylamide gels followed by immunoblotting with antibodies against UbE2E1 (Boston Biochem) or USP7 (Bethyl Laboratories). The immunoprecipitation experiments for endogenous UbE2E1 and USP7 were performed in a similar way. Lysates were immunoprecipitated with an antibody against UbE2E1 (BD Biosciences) and immunoblotted with an antibody against USP7 (Bethyl Laboratories).

**Immunofluorescence**—Immunostaining of endogenous UbE2E1 and USP7 was done using U2OS and HeLa cells. Cells were grown on coverslips and fixed in 2% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS. Cells were washed once with PBST (PBS with 0.1% Tween 20) and blocked with 1% BSA in PBST for 1 h at 37 °C. Endogenous USP7 was stained with rabbit anti-USP7 (Bethyl Laboratories) and CY3-labeled anti-rabbit IgG. Endogenous UbE2E1 was stained with UbcH6 goat polyclonal IgG (Santa Cruz Biotechnology) and FITC-labeled anti-goat IgG (Sigma). Staining of the nucleus of U2OS cells was done with DRAQ5 (Enzo Life Sciences International, Inc.). To test antibody specificity for USP7 and UbE2E1, 10 μg of recombinant GST-USP7 and His<sub>6</sub>-tagged UbE2E1 were incubated with each appropriate antibody respectively for 60 min at 37 °C before the staining procedures. Negative staining controls with 1 μl of normal rabbit IgG (Santa Cruz Biotechnology; control IgG) and CY3-labeled anti-rabbit IgG and 1 μl of normal mouse IgG (Santa Cruz Biotechnology; control IgG) and FITC-labeled anti-goat IgG were prepared to validate the specific USP7 and UbE2E1 staining, respectively. Images were obtained with Zeiss LSM 700 and Olympus FluoView 300 confocal laser-scanning microscopes. Images were analyzed and superimposed using ImageJ software.

**In Vivo UbE2E1 Ubiquitination and Deubiquitination**—293T cells were transfected with FLAG-tagged UbE2E1 and treated with 10 μM MG-132 for 6 h. Immunoprecipitation was
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FIGURE 1. Analysis of the in vitro interaction between USP7-NTD and UbE2E1. A, schematics of UbE2E1 and USP7 indicating domain organization of the two proteins and the putative USP7 binding motif within the N terminus of UbE2E1. CAT, catalytic domain. B, comparison of the substrate peptide sequences recognized by USP7-NTD, featuring the (P/A)XXS motif, which is also found in the N terminus of UbE2E1. C, GST pulldowns were performed using GST-USP7-NTD and UbE2E1. GST-USP7-NTD fusion protein was incubated with UbE2E1, loaded onto glutathione resin (load (L)), washed with wash buffer (wash (W)) and eluted with Laemmli SDS-PAGE loading dye (eluete (E)). A GST pulldown of UbE2E1 protein with GST alone served as a negative control. GST pulldown experiments also tested the interaction of GST-USP7-NTD and the N-terminal UbE2E1 deletion mutant (ΔN-UbE2E1) and between the GST-USP7-NTD double mutant USP7-NTDDW and UbE2E1.

performed on both MG-132-treated and untreated cells using anti-FLAG. Ubiquitinated UbE2E1 was visualized by immunoblotting using both antibodies against ubiquitin and UbE2E1. Myc-tagged wild type USP7 and the catalytic inactive mutant USP7-CS were immunoprecipitated from 293T cells and incubated with ubiquitinated UbE2E1 isolated from the MG-132-treated cell lysate for 30 min at 30 °C. The reactions were stopped by the addition of SDS-PAGE sample buffer and immunoblotted with a ubiquitin antibody.

**UbE2E1 Turnover Assays—**U2OS, HCT116 WT, or HCT116 USP7−/− cells were seeded in 6-cm dishes at 80% confluence. U2OS cells were transfected three times with siRNA for USP7 (5′-CCCAAAUUAAUCCCGGCAA as described in Tang et al. (29)) or a negative control siRNA (provided by GenePharma) as outlined previously (30). siRNA-transfected U2OS cells and untransfected HCT116 cells (WT and USP7−/−) were then treated with 10 μg/ml cycloheximide (Sigma) and harvested at various times after treatment. HCT116 USP7−/− cells were transfected with empty vector, Myc-USP7, and Myc-USP7-C233S and harvested after 48 h. Cells were then lysed in radioimmunoprecipitation assay buffer. 30 μg of total protein was subjected to SDS-PAGE and Western blotting using antibodies against USP7 (Bethyl Laboratories), actin and UbE2E1 (Boston Biochem). These experiments were repeated independently, and similar results were obtained. The levels of UbE2E1 were determined by normalizing the intensity of the UbE2E1 bands to those of the actin bands. Student’s t test was used to analyze the differences of the levels of UbE2E1 at each time point.

**RESULTS**

**USP7 Interacts with UbE2E1 in Vitro—**Previous studies have shown that the N-terminal domain of USP7 (USP7-NTD) interacts with substrates p53, Hdm2, and HdmX through their (P/A)XXS motifs (Fig. 1A) (24, 25). Alignment of the (P/A)XXS sequence motif among USP7 substrate proteins along with the UbE2E1 ASTS sequence suggests that UbE2E1 will interact with USP7 through this conserved motif (Fig. 1B). We therefore hypothesized that USP7 interacts with UbE2E1 and that this interaction is occurring through its N-terminal extension. Recombinant full-length UbE2E1 and a mutant lacking the N-terminal extension, ΔN-UbE2E1, were used to assess interaction with GST-USP7-NTD using GST pulldown assays (Fig. 1C). UbE2E1 was shown to form a complex with GST-USP7-NTD, but not GST alone (negative control), indicating that USP7-NTD interacts with UbE2E1. The interaction between GST-USP7-NTD and ΔN-UbE2E1 was disrupted, suggesting that the USP7 binding sequence is localized within the N-terminal extension of UbE2E1. A 164DWGF167 motif in USP7-NTD was previously shown to be essential for its interaction with substrate proteins containing the (P/A)XXS motif, and a double point mutant of USP7-NTD (USP7-NTDDW), in which Asp164 and Trp165 were changed to alanine residues, eliminated binding to previously reported (P/A)XXS-containing substrates (31). As shown in Fig. 1C, USP7-NTDDW failed to retain UbE2E1, suggesting that UbE2E1 shares the same USP7 binding surface as the other (P/A)XXS-containing substrates and that the Asp164 and Trp165 residues in USP7-NTD are critical in mediating interactions with UbE2E1. Collectively, USP7 and UbE2E1 proteins are able to directly interact in vitro through the (P/A)XXS motif in the UbE2E1 N-terminal extension and the 164DWGF167 motif of USP7-NTD.

**Molecular Analysis of the USP7-UbE2E1 Interaction—**To determine the binding affinity as well as to reveal the molecular basis of the interaction between USP7-NTD and UbE2E1, a peptide corresponding to the interaction motif (5DSRASTS15) in the UbE2E1 N-terminal extension and peptides with mutations in the binding motif were synthesized. The change in the intrinsic tryptophan fluorescence of USP7-NTD was monitored with increasing amounts of the UbE2E1 peptide to derive the dissociation constant between USP7-NTD and the peptide. The dissociation constant was calculated to be 9.4 ± 2.1 μM for 5DSRASTS15, whereas no USP7 binding was observed with

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the two mutant peptides (\textsuperscript{7}RAATASS\textsuperscript{13}, \textsuperscript{7}RAATAAS\textsuperscript{13}) at concentrations up to 150 \( \mu \text{M} \). The dissociation constant between USP7 and UbE2E1 is comparable with those previously reported for Hdm2 and p53 peptides (Fig. 2A) (24).

To gain further insight into the mode of interaction between USP7 and UbE2E1, USP7-NTD was co-crystallized with the UbE2E1\textsuperscript{5DSRASTSSSS14} peptide. The crystal structure of this USP7-NTD-UbE2E1\textsuperscript{ASTS} complex was determined using molecular replacement. The overall structure of USP7-NTD has previously been described (31). Briefly, USP7-NTD forms an eight-stranded antiparallel \( \beta \)-sandwich identical to the TRAF domain found in receptor-associated factors of tumor necrosis factor (32). The UbE2E1 peptide binds within a groove on the USP7-NTD surface adjacent to the \( \beta \)-sheet formed by strands \( \beta_2, \beta_3, \beta_4, \) and \( \beta_7 \) as previously seen with other USP7 binding peptides (Fig. 2B). The final model of USP7-NTD-UbE2E1\textsuperscript{ASTS} was refined to an \( R \) work of 0.197 and an \( R \) free of 0.217 at 1.75 \( \AA \) resolution with 75 water molecules. Residues 54 – 62 and 106 – 111 are disordered and were not built into the final model of the protein-peptide complex. The UbE2E1\textsuperscript{ASTS} peptide forms several interactions with USP7-NTD \( \beta \)-strand 7 (Fig. 2C). The side chain hydroxyl of UbE2E1 Ser\textsuperscript{11} makes the most contacts with USP7-NTD by forming H-bonds with the side and main chain of Asp\textsuperscript{164}. Ala\textsuperscript{8} of UbE2E1 participates in van der Waals interactions with the side chains of USP7-NTD Trp\textsuperscript{165} and Phe\textsuperscript{167}. The side chain hydroxyl of UbE2E1 Ser\textsuperscript{8} makes a water-mediated hydrogen bond to the main chain amide of USP7-NTD Ser\textsuperscript{168}. The main chain amide and carbonyl groups of UbE2E1 Ser\textsuperscript{9} interact with main chain amide and carbonyl groups of USP7-NTD Gly\textsuperscript{166}. UbE2E1 Thr\textsuperscript{10} does not make any interactions with USP7-NTD. The electron density for the remainder of the peptide is disordered, suggesting that it is not making any contacts with USP7-NTD. This is the second instance of a peptide containing an ASTS motif interacting with USP7-NTD. Viral interferon regulatory factor protein 4 (vIRF4) also interacts with USP7-NTD through an ASTS motif; however, the nature of the interaction between the vIRF4 peptide and USP7-NTD involved more residues than that seen between USP7-NTD and UbE2E1 (33). Comparison of the UbE2E1 and vIRF4 peptides indicated that the ASTS motif was completely superimposable and that the contacts made by ASTS from each peptide to USP7 were identical (Fig. 2D).

**Interaction of USP7 and UbE2E1 in Vivo**—We examined the subcellular localization of USP7 and UbE2E1 in U2OS cells. Endogenous USP7 and UbE2E1 were visualized by immunofluorescence using antibodies specific for USP7 and UbE2E1. Both USP7 and UbE2E1 exhibited predominantly nuclear staining (Fig. 3A). Similar results were obtained when studying USP7 and UbE2E1 subcellular localization in HeLa cells. The immunostaining was specific for both USP7 and UbE2E1; staining for both proteins was negligible in IgG control experiments and in experiments in which USP7 and UbE2E1 antibodies were incubated with purified USP7 or UbE2E1, respectively (data not shown). Immunoprecipitation experiments were conducted in HeLa cells to study the in vivo interaction between USP7 and UbE2E1. HeLa lysate transfected with FLAG-tagged UbE2E1 was immunoprecipitated with an anti-FLAG antibody and blotted for endogenous USP7. USP7 was readily detected in the complex of FLAG-tagged UbE2E1 (Fig. 3B). A reciprocal experiment was conducted using lysate of HeLa cells ectopically expressing FLAG-tagged USP7 and finding that USP7 is able to interact with UbE2E1.
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**FIGURE 3. USP7 and UbE2E1 localization and interaction in vivo.** A, immunofluorescence images of U2OS cells are shown after staining for endogenous UbE2E1 (Cy-3) and USP7 (Alexa Fluor 488) proteins. The merged image indicates a partially overlapping localization of USP7 and UbE2E1 in the nucleus. The nuclei were counterstained with DAPI. B, 293T cells transfected with FLAG-UbE2E1 were subject to immunoprecipitation (IP) using mouse IgG (negative control) and anti-FLAG followed by immunoblotting (IB) using antibodies against USP7 and UbE2E1. C, 293T cells transfected with Myc-USP7 were subject to immunoprecipitation using rabbit IgG and anti-Myc followed by immunoblotting against USP7 and UbE2E1. D, endogenous UbE2E1 from 293T cells was immunoprecipitated with a monoclonal antibody against UbE2E1 or mouse IgG followed by immunoblotting with polyclonal rabbit antibodies against USP7 or UbE2E1.

USP7 Attenuates UbE2E1-mediated Ubiquitination—The effect of USP7 on UbE2E1-mediated ubiquitination was tested using *in vitro* ubiquitination assays. NEDD-4L is a UbE2E1-interacting E3 ligase and known to catalyze ubiquitination in its presence (34–36). The level of total ubiquitination is greatly reduced in the presence of increasing amounts of full-length USP7 protein (Fig. 4A, lanes 1–4). However, when these assays were repeated using UbE2D2/UbcH5b, an E2 enzyme lacking the N-terminal extension seen in UbE2E1, no difference in the total level of ubiquitination in the presence of increasing amounts of USP7 was observed (Fig. 4A, lanes 5–8). To confirm that the decreased levels of total ubiquitination were due to the interaction between USP7 and UbE2E1, the *in vitro* ubiquitination assays were repeated using ΔN-UbE2E1, which lacks the N-terminal extension sequence and is unable to interact with USP7. Although ΔN-UbE2E1 was active in mediating ubiquitination, USP7 did not show any effect on the total level of ubiquitination as was seen with full-length UbE2E1 (Fig. 4A, lanes 9–12), suggesting that USP7 modulates UbE2E1-mediated ubiquitination through the interaction between the N-terminal domain of USP7 and the UbE2E1 N-terminal extension. In contrast to the effect of full-length USP7, increasing amounts of USP7-NTD did not have any effects on the level of ubiquitination in the presence of full-length UbE2E1 or ΔN-UbE2E1 (data not shown). The effect of USP7 on UbE2E1-mediated ubiquitination was further investigated using ITCH as an E3 ligase. Similar results were obtained, suggesting that the effect of USP7 on UbE2E1 is E3-independent (data not shown). Collectively, these results indicate that the interaction between USP7 and UbE2E1 attenuates the total levels of UbE2E1-mediated ubiquitination and that this effect requires an intact USP7 protein rather than USP7-NTD alone.

To examine whether the decreased levels of total ubiquitination were due to compromised ubiquitin loading of UbE2E1 by USP7, the ability of full-length UbE2E1 and ΔN-UbE2E1 to load ubiquitin was tested in the presence of E1 and ATP with increasing amounts of USP7. As shown in Fig. 4B, both full-length UbE2E1 and ΔN-UbE2E1 were able to load ubiquitin and the amount of loaded ubiquitin was not affected by the addition of USP7. Therefore, USP7 does not affect UbE2E1 ubiquitin loading.

To test whether the deubiquitination activity of USP7 was required for the observed reduction of UbE2E1-mediated ubiquitination, ubiquitin aldehyde was used to inhibit USP7 catalytic activity in an *in vitro* ubiquitination assay. As shown in Fig. 4C, untreated USP7 attenuated UbE2E1-mediated ubiquitination in a dose-dependent manner, whereas ubiquitin aldehyde-treated USP7 had no effect on ubiquitination. Similar results were obtained when USP7 was inactivated by preincubation with NEM, an alkylating reagent that irreversibly blocks cysteine thiol groups. NEM effectively abolishes the effect of USP7 on UbE2E1-mediated ubiquitination (data not shown). Collectively, these results indicate that the catalytic activity of USP7 is essential for the attenuation of UbE2E1 ubiquitination by USP7.

**USP7 Deubiquitinates UbE2E1**—Because UbE2E1 has been shown to undergo polyubiquitination in *in vitro* ubiquitination assays (37), we tested whether USP7 affects UbE2E1-specific
Consistent with the previous study, polyubiquitinated UbE2E1 was detected in the in vitro ubiquitination assays using a UbE2E1-specific antibody. The addition of USP7 reduced the levels of polyubiquitinated UbE2E1, even at the lowest tested concentration of USP7 (Fig. 5A). This suggests that UbE2E1 is a substrate of USP7 and that USP7 can deubiquitinate UbE2E1 in vitro. It has previously been shown that E2s are subject to regulation by ubiquitination, which leads to their degradation by the 26 S proteasome (38–40). Therefore, we wanted to establish whether UbE2E1 was polyubiquitinated and degraded in vivo. High molecular weight species indicative of UbE2E1 polyubiquitination were observed in the presence of the proteasomal inhibitor, MG-132, but not in its absence (Fig. 5B). The ability of USP7 to deubiquitinate UbE2E1 was further confirmed using Myc-tagged USP7 and a catalytically inactive mutant, Myc-tagged USP7-C233S (USP7-CS (13)), both expressed and purified from 293T cells. Polyubiquitinated UbE2E1 was enriched in the cells using the proteasome inhibitor MG-132, purified using a UbE2E1-specific antibody, and incubated with USP7 or USP7-CS. USP7 but not USP7-CS catalyzed the deubiquitination of UbE2E1 (Fig. 5C). These data strongly support that UbE2E1 is a bona fide deubiquitination substrate of USP7.

**USP7 is important for UbE2E1 stabilization in cells**—USP7 regulates the stability of many of its substrates by removing polyubiquitin chains and rescuing these substrates from proteasomal degradation. The fact that UbE2E1 is a substrate of USP7 prompted us to investigate whether USP7 regulates UbE2E1 stability in cells. We monitored UbE2E1 protein turnover in U2OS cells transfected with either control siRNA (siControl) or siRNA for USP7 (siUSP7). The cells were treated with either control siRNA or siRNA for USP7. The cells were treated
with cycloheximide to block de novo protein synthesis or left untreated (Fig. 6A). When compared with control siRNA transfection, transfection with USP7 siRNA noticeably decreased the level of endogenous USP7 (Fig. 6A, top panel). USP7 knockdown resulted in much faster UbE2E1 turnover when compared with control cells (Fig. 6A, middle panel), suggesting that USP7 is important for UbE2E1 stability. We also examined the turnover of UbE2E1 in the wild type colorectal carcinoma cell line HCT116 and a derivative cell line HCT116 USP7−/−, in which the USP7 gene is deleted (Fig. 6B, top panel). Consistent with our observations in U2OS cells, we found UbE2E1 to be stable for over 24 h in the WT HCT116 cells. However, in the HCT116 USP7−/− cells, UbE2E1 turned over much faster and was undetectable within 24 h (Fig. 6B, middle panel). It was also noted that the starting level of UbE2E1 was much lower in the absence of USP7. These observations show that USP7 is critical for the stability of UbE2E1 protein in vivo.

To further confirm that the observed decrease in UbE2E1 stability in the HCT116 USP7−/− cells was due to the lack of USP7 expression, we introduced USP7, USP7-CS, and USP7-DW into HCT116 USP7−/− cells. Expression of USP7, USP7-CS, and USP7-DW was readily detectable by Western blotting using an antibody against USP7, 48 h after transfection (Fig. 6C, top panel). When compared with untransfected cells, cells with ectopic USP7 expression showed a recovery in UbE2E1 levels (Fig. 6C, middle panel, compare lanes 1 and 2). This suggested that the lack of USP7 expression is in large part responsible for the lower stability of UbE2E1 in the HCT116 USP7−/− cells. Interestingly, expression of USP7-CS led to a further decrease in detectable UbE2E1 protein below the background levels observed in untransfected HCT116 USP7−/− cells (Fig. 6C, middle panel, compare lanes 1 and 3). Expression of USP7-DW showed similar results to USP7-CS, whereas levels of UbE2E1 were below background levels (Fig. 6C, middle panel, compare lanes 1 and 4). It should be noted that transfection efficiency was observed to be about 50%; thus the effect of USP7 on UbE2E1 levels would likely be even greater if the sample did not include untransfected cells.

FIGURE 6. USP7 stabilizes UbE2E1 in vivo. A, U2OS cells were transfected with nonspecific control siRNA (siControl) or siRNA targeting USP7 (siUSP7) and treated with 10 μg/ml cycloheximide (CHX) for the indicated number of hours or left untreated (lane 0). Cell lysates were subjected to SDS-PAGE and Western blot analysis using antibodies indicated. B, WT HCT116 cells or HCT116 USP7−/− were treated with cycloheximide and harvested for Western blotting as in A. The levels of UbE2E1 were normalized using actin as a loading control and presented as line graphs in the cycloheximide chase experiments. The asterisk represents the statistical analysis comparing levels of UbE2E1 after siRNA treatment with p < 0.01. Error bars indicate S.D. C, WT USP7 and the catalytically inactive mutant C223S, USP7-CS, were transfected into HCT116 USP7−/− cells. Cells were lysed and blotted for UbE2E1 48 h after transfection. The levels of UbE2E1 were normalized using actin as a loading control and presented in a bar graph (bottom). The asterisk represents the statistical analysis comparing levels of UbE2E1 after USP7 rescue with p < 0.01. Error bars indicate S.D.
DISCUSSION

The N-terminal domain of USP7 binds p53, Hdm2, and HdmX through distinct regions bearing the sequence motif (P/A)XXS, which make contact with a shallow groove lined by the $\beta$7 strand residues $^{164}$DWG$^{167}$ on the surface of USP7-NTD. Through biochemical, structural, and mutational analysis, USP7 was found to interact with the $^{8}$ASTS$^{11}$ motif harbored in the N-terminal extension of UbE2E1. GST pulldown assays indicated that USP7-NTD but not USP7-NTD$^{DW}$ was able to interact with UbE2E1. Conversely, UbE2E1 lacking its N-terminal extension was unable to interact with GST-USP7-NTD. The interactions formed between USP7 and UbE2E1 are similar to the previously described USP7-p53, USP7-Hdm2, and USP7-HdmX complex structures (24, 25, 31). Specifically, residue $^{8}$Ala$^{8}$ of the (P/A)XXS motif within UbE2E1 makes contact with Trp$^{165}$ of USP7, and Ser$^{11}$ forms two H-bonds with Asp$^{164}$ of USP7. Indeed, mutations at the Ser$^{11}$ residue of $^{8}$ASTS$^{11}$ in UbE2E1 or at the Asp and Trp residues of $^{164}$DWG$^{167}$ in USP7 abolish the interaction, suggesting that USP7-NTD $^{164}$DWG$^{167}$ is a general substrate binding site, responsible for recognizing a broad range of substrates including UbE2E1. This binding site is also targeted by the viral proteins, EBNA1 (31) and vIRF4 (33), to suppress USP7 function.

The effect of the USP7 UbE2E1 interaction on UbE2E1 was investigated by in vitro ubiquitination assays. UbE2E1-mediated polyubiquitination was found to be susceptible to the deubiquitination activity of USP7. This effect of USP7 was contingent upon the ability of USP7 to physically interact with UbE2E1 as USP7 was unable to affect the activity of ΔN-UbE2E1 and UbE2D2, both shown to be lacking USP7 binding. This observation suggests that USP7 has to be in complex with UbE2E1 to confer its regulatory effect rather than nonspecifically breaking down polyubiquitin chains formed in the ubiquitination reaction. USP7 did not negatively affect the ubiquitin loading activity of UbE2E1. Thus USP7 was conjectured to be attenuating UbE2E1 activity through noncanonical steric hindrance or through its deubiquitination activity. To explore these possibilities, we conducted ubiquitination experiments in the presence or absence of USP7 incubated with two different inhibitors, NEM and ubiquitin aldehyde, to render USP7 inactive. These experiments showed that USP7 catalytic activity is critical and required to negatively regulate UbE2E1 activity. The outcome of the USP7 UbE2E1 interaction offers an interesting contrast to the interaction between OTUB-1 and UbE2N, another DUB and E2, respectively. OTUB-1 binds to UbE2N and inhibits UBE2N-mediated ubiquitination (41). However, this inhibition does not require OTUB-1 catalytic activity because OTUB-1 prevents polyubiquitination through physical interaction and sequestering of Ub-charged UbE2N.

Because USP7 cleaves polyubiquitin from UbE2E1 and polyubiquitination of proteins in cells is generally associated with proteasomal degradation, this observation implies that USP7 plays a role in regulating UbE2E1 stability in cells. This role of USP7 was validated using both siRNA and gene knock-out approaches. Partial silencing of USP7 expression in U2OS cells led to a measurable decrease in the protein levels of UbE2E1. Lack of USP7 expression in HCT116 USP7$^{-/-}$ cells was accompanied by a substantial reduction in both levels and stability of the UbE2E1 protein. When USP7 was reintroduced using ectopic expression into HCT116 USP7$^{-/-}$ cells, UbE2E1 levels increased. Taken together, these observations are consistent with the idea that USP7 stabilizes UbE2E1. Note that neither partial silencing nor complete ablation of USP7 expression led to a complete absence of UbE2E1 from cells, suggesting that factors other than USP7 may also contribute to the stability of UbE2E1. This possibility is supported by our data that the expression of USP7-CS or USP7-DW in HCT116 USP7$^{-/-}$ cells led to a further decrease in UbE2E1 levels when compared with mock-transfected control cells. This suggests that other factors may contribute to the stability of UbE2E1; however, our observations clearly show USP7 to be a key regulator of UbE2E1 stability.

In this study, we describe a novel interaction between the class III ubiquitin conjugating enzyme UbE2E1 and the deubiquitinase USP7. Our study revealed that USP7-NTD interacts with the N-terminal extension of UbE2E1 and that the co-crystal structure of USP7-NTD with a UbE2E1 peptide resembles the complex structures of USP7 with its other three substrates, p53, Hdm2, and HdmX. The biological significance of the interaction between USP7 and UbE2E1 was also investigated. We showed that USP7 specifically attenuates UbE2E1-mediated ubiquitination and plays an important role in regulating the stability of UbE2E1 in cells (Fig. 7). To the best of our knowledge, this represents the first example of a DUB regulating the stability of an E2 enzyme and provides new insights into the complex network of the ubiquitin-proteasome pathway.

Although our studies were limited to the interaction between USP7 and UbE2E1, it is noteworthy that UbE2E2 and UbE2E3, members of the UbE2E subfamily, also contain a (P/A)XXS motif in their N-terminal extensions. We predict that USP7 will also interact with and regulate the levels of UbE2E2 and UbE2E3 as it does UbE2E1. Our studies with UbE2E1 as a representative of this subfamily clearly establish USP7 as an important regulator of the UbE2Es. E2 ubiquitination is often observed in vitro, but not in vivo. For an efficient ubiquitination machinery to target substrates, instead of targeting itself for degradation, cells need to develop a self-protecting and self-rescuing mechanism built into the ubiquitination machinery. In this regard, USP7 serves as a guardian of UbE2E1, ensuring its stability and availability for its biological function(s).

![USP7 is a Regulator of UbE2E1](image-url)
USP7 Is a Regulator of UbE2E1

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