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# Catalytically Important Residues of E6AP Ubiquitin Ligase Identified Using Acid-Cleavable Photo-Cross-Linkers

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Supporting Information

**ABSTRACT:** Inactivation of the E6AP E3 ubiquitin ligase (UBE3A gene) causes Angelman syndrome, while aberrant degradation of p53 by E6AP is implicated in cervical cancers. Herein, we describe the development of photo-cross-linkers to discover catalytic residues of E6AP. Using these cross-linkers, we identified covalent modifications of the E6AP catalytic cysteine and two lysines: $Lys^{847}$ and $Lys^{799}$. $Lys^{847}$ is required for the formation of $Lys^{48}$-linked polyubiquitin chains, while the K799A E6AP mutant was more active at producing $Lys^{48}$-linked polyubiquitin chains. Thus, opposing roles of $Lys^{799}$ and $Lys^{847}$ pave the path forward to pharmacological inhibitors or activators of E6AP for therapeutic purposes.

Protein ubiquitination is an important posttranslational modification that regulates many aspects of human biology.[1] E6AP (*UBE3A*) is the founding member of HECT E3 ubiquitin ligases, which form an obligatory thioester intermediate with ubiquitin prior to substrate ubiquitination.[2] In Angelman syndrome patients, the maternal allele of E6AP harbors inactivating mutations or a gene deletion,[3] while human papillomavirus hijacks E6AP to degrade the p53 tumor suppressor in cervical cancers.[4] Understanding the mechanisms that regulate the activity of E6AP is therefore fundamentally important. UbcH7, the E2 enzyme upstream of E6AP, transfers ubiquitin (Ub) from a UbcH7~Ub thioester onto the catalytic cysteine of E6AP.[5] The E6AP~Ub thioester can then catalyze the formation of an isopeptide bond between Ub and the substrate.[2] Importantly, E6AP forms efficient binding interactions with both UbcH7 and the UbcH7~Ub thioester.[6] However, it was recently suggested that E6AP harbors two distinct E2 enzyme binding sites[7] and may function as an oligomer.[8]

To gain more insights into E6AP catalysis, we became interested in developing robust and sterically small photo-cross-linking reagents to identify proximal and catalytically relevant residues at transient E2/E3 protein−protein interfaces (Figure 1A−C).[2] We envisioned that such photo-cross-linkers will act as useful and general tools for mapping protein−protein interactions *in vitro*.

To equip the E2 enzyme with cross-linkers that are sterically small and also suitable for MS analysis of cross-linked peptides, we envisioned a set of monocysteine mutants to be alkylated with iodoacetamide-diazirine photo-cross-linkers **1**−**3** (Figure 1 and Tables S1 and S2 of the Supporting Information).[9] The alkylated E2 enzyme can subsequently be purified and photo-cross-linked

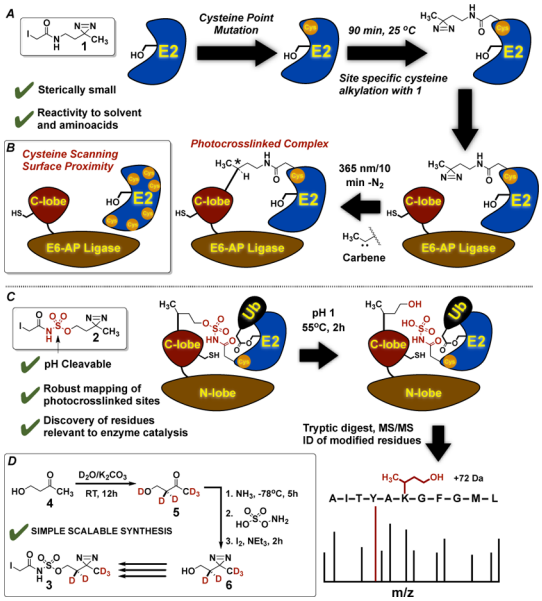


**Figure 1.** Identification of amino acid residues at a protein−protein interface. (A) Diazirine photo-cross-linker **1** can be site-specifically incorporated onto a protein surface to detect protein−protein interactions. (B) Multiple UbcH7 cysteine mutants can be equipped with **1** and then photo-cross-linked to detect which UbcH7 surface(s) interacts with E6AP. (C) Cross-linker **2** cleaves at low pH to allow detection of covalently modified residues by mass spectrometry (MS). (D) Synthesis of deuterated cross-linker **3** to allow further MS validation of modified residues.

to its downstream E3. The short linker lengths in **1**−**3** (~8 Å for **1** and ~11 Å for **2** and **3**) minimize disruption to the protein−protein interface, and the cysteine-reactive iodoacetamide allows site-specific installation on a protein surface using cysteine chemistry. After photo-cross-linking, we sought to facilitate MS/MS identification of modified residues by cleaving the cross-linker at acidic pH values to render unbound proteins with unique covalent modifications at cross-linked sites (Figure 1C). This step is desirable because noncleavable photo-cross-linkers produce reaction mixtures that demand complex analysis.[10] Although site-specific installation of photo-cross-linkers can be achieved using unnatural amino acid incorporation,[11] cysteine chemistry provides a much simpler and flexible approach *in vitro*.

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Importantly, commercially available heterobifunctional diazirine photo-cross-linkers predominantly contain lysine-reactive succinimide moieties. However, because of the abundance of lysine residues, it is difficult to achieve site-specific installation of these cross-linkers on protein surfaces.

To identify which UbcH7 surfaces interact with E6AP, photo-cross-linker **1** was site-specifically monoalkylated to a set of UbcH7 mutants that each had cysteine introduced at a distinct surface (Figure 2A). After desalting had been conducted, each

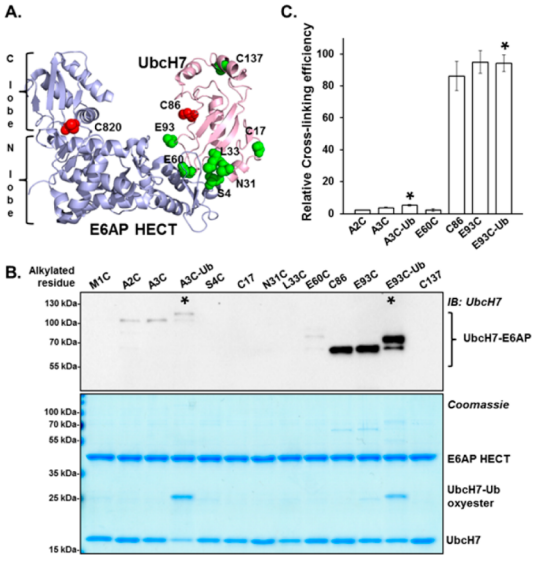


**Figure 2.** Cysteine scan identifies UbcH7 residues proximal to E6AP. (A) Eleven UbcH7 C17S C86S C137S (CΔS) mutants were expressed and equipped with **1** at the indicated residues (green). Protein Data Bank entry 1C4Z. (B) Different UbcH7 CΔS-**1** mutants (10 $\mu$M) and E6AP HECT (10 $\mu$M) were irradiated at 365 nm for 10 min and resolved via reducing SDS–PAGE, followed by Western blotting using UbcH7 antibody. (C) Triplicate reactions as in panel B were analyzed by Western blotting and quantitated with ImageJ to estimate relative cross-linking efficiency when **1** was placed at the indicated UbcH7 CΔS residue. An asterisk indicates that the cross-linker is on an UbcH7 CΔS-Ub oxyester.

photoreactive UbcH7 enzyme was investigated for its ability to undergo photo-cross-linking with the E6AP HECT domain (Figure 2B,C). Thus, the entire surface of the UbcH7 enzyme was scanned for proximity to E6AP in solution. To identify modified E6AP residues, the UbcH7 mutant that most efficiently cross-linked when modified by **1** was equipped with cross-linker **2**, which harbors an acid-cleavable *N*-acylsulfamate moiety.[12] Cleavage of photo-cross-linked peptides facilitates their subsequent MS and MS/MS analysis (Figure 1C).[10] Thus, cleavage of the UbcH7-E6AP cross-link prior to digestion and mass analysis provided the robust signal of E6AP peptides with a unique butanol covalent modification (+72.06 Da, Spectral Appendix I of the Supporting Information). The identification of these residues was further supported by an analogous experiment with deuterated diazirine **3**, for which we developed a simple synthetic protocol (Figure 1D, +77.09 Da butanol-$d_5$ modification, Spectral Appendix I of the Supporting Information). The developed synthesis of **3** features a facile H/D exchange on the readily available hydroxyl-ketone starting material with $D_2O$/$K_2CO_3$, followed by its conversion to diazirine. Importantly, $\alpha$-deuterated ketone survived subsequent conversion to $\alpha$-deuterated diazirine in liquid ammonia without the reverse D/H exchange (Spectral Appendix III of the Supporting Information). Cross-linker **2** and deuterated cross-linker **3** showed equal photo-cross-linking efficiency (Figure S1 of the Supporting Information).

While cross-linker **1** quantitatively labeled every UbcH7 cysteine mutant, **2** and **3** were not as reactive with some of the mutants (Tables S1 and S2 and Spectral Appendix II of the Supporting Information). This may be due to deprotonation of the acylsulfamate, which reduces the reactivity of **2** and **3** toward thiol nucleophiles.[12] Therefore, **1** was first used to rapidly survey the proximity of UbcH7 surfaces to an interface with E6AP. To avoid nonspecific alkylation of native UbcH7 cysteine residues Cys[17], Cys[86] (catalytic cysteine), and Cys[137], they were mutated to serine (termed UbcH7 CΔS). While Cys[86] is the key catalytic residue for mediating Ub transthiolation, UbcH7 CΔS and its alkylated analogues are competent to form UbcH7 C86S-Ub oxyester conjugates (UbcH7 CΔS-Ub) in the presence of ATP and ubiquitin-activating E1 enzyme (Figure S2A,B of the Supporting Information). Furthermore, UbcH7 CΔS-Ub and its alkylated analogues can transfer ubiquitin to E6AP (Figure S2C of the Supporting Information). These data suggest that mutagenesis and alkylation with cross-linkers **1**–**3** did not compromise the E2 enzyme structure.

An UbcH7/E6AP cocrystal structure[13] and an alanine scan of the protein–protein interface[14] initially guided our selection of UbcH7 residues near the interface to equip with a cross-linker. Ideally, a residue chosen for alkylation should not significantly contribute to the E6AP–UbcH7 binding interaction. Thus, we selected residues that did not markedly affect binding affinity when mutated to alanine.[14]

On the basis of this criterion, we alkylated UbcH7 CΔS mutants N31C, L33C, E60C, or E93C with photo-cross-linker **1**. Furthermore, the UbcH7/E6AP cocrystal structure indicates that the first three N-terminal UbcH7 residues positioned near the UbcH7–E6AP interface are disordered. We hypothesized that these residues do not contribute significantly to the UbcH7–E6AP binding interaction and could therefore serve as suitable residues for installing a photo-cross-linker. Thus, we chose UbcH7 residues M1C, A2C, A3C, or S4C as additional sites for cross-linker attachment. To explore the UbcH7–E6AP interaction landscape and to validate the specificity of cross-linking, we also made monocysteine UbcH7 mutants to place a cross-linker at native cysteines Cys[17], Cys[86], or Cys[137] (Figure 2). Alkylation of UbcH7 cysteine mutants occurred readily by incubating 50–300 $\mu$M UbcH7 with 5 mM cross-linker **1** at room temperature for 90 min before removing the free cross-linker with a desalting column. Intact protein mass spectrometry indicated near quantitative monoalkylation of all monocysteine UbcH7 mutants (Tables S1 and S2 and Spectral Appendix II of the Supporting Information). The UbcH7 CΔS-**1** mutants (10 $\mu$M) were then mixed with the E6AP catalytic HECT domain (10 $\mu$M) and irradiated at 365 nm for 10 min in a 96-well plate. The reaction mixtures were then resolved via reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed with an anti-UbcH7 Western blot. As expected, photo-cross-linking of UbcH7 to E6AP depends on the presence of both UV irradiation and E6AP (Figure S3 of the Supporting Information). Western blotting showed that UbcH7 CΔS mutants cross-link E6AP with varying degrees of efficiency. The most efficient cross-linking resulted when cross-linker **1** was placed at either UbcH7 CΔS E93C or the UbcH7 catalytic cysteine Cys[86] (Figure 2B,C). Because the UbcH7~Ub thioester is the true substrate of E6AP, we prepared the stable E2-Ub


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oxyesters UbcH7 CΔS E93C Ub-**1** and UbcH7 CΔS A3C Ub-**1** and observed cross-linking efficiency comparable to that of their nonubiquitinated analogues (Figure 2B,C).

Different cross-linking efficiencies indicate the high sensitivity and specificity of this technique to the cross-linker location on the UbcH7 surface. Because UbcH7 Phe$^{63}$ is critical for binding the N-lobe of the E6AP HECT domain, UbcH7 CΔS F63A mutants or their Ub oxyesters bearing **1** or **2** did not cross-link E6AP (Figure S4 of the Supporting Information). Furthermore, cross-linking is dose-dependent, has specificity that persists in the presence of HeLa cell lysate or detergents, and can be inhibited by wild-type UbcH7 in a dose-dependent manner (Figures S5−S8 of the Supporting Information).

To identify the photo-cross-linked sites of E6AP, we developed a facile and general mass spectrometry protocol. First, we photo-cross-linked UbcH7 and E6AP with acid-cleavable cross-linker **2** or **3**, separated the cross-linked UbcH7-E6AP complex from free UbcH7 and E6AP using SDS−PAGE, and then excised the gel band corresponding to the UbcH7-E6AP complex after minimal Coomassie staining of the gel (Figures S9 and 10 of the Supporting Information). Because an in-gel acid cleavage step proved to be problematic, we employed a customized electroelution apparatus to first extract photo-cross-linked protein complexes for subsequent acid cleavage of the cross-linker and proteolytic digestion (Figure S11 and Supplementary Methods of the Supporting Information).[15] Following electroelution of the cross-linked complexes into Tris-glycine-SDS buffer, we cleaved them by acidifying the solution with aqueous HCl to pH 1 and incubating them at 55 °C for 120 min. We found that performing the acidification in Tris-glycine-SDS buffer as opposed to HEPES buffer prevents hydrolysis of the protein backbone (Figure S9B,C of the Supporting Information). After this step, the solution was neutralized with aqueous NaOH to pH 9. Finally, we precipitated protein with acetone, digested protein with trypsin, and then analyzed the peptides with an Orbitrap Velos instrument.

Mass analysis was performed via cross-linking experiments between E6AP HECT and E2 enzyme alone (UbcH7 CΔS E93C-**2** or UbcH7 CΔS E93C-**3**) or E2-Ub oxyester [UbcH7 CΔS E93C Ub-**2** or UbcH7 CΔS E93C Ub-**3** (Tables S1 and S2 and Spectral Appendix I of the Supporting Information)]. As expected, UbcH7 was modified at E93C with the sulfamic acid that remains following cleavage of the cross-linker [+136.98 Da for **2** or **3** (Spectral Appendix I of the Supporting Information)]. Covalent modifications (72.06 Da for **2** and 77.09 Da for **3**) on E6AP were localized to the HECT domain C-lobe at the catalytic cysteine Cys$^{820}$, Lys$^{847}$, and Lys$^{799}$ (Figure S9D,E of the Supporting Information). While no modifications were found on the E6AP N-lobe where the E2 binding site is located, this was not entirely unexpected because the cross-linker on UbcH7 E93C is 10−11 Å from the UbcH7 catalytic site, which encounters the flexible HECT C-lobe to undergo trans-thiolation.[16] Thus, placing the cross-linker on UbcH7 E93C allowed us to interrogate the catalytic environment near the E2 and HECT E3 catalytic cysteines (Figure 3A).

Cys$^{820}$ is the catalytic cysteine of E6AP located on the HECT C-lobe, and its modification by a cross-linker suggests that it approaches the UbcH7 catalytic Cys$^{86}$. Although the C-terminal Lys$^{847}$ of E6AP is disordered in X-ray crystal structures, our developed photo-cross-linkers were also able to locate the proximity of this residue to the active site of the E2 enzyme. Furthermore, covalent modification at Lys$^{799}$ is an interesting finding because inactivating mutations of Angelman syndrome

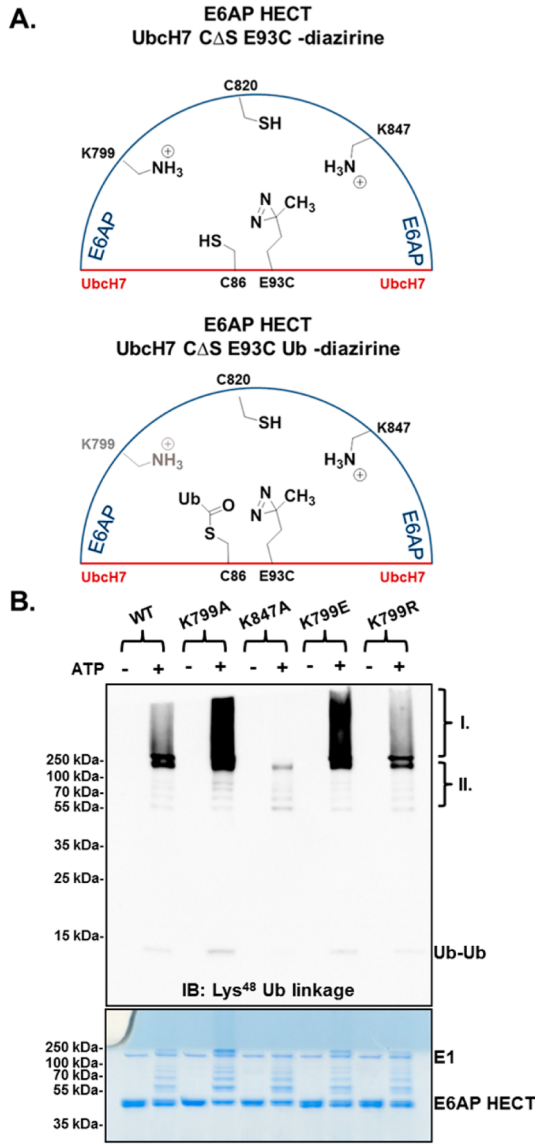


**Figure 3.** E6AP Lys$^{799}$ and Lys$^{847}$ affect the formation of Lys$^{48}$-linked polyubiquitin chains. (A) Schematic representations of residues spatially proximal to UbcH7 E93C. Cross-linking UbcH7 CΔS E93C-**2** or -**3** or UbcH7 CΔS E93C-Ub-**2**- or **3** interrogates the UbcH7/E6AP catalytic microenvironment. Black E6AP residues were labeled by cross-linkers **2** and **3**. E6AP K799 was only labeled by **3** in the experiments with UbcH7 CΔS E93C-Ub oxyester. (B) WT UbcH7 (2 $\mu$M), E6AP HECT (indicated mutant, 2 $\mu$M), Uba1 (0.2 $\mu$M), Ub (1500 $\mu$M), and ATP (2 mM) were incubated for 60 min in 25 mM HEPES (pH 7.6), 100 mM NaCl, and 4 mM $MgCl_2$ at 37 °C before being resolved via SDS−PAGE, and analyzing the reaction mixtures with Lys$^{48}$-linkage-specific antibody. Section I indicates free polyubiquitin chains, and section II indicates autoubiquitinated E6AP. This assignment is based on experiments with isopeptidase T, which preferentially disassembles free polyubiquitin chains (Figure S13A of the Supporting Information).

are located just C-terminal to this residue ($^{801}$KMII$^{804}$).[17] Overall, this suggested to us that Lys$^{799}$ and Lys$^{847}$ of E6AP are important for enzyme catalysis.

Because the catalytic roles of Lys$^{847}$ or Lys$^{799}$ have not been previously known, we investigated how alanine mutations affect the ability of E6AP to assemble Lys$^{48}$-linked polyubiquitin chains under standard conditions. (Figure 3B and Figure S12 of the Supporting Information). Interestingly, we found that the E6AP



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K847A mutant is deficient in synthesizing $Lys^{48}$-linked polyubiquitin chains, suggesting that $Lys^{847}$ participates in enzyme catalysis. Moreover, the catalytic activity of E6AP is restored (and is even more active) with the E6AP K847R mutant, but not to the same degree with the E6AP K847E mutant (Figure S12 of the Supporting Information). To verify that the K847A mutation does not affect the folding of E6AP HECT or its ability to bind UbcH7, we conducted a photo-cross-linking assay with UbcH7 CΔS E93C-**2**, which showed that UbcH7 can still efficiently photo-cross-link such an E6AP mutant (Figure S12 of the Supporting Information). Moreover, E6AP HECT K847A retains wild-type activity in forming the E6AP∼Ub thioester (Figure S14 of the Supporting Information).

Unexpectedly, however, E6AP K799A and K799E mutants were more active at producing $Lys^{48}$-linked polyubiquitin chains than wild-type E6AP or its K799R mutant (Figure 3B and Figures S15 and S16 of the Supporting Information). The mechanistic basis behind such activation requires further investigations. Importantly, K799A or K847A activates or deactivates, respectively, both free polyubiquitin chain formation and E6AP autoubiquitination in the presence or absence of the universal S5a substrate (Figure S13A of the Supporting Information).[18] Taken together, our data show that photo-cross-linker **2** and its deuterated analogue **3** can be used to scan an E2/E3 interface and identify catalytically relevant residues on E3 ubiquitin ligases.

In summary, we have identified previously unrecognized catalytic lysine residues ($Lys^{799}$ and $Lys^{847}$) of the E6AP ligase with opposing roles in catalysis. E6AP $Lys^{847}$ is required for the formation of $Lys^{48}$-linked polyubiquitin chains, while the K799A mutation increases the activity of the catalytic E6AP HECT domain, which was an unexpected finding. Discovery of these two residues is significant, because small molecules that mimic these biochemical point mutations can be used to inactivate or activate the catalytic E6AP HECT domain for therapeutic purposes. Further mechanistic investigations are needed to understand the precise catalytic roles of these residues and will be reported in the future. To discover these residues, we developed a novel class of sterically small noncleavable and acid-cleavable photo-cross-linkers that can be site-specifically installed on a protein surface using simple cysteine chemistry. Furthermore, we developed a simple and accessible synthesis of their isotopically labeled analogues and applied these cross-linkers to interrogate a protein−protein interface between the UbcH7 E2 enzyme and the E6AP E3 ligase.

We envision that the developed photo-cross-linkers coupled with the unique sample preparation protocol may find widespread use as general *in vitro* biochemical tools for studying protein−protein interactions such as the ∼24000 possible E2/E3 enzyme interactions, as well as histone/nucleosome-protein/enzyme complexes.[19] Such cross-linkers may also be advantageous in cases in which large protein size precludes analysis with nuclear magnetic resonance and X-ray crystallography methods.

## ■ ASSOCIATED CONTENT

### Supporting Information

Supporting data, biochemical procedures, chemical syntheses, spectral appendices, and protein sequences. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00625.

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### Notes

The authors declare no competing financial interest.

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