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Activation of E6AP/UBE3A-Mediated Protein Ubiquitination and Degradation Pathways by a Cyclic γ-AA Peptide

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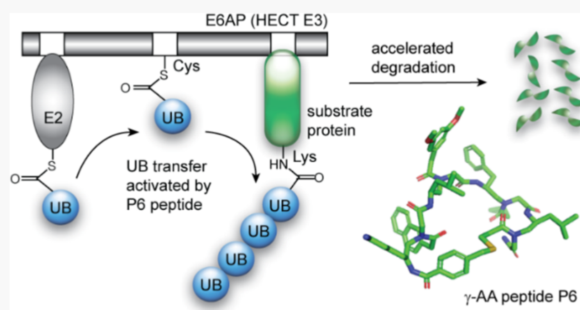
ABSTRACT: Manipulating the activities of E3 ubiquitin ligases with chemical ligands holds promise for correcting E3 malfunctions and repurposing the E3s for induced protein degradation in the cell. Herein, we report an alternative strategy to proteolysis-targeting chimeras (PROTACs) and molecular glues to induce protein degradation by constructing and screening a γ-AA peptide library for cyclic peptidomimetics binding to the HECT domain of E6AP, an E3 ubiquitinating p53 coerced by the human papillomavirus and regulating pathways implicated in neurodevelopmental disorders such as Angelman syndrome. We found that a γ-AA peptide **P6**, discovered from the affinity-based screening with the E6AP HECT domain, can significantly stimulate the ubiquitin ligase activity of E6AP to ubiquitinate its substrate proteins UbxD8, HHR23A, and β-catenin in reconstituted reactions and HEK293T cells. Furthermore, **P6** can accelerate the degradation of E6AP substrates in the cell by enhancing the catalytic activities of E6AP. Our work demonstrates the feasibility of using synthetic ligands to stimulate E3 activities in the cell. The E3 stimulators could be developed alongside E3 inhibitors and substrate recruiters such as PROTACs and molecular glues to leverage the full potential of protein ubiquitination pathways for drug development.



INTRODUCTION

E3 ubiquitin (UB) ligases are promising drug discovery targets due to their essential regulatory roles in diverse cellular processes such as protein degradation, gene activation, DNA repair, autophagy, and cell cycle and differentiation.[1−4] Recent work on repurposing E3s for induced protein degradation further fuels the effort for designing and screening E3 ligands for the assembly of bifunctional proteolysis-targeting chimeras (PROTACs) or monovalent molecular glues to control protein stability in the cell.[5,6] E6 associated protein (E6AP) has been a prototypical E3 for probing the catalytic mechanism of the UB transfer reaction and the roles of E3s in cell regulation[7−11] (Figure 1). E6AP was identified for its association with the E6 protein of the human papillomavirus (HPV) that would stir E6AP to ubiquitinate p53 for its degradation by the proteasome. This would allow the virus to subvert the antiviral response of the host cells and promote viral infection that eventually leads to tumorigenesis.[12−14] For counteracting E6AP that coalesces with HPV E6 to manifest its oncogenic activity, efforts have been devoted to the screening of cyclic peptides that would bind to E6AP and inhibit p53 ubiquitination[15] and small molecules and peptide ligands that would bind to E6 to prevent the formation of the E6-E6AP complex.[16−19] On the other hand, the deletion and mutation of *UBE3A*, the gene encoding E6AP, is implicated in neurodevelopmental disorders such as Angelman syndrome.[20,21] A variety of approaches have been developed to replenish E6AP activity in Angelman patients, including the use of a topoisomerase inhibitor, antisense oligonucleotides, and Cas9-mediated gene editing to activate the expression of

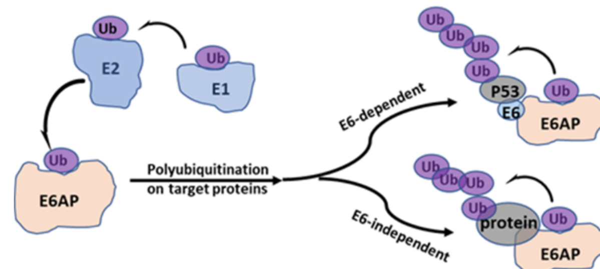


Figure 1. E6AP-catalyzing polyubiqutination of target proteins in E6-independent and E6-dependent manners.

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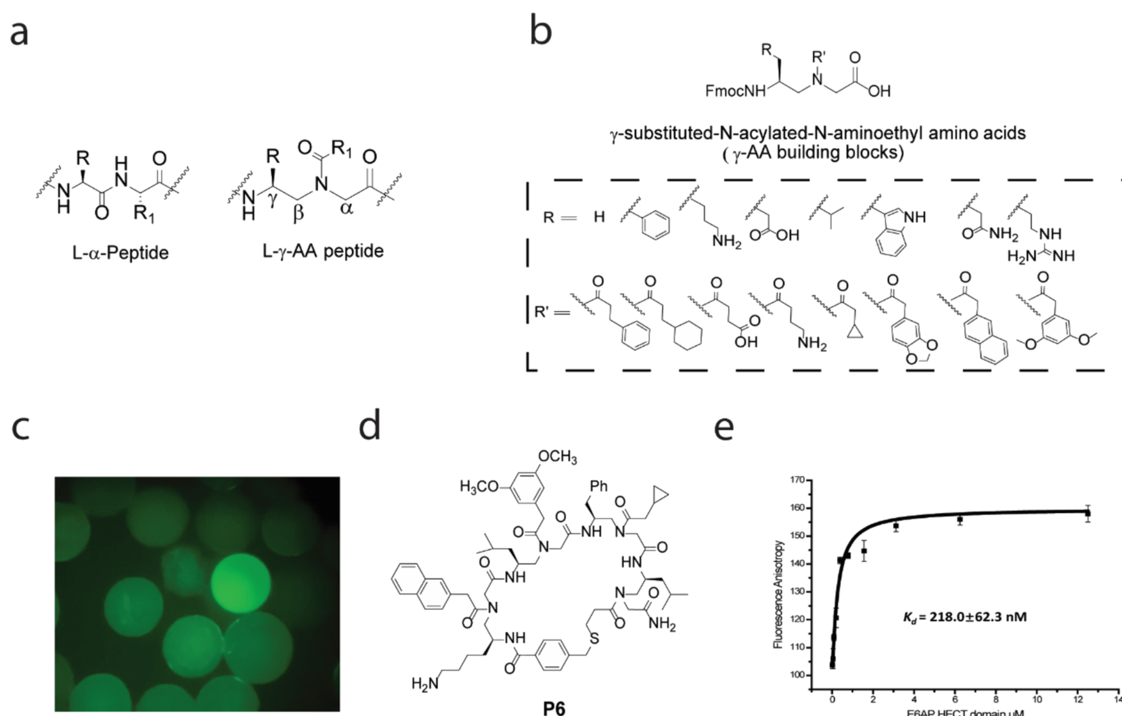


Figure 2. Screening of γ-AA peptide ligands with binding affinities with the E6AP HECT domain. (a) General structures of L-α-peptide and L-γ-AA peptide. (b) γ-AA building blocks used for library preparation. (c) Beads bound with AlexaFluor488-conjugated anti-Flag antibody emitting green fluorescence. (d) Structure of the **P6** ligand from the affinity screen. (e) Fluorescence polarization assay for measuring the binding affinity of **P6** with the HECT domain of E6AP. The **P6** peptide was labeled with the FITC fluorophore and the K_d of the **P6**-HECT complex was determined to be 218.0 ± 62.3 nM. Error bars = standard deviation from three independent experiments.

UBE3A gene.[22−24] Furthermore, a recent screening yielded small molecules that can stimulate the UB ligase activity of E6AP as demonstrated by *in vitro* ubiquitination assays.[25]

We believed that identification of activators of a specific E3 to stimulate the ubiquitination and degradation of the native E3 targets could be a viable alternative strategy to PROTACs and molecular glues. It is well recognized that targeted protein degradation is an emerging field in chemical biology and therapeutic development. So far, PROTACs and molecular glues have been the focus of study to induce the degradation of target proteins in the cell. Both strategies require the development of bi-functional ligands binding simultaneously with E3 ubiquitin ligases and the target proteins. To induce the degradation of new targets by PROTACs or molecular glues, one has to acquire affinity ligands with the target proteins through either rational design or library screening. In contrast, an E3 activator could be a mono-functional ligand but stimulate degradation of multiple protein substrates, which could achieve a synergistic effect in treating a variety of diseases in the future. Such an activator may also be used to restore the function of an E3 in the cell or tissues when the native activity of an E3 is suppressed by certain diseases. To this end, in this study, we screened a γ-AA peptide library for ligands binding to the HECT domain of E6AP that is the catalytic unit of the E3 responsible for transferring UB to the substrate proteins. γ-AA peptides (Figure 2a), named for the oligomers of γ-substituted-*N*-acylated-*N*-aminoethyl amino acids, are derived from the backbone of chiral peptide nucleic acids (PNA). This new class of unnatural peptidomimetics possesses enormous chemical diversity, remarkable resistance to proteolytic degradation, and excellent capacities for cell delivery.[26] We developed an affinity-based screening for peptides binding to the HECT domain of E6AP, and emerged from the screening are a series of γ-AA cyclic peptides with submicromolar affinity with the target HECT domain. We found one peptide ligand, known as **P6**, that can significantly stimulate the activity of E6AP in ubiquitinating substrate proteins UbxD8, HHR23A, and β-catenin in reconstituted reactions. Interestingly, **P6** could enhance the ubiquitination of E6AP substrates in the cell and accelerate their degradation by the proteasome. The discovery of the γ-AA peptide ligand for E6AP activation attests to the malleability of the non-conventional peptide scaffold for ligand discovery to target the protein ubiquitination cascade. Moreover, our results suggest a new therapeutic landscape based on the identification of E3-activating ligands and confirm the feasibility of using E3 activators alongside inhibitors and substrate recruiters for manipulating protein degradation pathways in the cell.

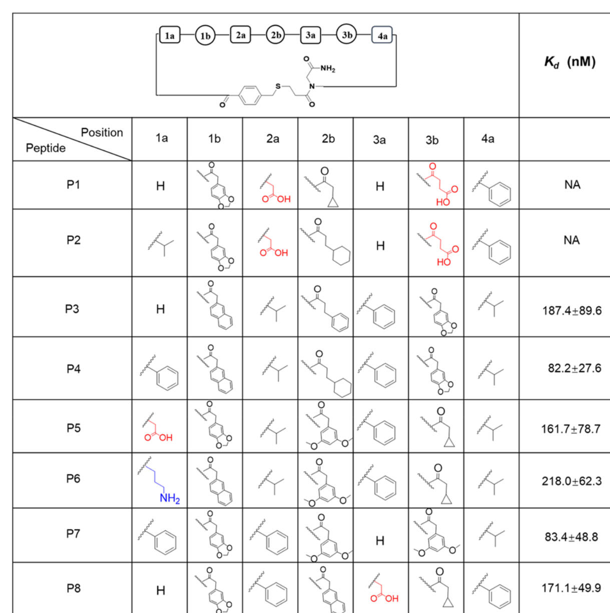
■ RESULTS AND DISCUSSION

Library Screening and Characterizing the Affinities of the γ-AA Peptide Ligands with the E6AP HECT Domain. E6AP runs the third relay of the E1-E2-E3 cascade that passes UB to the substrate proteins to form an isopeptide bond between the C-terminal carboxylate of UB and the Lys residues of the substrates. The HECT domain of E6AP engages the UB−E2 thioester conjugate to facilitate the delivery of UB from E2 to a catalytic Cys residue of the HECT domain before passing UB to the substrate proteins.[27,28] We recently engineered the HECT domain of E6AP for the assembly of an orthogonal UB transfer cascade (OUT) to profile the substrate specificity of the E3.[7] We posited that synthetic ligand binding to the HECT domain of E6AP might affect its catalytic activities, so they could be further developed as

inhibitors or activators of the E3. Toward such a goal, we designed a cyclic γ-AA peptide library composed of a variety of γ-AA building blocks (Figure 2b). Each cyclic γ-AA peptide contained four γ-AA building blocks, and it matched the size of an 8-residue cyclic peptide. The choice of the macrocyclic ring size was based on our previous work that demonstrated the high binding affinities of cyclic γ-AA peptides of four units with diverse biological targets.[29−31] In the current library design, both side chains of γ-AA building blocks (R and R′) were selected from a pool of hydrophobic and charged groups, and a random combination of the building blocks would constitute a library of greater than 2×10^6 in diversity (Figure 2b). A detailed protocol for the preparation of the one-bead-two-compound (OBTC) library of the γ-AA peptides is included in the Supporting Information (Figure S1).

The library was screened based on the binding between the bead-anchored peptides with the HECT domain of E6AP with an N-terminal Flag tag that was recognized by an anti-Flag antibody labeled with AlexaFluor 488 that would emit green fluorescence (Figure S2). Five beads with strong green fluorescence were picked for their positive response to the binding of the E6AP HECT domain (Figure 2c), and the corresponding structures of the γ-AA peptides anchored on the beads were elucidated by the tandem MS/MS of MALDI (Figure 2d and Figure S3). We found two beads, each yielding a single unambiguous structure, whereas for the remaining three beads, each was associated with two possible structures, leading to a total of eight γ-AA peptides as putative binders of the E6AP HECT domain. The peptide ligands from the screening were named **P1−P8**, and they were resynthesized to measure their individual binding affinities with the HECT domain by fluorescence polarization (FP). **P3−P8** were found to have submicromolar binding affinities with the E6AP HECT with K_d's ranging from 80 to 218 nM, while **P1** and **P2** did not show any measurable binding with the HECT domain (Table 1, Figure 2e, and Figure S4). Alignment of the selected peptide sequences revealed a preference for bulky hydrophobic residues such as naphthyl and 3,4-(methylenedioxy) phenyl at positions 1b, 2b, and 3b and small hydrophobic residues such as isopropyl and phenyl at positions 2a and 4a (Table 1). **P4** showed a two-fold higher affinity than **P3** with the HECT domain, while the main difference between the two peptides is that **P4** has a phenyl residue at position 1a while **P3** has a hydrogen atom at the corresponding position. Similarly, **P7** with a phenyl residue at position 1a has a two-fold higher affinity than **P8** of a similar structure but with a hydrogen atom at the same position. Insights from such a comparison may improve the design of the γ-AA peptide ligands of the E6AP HECT domain in the future.

Table 1. Alignment of the Peptide Sequences from the Library Screening and the Binding Affinities of the Identified γ-AA Peptide Ligands P1−P8 with the HECT Domain of E6AP as Measured by a Fluorescence Polarization Assay[a]



[a]Positively charged residues are shown in blue, and negatively charged residues are shown in red. a and b denote the chiral and achiral side chains in a γ-AA building block, respectively.

Effects of γ-AA Peptides on E6AP Activity Assayed by Self-Ubiquitination. We first assayed the activities of the γ-AA peptides based on their effects on the self-ubiquitination of the HECT domain and full-length E6AP. We incubated E6AP HECT with each peptide (10 and 100 μM) from the affinity screening and added Uba1 (E1), UbcH7 (E2), and HA-tagged UB (HA-UB) to initiate the ubiquitination reaction. We found that peptides **P1−P5**, **P7**, and **P8** had little effect on the formation of the UB-HECT conjugate while the **P6** peptide enhanced the formation of polyubiquitinated HECT species in the high molecular weight range (Figure 3a). This suggests the stimulatory effect of **P6** on the catalytic activity of the HECT domain. We then repeated the assay on the full-length E6AP with the chosen condition that mainly generated mono-ubiquitinated species of the E3 without the addition of the peptides. Similar to the assay with the HECT domain, there was not much effect of **P1−P5**, **P7**, and **P8** on the self-ubiquitination of full-length E6AP except that **P3** and **P4** showed a weak inhibitory effect at 100 μM concentration. **P6** again stimulated the E6AP self-ubiquitination, demonstrating its unique effect on E6AP activation (Figure 3b). We then assayed the self-ubiquitination of HECT and full-length E6AP in the presence of the **P6** peptide of varying concentrations and found that the peptide can stimulate E3 self-ubiquitination at a concentration of 5 μM (Figure 3c). Since the HECT domain and the full-length E6AP showed a similar response to **P6**, it is likely that **P6** activated E6AP by binding and stimulating the catalytic activity of the HECT domain. It is intriguing that **P6** exhibited the weakest binding affinity (218 nM) for the E6AP HECT domain among **P3−P8** (Figure 2e), but it has a unique stimulatory effect on the UB transfer reaction catalyzed by the HECT domain. Alignment of the sequences of **P6** and other peptides showed that **P6** has a distinctive positively charged Lys side chain at position 1a while there is a hydrogen (**P3** and **P8**), phenyl (**P4** and **P7**), or Asp side chain (**P5**) at the same position (Table 1). The Lys side chain at 1a may contribute to the different binding mode of **P6** with the HECT domain to activate the catalytic activity of E6AP.

Effect of γ-AA Peptides on Substrate Ubiquitination Catalyzed by E6AP. **P6** stimulation of E6AP self-ubiquitination prompted us to assay the effect of **P6** on the ubiquitination of E6AP substrates. We previously used an engineered-OUT cascade of E6AP to identify UbxD8, an adaptor protein regulating lipid droplet formation, and β-catenin, a transcription factor, as E6AP substrates.[7] Other

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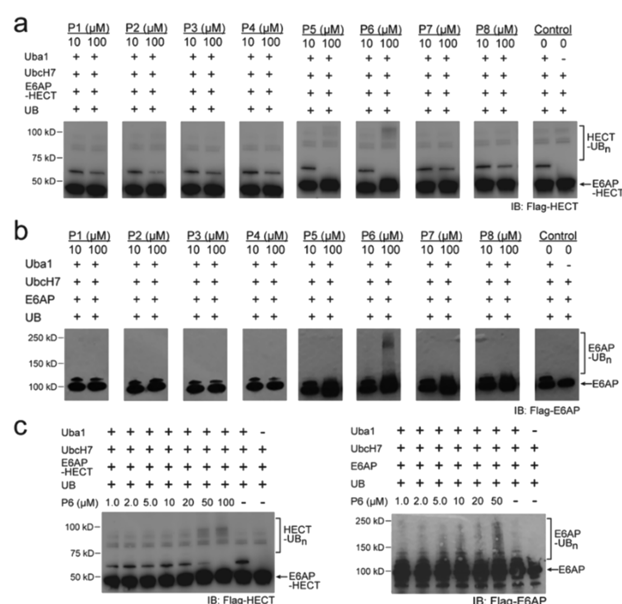

Figure 3. Effect of γ-AA peptide ligands on the UB ligase activity of E6AP assayed by self-ubiquitination. (a) Assaying the effects of ligands **P1−P8** on E6AP activity based on the self-ubiquitination of the HECT domain of E6AP. HECT domain self-ubiquitination was measured by Western blot probed with an anti-Flag antibody that binds to the N-terminal Flag tag fused to the HECT domain. The **P6** ligand showed a stimulatory effect on the HECT domain activity. (b) Assaying the effect of **P1−P8** on E6AP activity based on the self-ubiquitination of the full-length E3. Ubiquitination of E6AP was followed by probing the N-terminal Flag tag fused to the E3 on the Western blot. (c) Dose-dependent activation of the HECT domain (left panel) and full-length E6AP (right panel) by the **P6** ligand as measured by the self-ubiquitination reaction.

reports also verified β-catenin and HHR23A, a protein involved in DNA repair, as E6AP substrates.[32−34] We thus measured the effect of the peptide ligands on the E6AP-catalyzed ubiquitination of UbxD8, HHR23A, and β-catenin. We first assayed the E6AP ubiquitination of UbxD8 in the presence of 10 and 100 μM peptides. **P1−P4** did not show much effect on UbxD8 ubiquitination compared to the control reactions with no addition of the peptides. In contrast, **P6** showed a distinctive stimulatory effect on UbxD8 ubiquitination at both concentrations of the peptide, while **P5**, **P7**, and **P8** showed a weaker stimulatory effect (Figure 4a). When the ubiquitination assay was performed with varying concentrations of the **P6** peptide, all three substrates, namely, UbxD8, HHR23A, and β-catenin, showed enhanced ubiquitination in a dose-dependent manner in response to the amount of **P6** in the reconstituted reaction (Figure 4b). These results suggest that **P6** would enhance the UB-transfer activity of E6AP to a broad range of substrates. The E6 protein from HPV virus can both enhance the activity of E6AP in substrate ubiquitination and stir its substrate specificity to new targets such as p53.[11,35] We thus assayed if the γ-AA peptides from the affinity screen would affect p53 ubiquitination catalyzed by E6AP. We set up the ubiquitination reaction with the peptides (either 10 or 100 μM) and found that none of the peptides, including **P6**, affected p53 ubiquitination with HPV E6 in the reconstituted reaction (Figure 4c). Such a result suggests that HPV E6 may override the stimulatory effect of **P6** on E6AP, so no additional enhancement of p53 ubiquitination by **P6** was observed when both the **P6** peptide and HPV E6 protein were added to the reaction.

P6-Mediated Enhancement of Ubiquitination and Accelerated Degradation of E6AP Substrates in HEK293T Cells. We then assayed if **P6** would affect the ubiquitination of E6AP substrates and their stabilities in the cell. We incubated HEK293T cells with the 25 and 50 μM **P6** peptide for 12 h in the presence of MG132, a proteasome inhibitor to suppress the degradation of ubiquitinated proteins in the cell. We then lysed the cell, immunoprecipitated UbxD8 and HHR23A as E6AP substrates with specific antibodies, and analyzed the ubiquitination levels of substrate proteins by Western blotting probed with an anti-UB antibody (Figure 5a). We found that both UbxD8 and HHR23A showed an enhanced level of protein ubiquitination in cells with the addition of **P6** compared to the control cells with no **P6** added. This result suggests that the **P6** peptide can enhance the ubiquitination of E6AP substrates in the cell.

To measure if the enhanced E6AP activity due to **P6** stimulation would accelerate the degradation of the substrate proteins in the cell, we carried out a cycloheximide (CHX) chase assay to follow the stability of UbxD8 and HHR23A in the presence of **P6**. We pretreated HEK293T cells with various concentrations of **P6** to stimulate the E6AP activity and then added CHX, a ribosome inhibitor to block the synthesis of new proteins. At various time points of CHX chase, we lysed the cells and probed the levels of UbxD8 and HHR23A to follow their degradation (Figure 5b). We found that both substrates showed a faster degradation pattern in cells cultured with 50 μM **P6** than in cells with no **P6** added. HHR23A also showed accelerated degradation in cells treated with 1 or 5 μM **P6** (Figure 5c). These results confirm that **P6** can promote the degradation of E6AP substrates by stimulating the catalytic activity of E6AP in the cell.

Stability of P6. A distinctive characteristic of γ-AA peptides compared with canonical peptides is their remarkable resistance to protease. To assay the stability of **P6** in the presence of protease, we incubated it with 0.1 mg/mL pronase at 37 °C for 24 h. High-performance liquid chromatography and mass spectrometry of the **P6** peptide before and after the exposure to pronase showed that **P6** was resistant to protease cleavage (Figure S5a). We also incubated **P6** in the human serum for 24 h and confirmed its stability (Figure S5b). The superior stability of **P6** potentiates its use as a molecular probe for cell-based studies and therapeutic development.

■ CONCLUSIONS

γ-AA peptides have exhibited promising potential for biological application and drug discovery. Due to their propensity to form helical structures akin to α-helices of proteins,[38−41] γ-AA peptides could mimic host-defense peptides[36,37] and modulate disease-related protein−protein interactions such as p53/MDM2, β-catenin/BCL9, and GLP-1/GLP-1R *in vitro* and *in vivo*.[42−45] Additionally, owing to the convenience for incorporating unnatural functionalities to their scaffolds with the modular synthesis protocol, γ-AA peptides are ideal for generating diverse libraries to screen for ligands of biological targets.[29−31] In this study, we carried out an affinity-based screen to identify γ-AA peptides that can bind to the HECT domain of E6AP with submicromolar binding affinity. Among the ligands that we deduced from the library, one peptide, **P6**, stands out as a potent activator of E6AP—not only that **P6** can stimulate the self-ubiquitination of E6AP and E6AP-catalyzed

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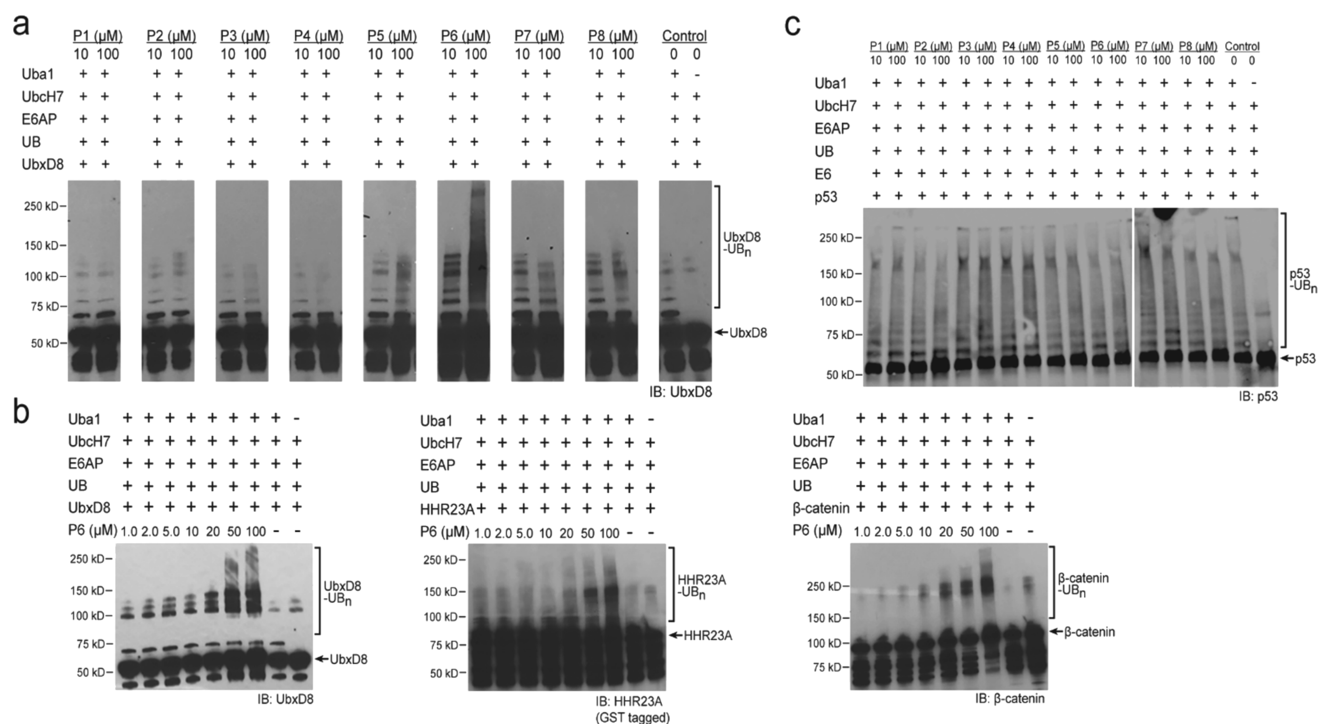

Figure 4. Effect of the γ-AA peptide ligands on the UB ligase activity of E6AP assayed by substrate ubiquitination. (a) Measuring the effects of ligands **P1−P8** on UbxD8 ubiquitination catalyzed by E6AP. The ubiquitination reaction contains ligands (10 or 100 μM) incubating with the Uba1-UbcH7-E6AP cascade and UbxD8 as the E6AP substrate. Ligand **P6** showed the most significant stimulatory effect on UbxD8 ubiquitination catalyzed by E6AP. (b) Dose-dependent activation of E6AP-catalyzed substrate ubiquitination by the **P6** ligand. Varying concentrations of **P6** were added to the ubiquitination reaction containing the Uba1-UbcH7-E6AP cascade and the E6AP substrates UbxD8 (left panel), HHR23A (middle panel), and β-catenin (right panel). Ubiquitination of the substrates was measured by Western blots of the reaction probed with antibodies specific for each substrate. (c) Ligands **P1−P8** have no stimulatory effect on p53 ubiquitination catalyzed by E6AP pairing with the E6 protein of HPV. The ligands (10 or 100 μM) were incubated with the E6AP enzymatic cascade, the viral E6 protein, and p53. The ubiquitination of p53 was followed by a Western blot of the reaction mixture probed with an anti-p53 antibody.

substrate ubiquitination in reconstituted reactions *in vitro*, but it can also enhance the ubiquitination of E6AP substrates in the cell and accelerate their degradation by the proteasome. Such an E3 ligand that can stimulate the UB ligase activity of an E3 is unique in that it can occupy a distinctive therapeutic landscape from the many E3 inhibitors that are being moved through the drug discovery pipeline.[4] Both an overactive E3 due to dysregulation or an underperforming E3 due to genetic mutations can be causative of diseases. The discovery of E3-stimulating ligands such as **P6** for E6AP may boost the activity of mutated E3s in the cell to restore their normal functions. It would be interesting to assay if **P6** and other peptide ligands from the screen may activate mutated E6AP that are causative for Angelman syndrome. Alternatively, the screen of the γ-AA peptide library could be repeated with the mutated E6AP to identify ligands that can restore the activity of the E3 implicated in Angelman syndrome. We also found that the **P6** peptide would not further activate E6AP when there was a viral E6 protein from HPV present. Whether **P6** and E6 share the same binding mode with E6AP warrants further study.

Previously, phage-displayed libraries of UB variants (UbVs) have been selected for binding to the HECT domains of various E3s, and some of the UbVs from the selection were found to activate HECT E3s Nedd4 and Nedd4L.[46] Peptide and small molecule ligands of E3 would be better leads for drug development, so *N*-methyl-cyclic peptides were identified for binding to the HECT domain of E6AP and inhibiting its UB ligase activity.[15] However, the activity of the *N*-methyl-peptide in the cell was not characterized. In another report, small molecule ligands of flavin derivatives were found to activate E6AP to enhance its ubiquitination of substrate proteins.[25] Interestingly, the flavin-like ligands would induce a conformational change of E6AP close to the effect of HPV E6 on E6AP.[25] The effects of the flavin-like ligands on E6AP activity in the cell were not characterized. Still, it would be interesting to test if the **P6** γ-AA peptide would target the same binding site of the flavin ligand in E6AP and if **P6** would induce a similar conformational change in E6AP to activate its ligase activity.

Our study also demonstrated the advantage of using the γ-AA peptide scaffold for developing ligands to affect E3 activities in the cell. The enzymatic cascades of UB transfer rely on sophisticated protein−protein interactions to deliver UB to the substrate proteins. Peptides and their structural mimics such as γ-AA peptides would have a better chance than small molecules to manipulate protein−protein interactions to inhibit or activate enzymes of the UB-transfer cascades. Indeed, recent success in designing stapled peptides to perturb E1-E2 interactions demonstrates the potential of peptide ligands as a privileged scaffold to target protein ubiquitinating enzymes.[47] The remarkable stability of the γ-AA peptides under physiological conditions would provide another advantage for developing them to activate or inhibit protein ubiquitination pathways.

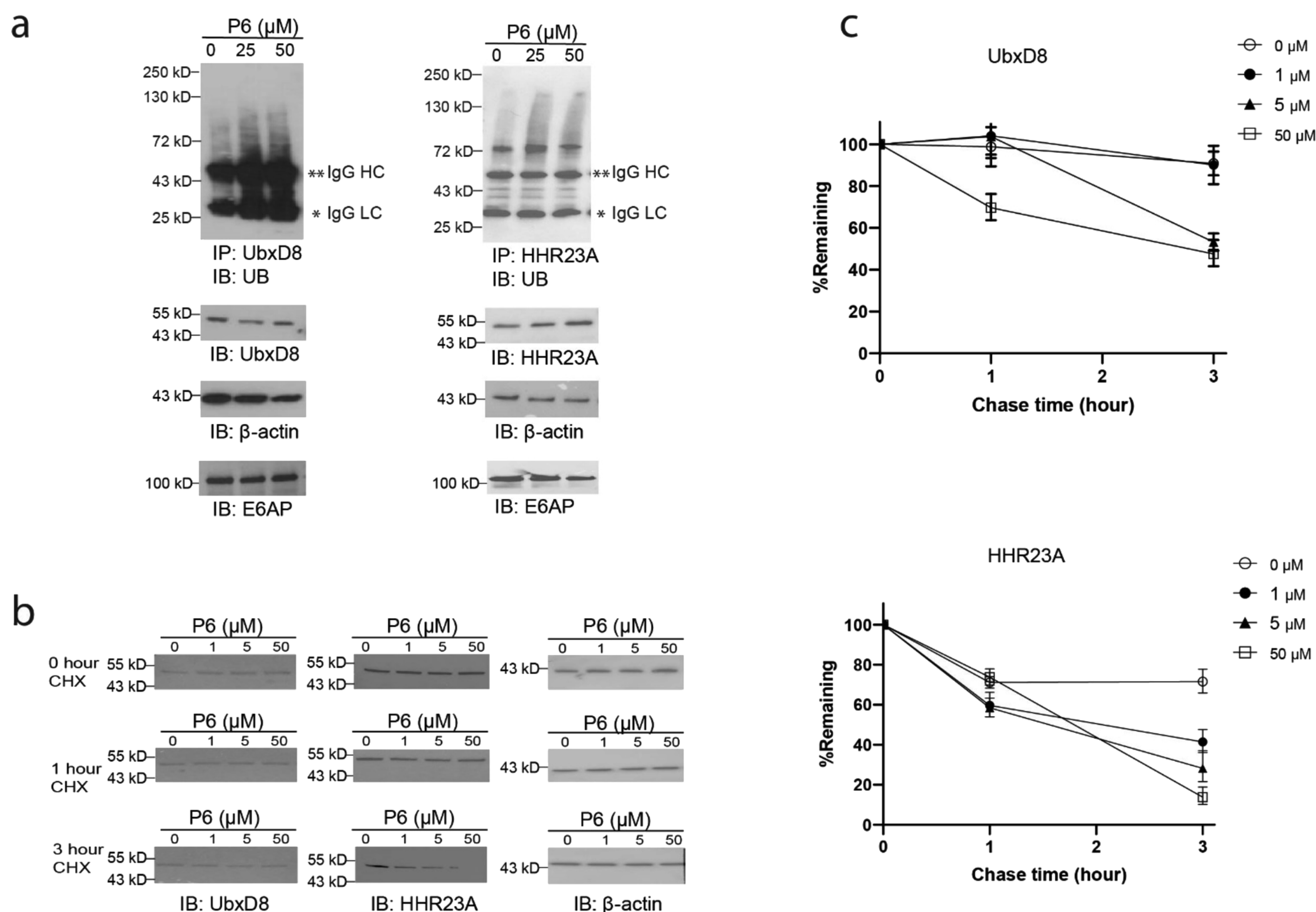

Figure 5. Stimulatory effect of the **P6** ligand on E6AP-catalyzed substrate ubiquitin in the cell and acceleration of the degradation of E6AP substrates. (a) **P6** enhanced the ubiquitination of E6AP substrates UbxD8 (left panel) and HHR23A (right panel) in HEK293T cells. The **P6** peptide of 0, 25, and 50 μM was incubated with HEK293T cells for 14 h, and the cells were treated with proteasome inhibitor MG132 for another 12 h. The cells were harvested for immunoprecipitating the substrate proteins with specific antibodies from the cell lysate, and the ubiquitination levels of the substrate proteins were measured by Western blots probed with an anti-UB antibody. *, IgG light chain (LC); **, IgG heavy chain (HC). (b) Cycloheximide (CHX) chase assay to measure the accelerated degradation of UbxD8 (left panels) and HHR23A (middle panels) in the presence of varying concentrations of **P6**. β-actin levels in the cell were measured in parallel as a control (right panels). Cells were incubated with varying concentrations of the **P6** peptide for 14 h and treated with ribosome inhibitor CHX for 0, 1, and 3 h before harvesting to collect cell lysates. Levels of UbxD8, HHR23A, and β-actin in the cell lysates were measured by Western blot probed with specific antibodies. (c) Levels of the E6AP substrates UbxD8 (top panel) and HHR23A (bottom panel) were plotted against the chase time after the cells were incubated with varying concentrations of the **P6** peptide. **P6** accelerated UbxD8 degradation at a concentration of 5 μM and accelerated HHR23A degradation at a concentration of 1 μM. Error bars = standard deviation from three independent experiments.

■ EXPERIMENTAL SECTION

Reagents. Rink amide resin (loading: 0.4 mmoL/g) was used for the solid-phase synthesis of γ-AA peptides and was purchased from GL Biochem. TentaGel resin (0.23 mmoL/g) was purchased from RAPP Polymere and used for preparation of the OBTC library. All solvents and other chemical reagents used for building block synthesis were obtained from commercial suppliers and used without purification unless otherwise indicated. All the building blocks used for the library preparation were synthesized according to our previous research.[48,49] The cyclic peptides were purified and analyzed on a Waters Breeze 2 HPLC system installed with both an analytic module (1 mL/min) and a preparative module (16 mL/min) by employing a method using a 5−100% linear gradient of solvent B (0.1% TFA in MeCN) in solvent A (0.1% TFA in H_2O) over 45 min followed by 100% solvent B over 5 min. All compounds are >95% pure by analytical HPLC. The molecular weight of each peptide was confirmed by high-resolution mass spectrometry obtained from an Agilent 6220 using electrospray ionization time-of-flight (ESI-TOF). MS/MS analysis was performed with an Applied Biosystems 4700 Proteomics Analyzer. HEK293T cells were from American Tissue Culture Collection (ATCC), and the AlexaFluor488-conjugated FLAG Epitope tag monoclonal antibody was purchased from Sigma-Aldrich.

XL1 Blue cells were from Agilent Technologies (Santa Clara, CA, USA). BL21 (DE3) pLysS chemical competent cells were from Invitrogen for protein expression. pET-15b and pET-28a plasmids for protein expression were from Novagen (Madison, WI, USA). Uba1, UbcH7, the HECT domain, and full-length proteins of E6AP, UB, and UbxD8 were expressed from pET28a-Uba1, pET15b-UbcH7, pET28a-E6AP HECT, full-length pET28a-E6AP, pET15b-UB, and pET28a-UbxD8 plasmids, respectively. β-Catenin and HHR23A were expressed from pGEX-β-catenin and pGEX-HHR23A plasmids, respectively. HEK293T cells were from American Tissue Culture Collection (ATCC) and cultured in high-glucose Dulbecco's modified Eagles medium (DMEM) (Life Technologies, Carlsbad, CA, USA, 11965092) with 10% (v/v) Fetal bovine serum (FBS) (Life Technologies, 11965092). The anti-HHR23A antibody (sc-365669), anti-UB antibody (sc-8017), anti-UbxD8 antibody (sc-374098), anti-E6AP antibody (sc-25509), anti-β-actin (sc-47778), anti-β-catenin antibody(sc-65480), and anti-p53 antibody (sc-126) were from Santa Cruz Biotechnology. These antibodies were diluted between 500- and 1000-fold to probe the Western blots. The anti-Flag M2 antibody

(F3165) was from Sigma-Aldrich and was diluted 2000-fold for Western blotting.

Library Preparation. A detailed protocol is described in the Supporting Information (Figure S1). Briefly, split and pool methods were used to prepare the γ-AA peptide-based OTBC library. Each bead was manipulated to have two layers: inner and outer layers. The γ-AA peptide was synthesized on the outer layer, in which the Fmoc protecting group of the γ-AA peptide building block was removed by 20% piperidine in DMF, and the exposed amino group reacted with the next γ-AA peptide building block using HOBt/DIC (6:6 equiv) as the activation agents in DMF for 6 h. The Alloc protecting group in the γ-AA peptide building block was removed by 1% $Pd(PPh_3)_4$ and 10% $Me_2NH \cdot BH_3$ in CH_2Cl_2, and the deprotected building block reacted with carboxylic acids in the presence of HOBt/DIC (6:6 equiv) to introduce side chains. The decoding peptide was synthesized on the inner layer, in which the Dde group was removed using deprotection solution according to the previous report.[50] The Dde protected amino acids were coupled onto the solid phase in DMF for 4 h in the presence of PyBop (6 equiv) and NEM (6 equiv).

Library Screening. The TentaGel beads (200−250 μm, 13.67 g) were swollen in DMF for 1 h. After being washed with Tris buffer three times, the beads were equilibrated in Tris buffer overnight at room temperature.

Prescreening. First, the TentaGel beads were incubated with blocking buffer (1% BSA in Tris buffer with a 1000× excess of *Escherichia coli* lysate) for 1 h, and then, they were washed thoroughly with Tris buffer followed by incubation with the AlexaFluor488-conjugated FLAG Epitope tag monoclonal antibody at a dilution of 1:500 for 2 h at room temperature. Then, the beads were washed with Tris buffer three times and transferred into a six-well plate to be observed under a fluorescence microscope. The beads emitting green fluorescence were picked up and excluded from formal screening. The rest of the beads were pooled into a peptide vessel. After being washed with Tris buffer, the beads were treated with 8 M guandine·HCl for 1 h to remove the bond protein at room temperature. Finally, the guandine·HCl was washed away with water and Tris buffer. The beads were then incubated in DMF for 1 h followed by washing and equilibration in Tris buffer overnight.

Screening. The beads were incubated with blocking buffer (1% BSA in Tris buffer with a 1000× excess of *E. coli* lysate) for 1 h at room temperature. After being washed with Tris buffer three times, the beads were incubated with the Flag-tagged E6AP domain at a concentration of 50 nM for 4 h with 1% BSA in Tris buffer and 1000× excess of *E. coli* lysate. After the thorough wash with Tris buffer, the library beads were incubated with 20 μL of the AlexaFluor488-conjugated FLAG Epitope tag monoclonal antibody in 10 mL of Tris buffer at room temperature for 2 h. Then, the beads were washed with Tris buffer three times and transferred into a six-well plate to be observed under a fluorescence microscope. The positive beads were picked up based on binding between the bead-anchored peptides and the HECT domain of E6AP, which have a Flag tag that was recognized by an anti-Flag antibody labeled with AlexaFluor 488 that would emit green fluorescence. The beads emitting green fluorescence were picked up as putative hits.

Cleavage and Analysis. Each bead identified was transferred to a 1.5 mL microtube and denatured with 100 μL of 8 M guandine·HCl for 1 h at room temperature. Then, the bead was rinsed with Tris buffer, water, DMF, and ACN three times in sequence. At last, the bead was placed in ACN overnight and then the ACN was evaporated. The bead was incubated in the solution of ACN:glacial acetic acid:H_2O containing cyanogen bromide (CNBr) (v:v:v = 5:4:1) at a concentration of 50 mg/mL overnight at room temperature. After the cleavage of the peptides from the bead, the solution was evaporated, and the cleaved peptide was dissolved in ACN:H_2O (4:1) and decoded by MALDI/MS.

FITC-Labeled Peptide Preparation. Fmoc-Lys(Dde)-OH was first attached to the Rink amide resin. Then, the Fmoc-protecting group was removed followed by coupling with the desired building blocks. After cyclization, the Dde group was removed. FITC (2 equiv) and DIPEA (10 equiv) in DMF were added to the vessel and shaken overnight at room temperature. Then, the FITC-labeled cyclic peptide was cleaved by 1:1 (v/v) DCM/TFA containing 2% triisopropylsilane. The crude was purified by a Waters HPLC, and the detailed structures can be found in the Supporting Information.

Binding Affinity. The binding affinity (K_d) of the peptides was measured by fluorescence polarization. Briefly, a constant amount of the 100 nM FITC-labeled cyclic peptide was incubated with a serial dilution of the E6AP HECT domain. The K_d values were calculated using the following equation, in which L_{st} and x refer to the concentration of the peptide and protein, respectively.

$$y = [FP\text{min} + (FP\text{max} - FP\text{min})] \frac{(K_d + L_{st} + x) - \sqrt{(K_d + L_{st} + x)^2 - 4L_{st} \times x}}{2L_{st}}$$

Protein Expression from Recombinant pET Plasmids. Recombinant pET plasmids for the expression of Uba1, UbcH7, E6AP HECT domain, E6AP full-length, UB, and UbxD8 were transformed into BL21 cells and cultured in 2XYT broth with antibiotics under 37 °C until the OD value of the media was within the range of 0.6−0.8. IPTG (1 mM) was added to the cell culture to induce the expression, and the cell culture was incubated overnight under 20 °C with agitation (220 rpm) before the cells were harvested by centrifugation (5500 rpm, 4 °C, 20 min). Cells were resuspended in 20 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) with the addition of 40 mg of lysozyme and 1 mM PMSF, and the mixture was incubated on ice for 30 min. The cell resuspension was sonicated on ice, the resulting cell lysate was centrifuged (10,000 rpm, 4 °C, 30 min), and the supernatant was collected to bind with Ni-NTA beads overnight at 4 °C. The protein was purified by a gravity-flow column with washes by 20 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, pH 8.0) once and 20 mL wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) twice followed by elution with 5 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted protein solution was further dialyzed in a dialysis buffer (50 mM Tris, 50 mM NaCl, 1 mM DTT, pH 8.0) and concentrated.

***In Vitro* Assay to Measure the Effect of the Peptide Ligands on E6AP Self-Ubiquitination.** All assays were set up in 50 μL of reaction buffer supplemented with 50 mM Tris, 5 mM $MgCl_2$, 5 mM ATP, and 1 mM DTT. Each peptide (10 or 100 μM) was incubated with 0.5 μM wt Uba1, 0.5 μM wt UbcH7, and 0.5 μM wt N-terminal Flag-tagged E6AP HECT domain or full-length protein at 37 °C for 30 min before 5 μM wt UB was added to start the UB-transfer reaction. The reactions were incubated for another 2 h at 37 °C and then quenched by boiling in the sample loading buffer of SDS-PAGE with DTT for 5 min and analyzed by SDS-PAGE and Western blot probed with the anti-Flag antibody. In another experiment, **P6** of gradient concentrations from 1 to 100 μM was added to the ubiquitination reaction mixture and the reactions proceeded similar to assay substrate ubiquitination.

***In Vitro* Assay to Measure the Effect of the Peptide Ligands on Substrate Ubiquitination by E6AP.** All assays were set up in 50 μL of reaction buffer supplemented with 50 mM Tris, 5 mM $MgCl_2$, 5 mM ATP, and 1 mM DTT. Each peptide (10 or 100 μM) was incubated with 0.5 μM wt Uba1, 0.5 μM wt UbcH7, 0.5 μM wt N-terminal Flag-tagged E6AP, and 2 μM substrates (UbxD8, β-catenin, or HHR23A) at 37 °C for 30 min before 5 μM wt UB was added to start the UB-transfer reaction. The reactions were incubated for another 2 h at 37 °C and then quenched by boiling in the sample loading buffer of SDS-PAGE with DTT for 5 min and analyzed by SDS-PAGE and Western blot probed with substrate-specific antibodies. In another experiment, **P6** of varying concentrations from 1 to 100 μM was added to the ubiquitination reaction mixture and the reactions proceeded similar to assay substrate ubiquitination. The p53 ubiquitination assay was set up with the same concentrations of Uba1, UbcH7, and E6AP with the addition of 0.5 μM E6 protein. The enzymes and the peptide ligands were pre-incubated for 30 min

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before 5 μM wt UB was added to start the reaction. The reactions were incubated for another 2 h at 37 °C and then quenched by boiling in the sample loading buffer with DTT for 5 min and analyzed by SDS-PAGE. The Western blot was probed with an anti-p53 antibody.

Cellular Assays to Measure the Stimulatory Effects of P6 on Ubiquitination of E6AP Substrates. HEK293T cells were preincubated with **P6** at 0, 25, and 50 μM for 14 h and with 0.5 μM MG132 for an additional 12 h. Cells were then washed twice with ice-cold PBS, pH 7.4, and 1 mL of ice-cold RIPA buffer was added and incubated with the cells at 4 °C for 10 min. The cells were disrupted by repeated aspiration through a 21-gauge needle to induce cell lysis, and the cell lysate was transferred to a 1.5 mL tube. The cell debris was pelleted by centrifugation at 13,000 rpm. for 20 min at 4 °C, and the supernatant was transferred to a new tube and precleared by adding 1.0 μg of the appropriate control IgG (normal mouse or rabbit IgG corresponding to the host species of the primary antibody). A total of 20 μL of suspended Protein A/G PLUS-agarose was added to the supernatant, and incubation was continued for 30 min at 4 °C. After this, the cell lysate containing a 2 mg total protein was transferred to a new tube and a 30 μL (i.e., 6 μg) primary antibody specific for UbxD8 or HHR23A was added. Incubation was continued for 1 h at 4 °C, and 50 μL of resuspended Protein A/G PLUS-Agarose was added. The tubes were capped and incubated at 4 °C on a rocking platform overnight. The next day, the agarose beads were pelleted by centrifugation at 350*g* for 5 min at 4 °C. The beads were then washed three times, each time with 1.0 mL of PBS. After the final wash, the beads were resuspended in 40 μL of 1× Laemmli buffer with β-mercaptoethanol. The samples were boiled for 5 min and analyzed by SDS-PAGE and Western blotting probed with an anti-UB antibody.

E6AP-Induced Protein Degradation in HEK293T Cells. Cycloheximide (CHX) chase assays were performed with HEK293T cells (5×10^6 cells) incubated with **P6** at 0, 1, 5, and 50 μM for 14 h. After incubation, cells were treated with 100 μg/mL CHX to block *de novo* protein synthesis and harvested after various incubation times with CHX. The levels of substrate proteins in the cell were assayed by immunoblotting with antibodies specific for UbxD8 and HHR23A. Protein levels were normalized to β-actin.

Enzymatic Stability Study. **P6** (0.01 mg/mL) was incubated with 0.1 mg/mL pronase in 100 mM ammonium bicarbonate buffer (pH 7.8) at 37 °C for 24 h. The reaction mixture was concentrated in a speed vacuum to remove water and ammonium bicarbonate. The remaining were dissolved in 100 μL of H_2O/CH_3CN and analyzed by a Waters analytical HPLC system with a 1 mL/min flow rate and 5−100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over a duration of 50 min. The UV detector was set to 215 nm.

Serum Stability Assay. The serum stabilities of peptides were determined in 25% (v/v) aqueous pooled serum from human male AB plasma (Sigma-Aldrich, Milan, Italy). **P6** (1 mg) was dissolved in 50 μL of CH_3CN/H_2O (70:30, v/v) and then diluted in serum and incubated at 37 °C for 24 h. Then, 100 μL of solution was added to 100 μL of CH_3CN on ice for 15 min and was centrifuged at 4 °C for 10 min. The supernatant was then analyzed by a Waters analytical HPLC system with a 1 mL/min flow rate and 5−100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over a duration of 50 min. The UV detector was set to 215 nm.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c01922.

> Procedure to synthesize the OBTC γ-AA peptide library; a schematic illustration of the screening method; determination of decoding sequence by the tandem MS/MS of MALDI; binding affinity experiments; analytic HPLC traces of enzymatic and serum stability; structures of FITC-labeled **P1** to **P8**; HRMS of all peptides and FITC-labeled peptides; and HPLC trace of all compounds (PDF)
>
> SMILES of **P1** to **P8** and FITC-labeled **P1** to **P8** (CSV)

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Author Contributions

[§]B.H., L.Z., and R.L. contributed equally. J.Y. and J.C. conceived the idea and designed the experiments. B.H. constructed the γ-AA peptide library and carried out affinity screens of the library. L.Z. performed the cellular assay of the ligands. R.L. assayed the ligand activities in reconstituted ubiquitination reactions. G.H.J. and I.H.J. helped with the ubiquitination assays. L.W., S.X., Y.S., and S.L. helped with the library preparation. B.H., L.Z., R.L., J.Y., and J.C. analyzed the data and interpreted the results. B.H., L.Z., R.L., J.Y., and J.C. wrote and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PROTACs, proteolysis-targeting chimeras; E6AP, E6 associated protein; HPV, human papillomavirus; γ-AA peptide, γ-substituted-*N*-acylated-*N*-aminoethyl peptide; PNA, peptide nucleic acids; HECT domain, homologous to the E6AP carboxyl terminus; OBTC, one-bead-two-compound; MALDI, matrix-assisted laser desorption/ionization; MS/MS, tandem mass spectrometry; Uba1, ubiquitin-like modifier activating enzyme 1; UbcH7, ubiquitin-conjugating enzyme E2; MG132, carbobenzoxy-Leu-Leu-leucinal; CHX chase, cycloheximide chase; GLP-1, glucagon-like peptide 1; MDM2, mouse double minute 2 homolog; BCL9, B cell lyphoma 9; UB, ubiquitin; HOBt, hydroxybenzotriazole; DMF, dimethylformamide; DIC, *N*,*N*′-diisopropylcarbodiimide; Alloc, allyloxycarbonyl; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; PyBop, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; NEM, *N*-ethylmaleimide; Tris buffer, tris(hydroxymethyl) aminomethane buffer; BSA, bovine serum albumin; ACN, acetonitrile; FITC, fluorescein isothiocyanate; DIPEA, *N*,*N*-diisopropylethylamine; DCM, dichloromethane; TFA, trifluoroacetic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol

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