Supplemental Information

Orthogonal Ubiquitin Transfer through Engineered E1-E2 Cascade for Protein Ubiquitination

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Supplemental Results

Model selection of UB displayed phage.

We used the pJF3H phagemid (Barbas, et al., 2000; Crameri and Suter, 1993) to display UB on the phage with a free C-terminus (Figure S4A and S4B). The pJF3H phagemid encodes two fusion proteins – a Jun-pIII fusion with the Jun peptide fused to the N-terminus of M13 phage capsid protein pIII, and a Fos-target protein fusion with the Fos peptide fused to the N-terminus of the displayed protein. When the Jun-pIII and Fos-target protein are both expressed in bacterial cells, they form a heterodimer due to the binding of Jun-Fos coiled coil. The heterodimer can be further stabilized by two disulfide bonds between the Cys residues flanking the Fos and Jun peptide sequence. Phage assembly anchors the Jun-Fos dimer on the phage surface and the displayed protein is fused to the dimer with an exposed C-terminus. We cloned the UB gene as a fusion to the Fos peptide in the pJF vector. We also inserted an HA tag in between Fos and the N-terminus of UB to allow the detection of UB on phage. We refer to the pJF plasmid for UB display as “pJF-HA-UB” (Figure S4B).

Phage prepared with pJF-HA-UB phagemid were analyzed by enzyme linked immunosorbent assay (ELISA) (Barbas, et al., 2000; Kay, et al., 1996) and western blot to confirm the display of UB. In this assay, an immuno plate was coated with an anti-HA antibody and bound with UB displayed phage with a 10-fold dilution across the plate. Phage bound to the HA antibody on the plate were detected with an anti-M13 antibody conjugated with horse radish peroxidase (HRP). We observed strong signals of phage ELISA suggesting the efficient display of HA-tagged UB on the phage surface (Figure S4C). We also lysed the phage particles and separated the phage capsid proteins by polyacrylamide gel electrophoresis (PAGE). Western blot of the gel was probed with a mouse anti-HA antibody and an anti-mouse antibody-HRP conjugate (Figure S4D). A band of 15 kDa corresponding to the size of Fos-HA-UB fusion was detected on the blot. These experiments confirm the display of HA-UB on phage surface by the pJF-HA-UB phagemid.

We then tested if UB displayed phage can be activated and conjugated to wtUba1 on the streptavidin plate for the selection of catalytically active UB clones. We expressed the PCP-Uba1 fusion and labeled the fusion protein with biotin-CoA catalyzed by Sfp. Biotin conjugated PCP-Uba1 was then bound to the streptavidin plate. We then reacted phage displayed UB with PCP-Uba1 on the plate in the presence of ATP. Control reactions were also set up excluding the addition of ATP or using a streptavidin plate without the binding of PCP-Uba1 to the plate (Figure S4E). We also used the same number of phage displaying a virus protein SV5V (Precious, et al., 2005; Ulane and Horvath, 2002) to react with Uba1-PCP on the plate. SV5V has no reactivity with Uba1 so SV5V displayed phage should only give background binding to the plate. After reaction, the plate was washed and phage bound to the plate were detected with an anti-M13 phage antibody conjugated with HRP (Figure S4E). We observed strong binding of UB phage to the PCP-Uba1 plate based on the phage ELISA signal (Figure S4E, lane 1) suggesting the formation of UB~Uba1 conjugate. The binding of UB phage to the plate was dependent on the addition of ATP and the immobilization of PCP-Uba1 in the well (Figure S4E, lanes 2 and 3). When UB displayed phage were replaced with SV5V displayed phage, there was very little binding of the phage

to the plate (Figure S4E, lanes 4-6). These results suggest that UB on the phage surface was reactive with Uba1 immobilized on the plate and after the reaction, UB displayed phage were bound to the plate through the formation of UB~Uba1 conjugate. Western blot of the reaction of UB-phage with PCP-Uba1 also confirms the formation of the UB~Uba1 conjugate of 160 kDa that corresponds to the combined size of PCP-Uba1 fusion and the Fos-HA-UB / Jun-pIII heterodimer (Figure S4F, lane 1). The band for the Fos-HA-UB / Jun-pIII heterodimer was also detected on the blot with a size of 40 kDa.

In another experiment, we eluted phage from the plate with DTT after the reaction. The titer of the eluted phage showed that there were approximately 1000-fold more UB phage eluted from the plate than the SV5V phage (Figure S4E, lanes 1 and 4). Control reactions without ATP or Uba1-PCP binding to the plate showed no phage enrichment. These results match with the phage ELISA assay suggesting that we can use phage display to select for UB mutants that are reactive with mutant Uba1.

To assay the selection efficiency, we mixed UB and SV5V displayed phage at a ratio of 1/50, 1/500 and 1/5000, respectively and reacted the phage mixture with Uba1-PCP on the plate in the presence of ATP. After the reaction, phage bound to the plate were eluted with DTT and the eluted phage were used to infect *E coli* cells. The composition of the phage mixture was analyzed by colony PCR with a pair of primers that were bound to the backbone sequence of pJF3H plasmid to amplify the genes of either UB or SV5V. The UB phage clone gave a PCR fragment of 1 kbp while the SV5V clone gave a fragment of 1.5 kbp. Based on the size of the PCR product, we could differentiate the two types of phage clones. After phage selection, we found that all of the eluted phage were UB clones from selection of the 1/50 mixed pool. Seven out of 10 clones selected from the 1/500 phage mixture were UB clones and three out of 10 clones from the 1/5000 mixture were UB clones (Figure S4G). These results suggest that the enrichment of UB clones from each round of phage selection would be at least 500-fold. Thus in the selection experiments with a library of UB, we would be able to follow the same protocol to enrich UB mutants that were reactive with the triple mutant of Uba1.

Model selection of Ubc1 displayed phage

We validated the phage display method to engineer xE1-xE2 pairs by displaying wtUbc1 (wtUbc1) on phage and used wtUba1 to catalyze the conjugation of biotin-UB. Enzyme-linked immunosorbent assay (ELISA) showed that biotin-UB conjugated Ubc1 phage could be specifically bound to a streptavidin-coated plate and that phage binding was dependent on Uba1 catalyzed transfer of biotin-UB (Figure S6A and S6B). To measure the selection efficiency, we mixed Ubc1 displayed phage at 1/100 and 1/500 ratio with a phage displaying the EntE protein with no E2 activity (Ehmann, et al., 2000). We carried out model selection of the mixed phage pool. Phage colonies after selection were PCR amplified with a pair of primers binding to the pComb3H plasmid flanking the Ubc1 or EntE gene. There should be a 1 kbp fragment amplified from the Ubc1 clone and a 1.5 kbp fragment from the EntE clone. Colony PCR showed that after one round of selection, nine out of ten phage clones were Ubc1 in the 1/100 mixed pool and three out of ten clones were Ubc1 in the 1/500 mixed pool (Figure S6C). This suggests that Ubc1 displayed phage could be enriched by more than 100 fold in just one round of selection.

Supplemental Figures

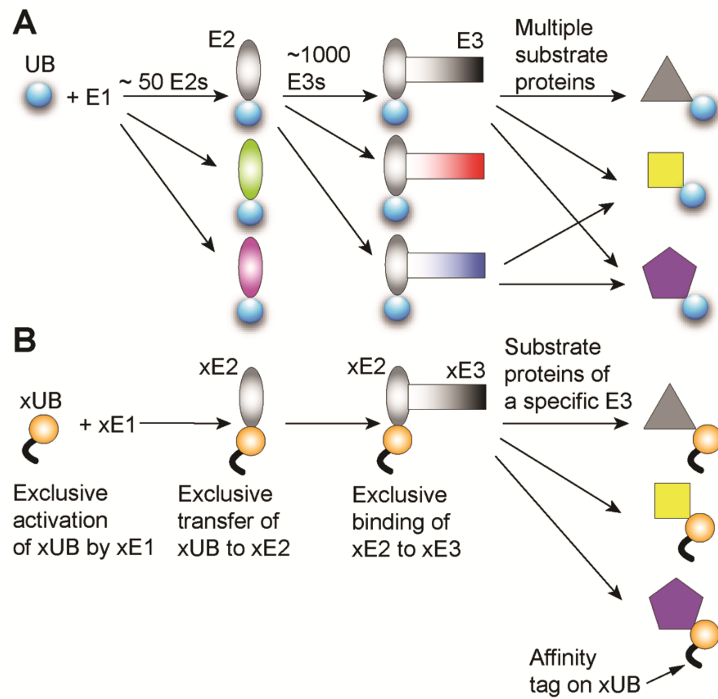

Figure S1. UB transfer reactions through the E1, E2 and E3 enzymes. (A) Native UB transfer cascade with a complex network of E1, E2 and E3 enzymes. (B) Engineered orthogonal UB transfer (OUT) cascade that enables an affinity tagged xUB to be transferred through engineered xE1, xE2 and xE3 enzymes to the substrate proteins of a specific E3.

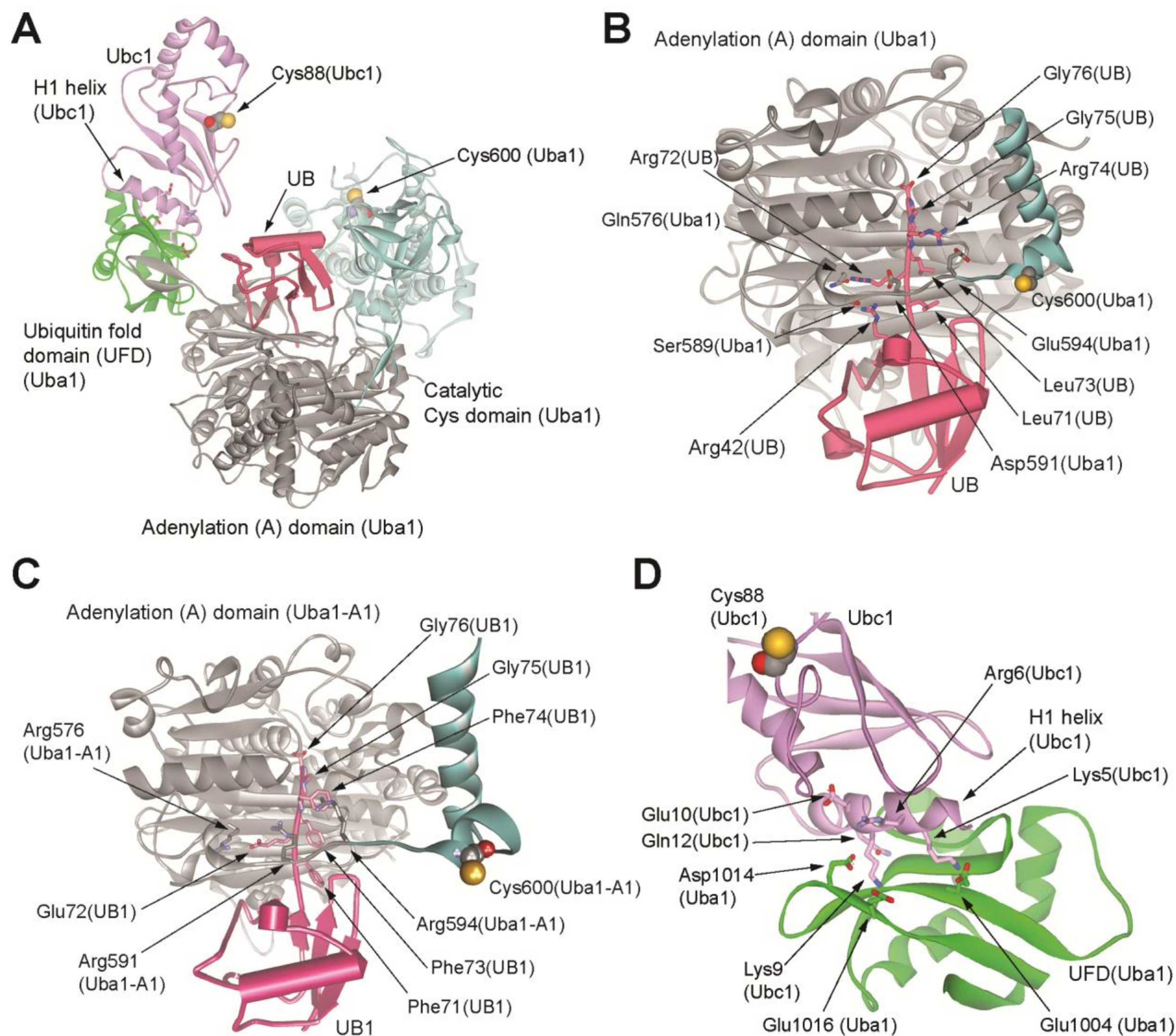

Figure S2. Structural analysis of the binding interfaces between Uba1 and UB, and Uba1 and Ubc1. (A) Modeled structure of Uba1 in complex with UB and Ubc1. The crystal structure of UB-Uba1 complex (PDB ID 3CMM) was used to generate the model (Lee and Schindelin, 2008). The adenylation (A) domain of Uba1 is colored in gray, the catalytic Cys domain in cyan and the ubiquitin fold domain (UFD) in green. The UB molecule is in red and Ubc1 in purple. Catalytic Cys residues in Uba1 (Cys600) and Ubc1 (Cys88) are the site of thioester formation with UB in the UB~Uba1 and UB~Ubc1 complex. The thiol groups of the Cys residues are shown as golden balls. (B) The UB-Uba1 binding site is shown with the C-terminus of UB extending into the adenylation domain of Uba1. Residues of UB and Uba1 involved in critical interactions are shown. (C) In silico generated model of the UB mutant UB1 with the C-

terminal sequence ^{71}FEFFGG^{76} bound to the Uba1-A1 with Gln576Arg, Asp591Arg and Glu594Arg mutations. UB1 was identified by phage selection with the A1 mutant. (D) The binding interface between Uba1 and Ubc1 in the modeled complex. Key residues engaged in electrostatic interactions between the UFD domain of Uba1 and H1 helix of Ubc1 are shown.

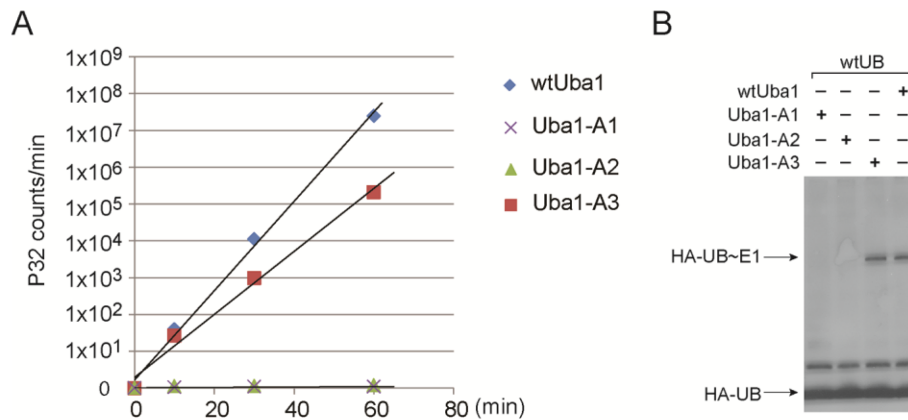

Figure S3. Reactivities of Uba1 mutants with wtUB. (A) Comparison of the ATP-PPi exchange rates for wtUB activation by wtUba1 and Uba1 mutants A1, A2 and A3. (B) Western blot to show that A1 and A2 cannot form thioester conjugates with wtUB, but A3 can for wtUB~A3 conjugate.

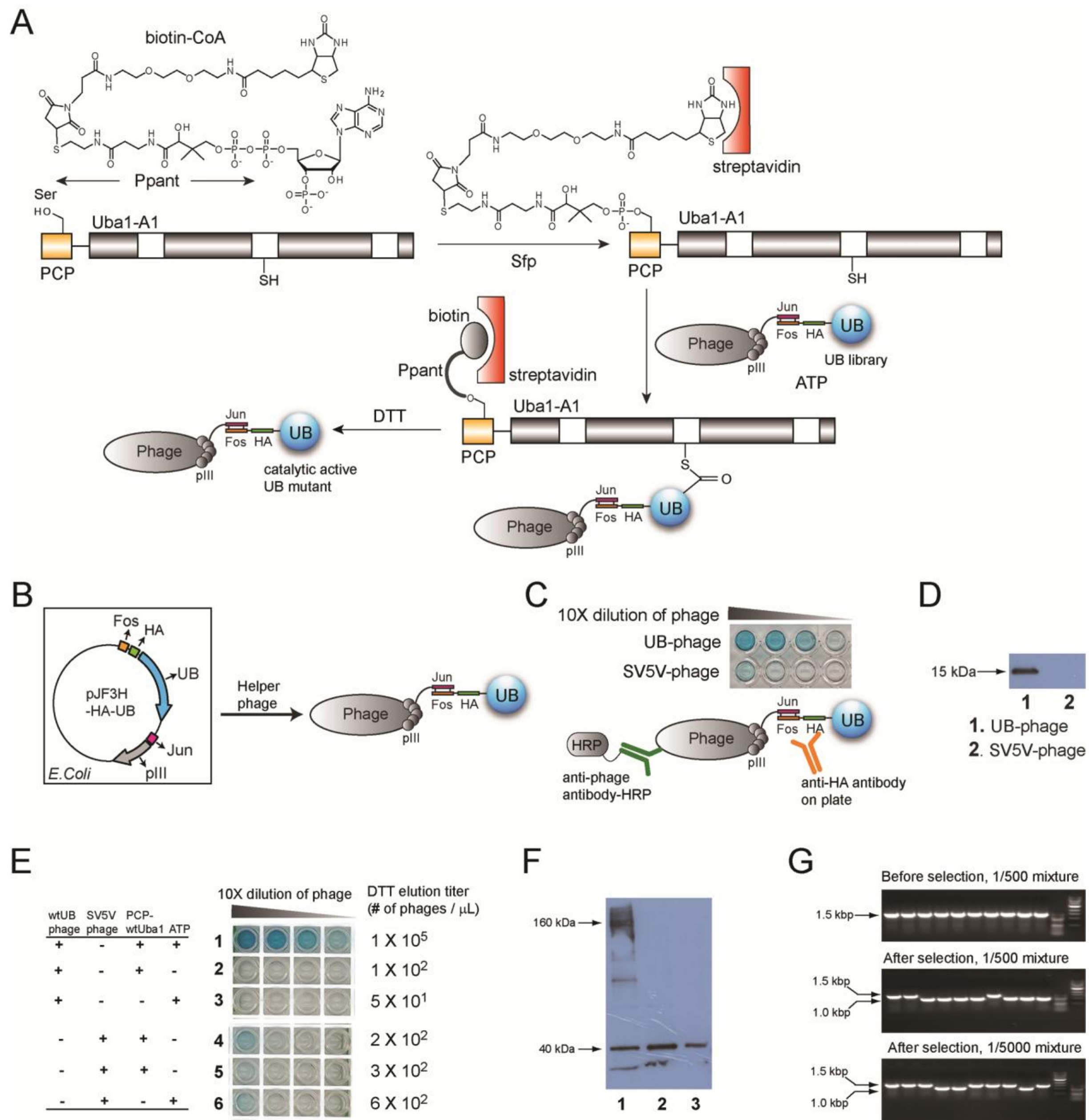

Figure S4. Selection of phage displayed UB library based on the reactivity with the E1 enzyme. (A) Selection of phage library of UB with A1 immobilized on the streptavidin plate. The PCP domain fused to the A1 mutant was first labeled with biotin catalyzed by Sfp. (B) Phage display of UB with a free C-terminus by the pJF-HA-UB plasmid. (C) Phage ELISA to confirm the display of UB with an anti-HA antibody coated on the plate. (D) Western blot of the UB phage probed with an anti-HA antibody. (E)-(G),

Model selection of UB phage with PCP-Uba1 immobilized on the streptavidin plate. (E) Phage ELISA to detect the phage binding to the plate and the titers of the eluted phage after DTT cleavage. (F) Western blot of the UB phage after reaction with Uba1-PCP. Lane assignment is the same as in (E). The blot was probed with an anti-HA antibody and an anti-mouse antibody-HRP conjugate. (G) Colony PCR for the phage clones from the model selection of UB phage / SV5V phage mixture.

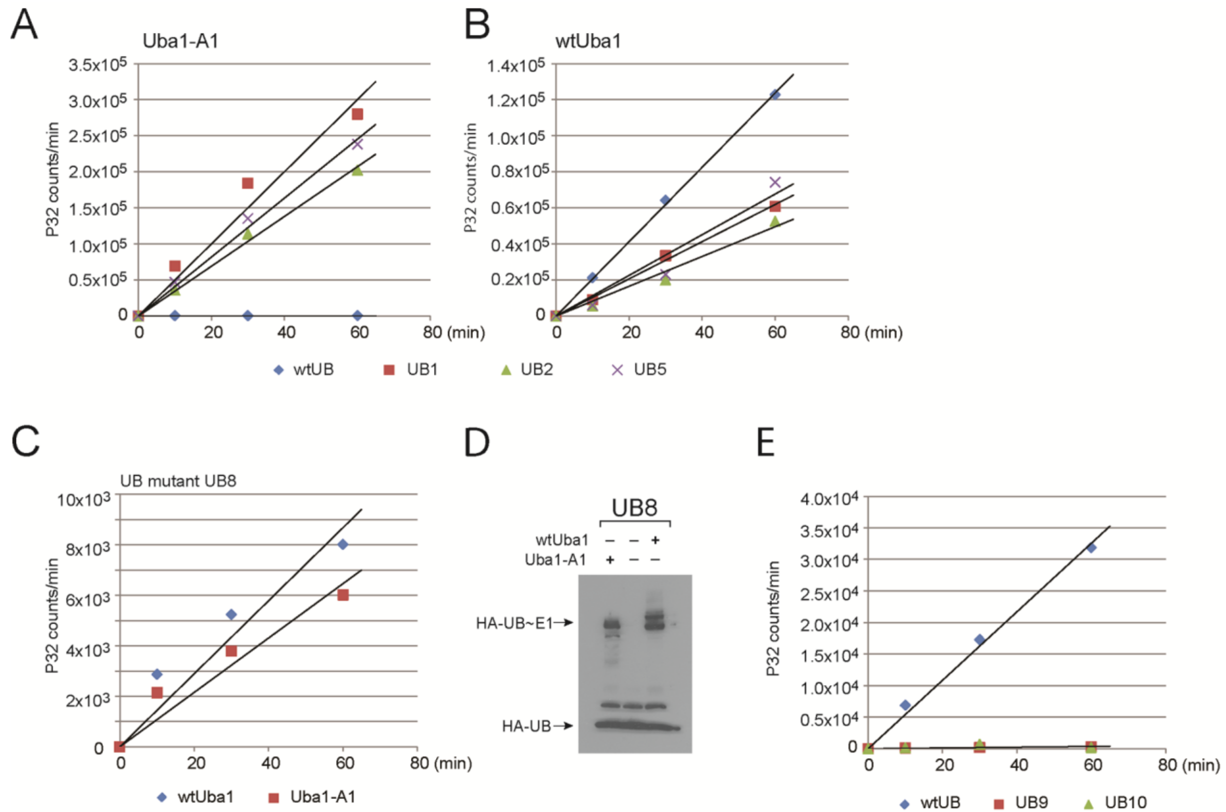

Figure S5. Reactivities of the UB mutants with wtUba1 and A1. (A) ATP-PPi exchange for the activation of UB1, UB2 and UB5 catalyzed by Uba1-A1. (B) ATP-PPi exchange for the activation of UB1, UB2 and UB5 catalyzed by wtUba1. (C) ATP-PPi exchange for the activation of UB8 by wtUba1 and A1. (D) Western blot to show the formation of UB~E1 conjugates of UB8 with wtUba1 and A1. (E) Reactivity of UB9 and UB10 with wtUba1 assayed by ATP-PPi exchange.

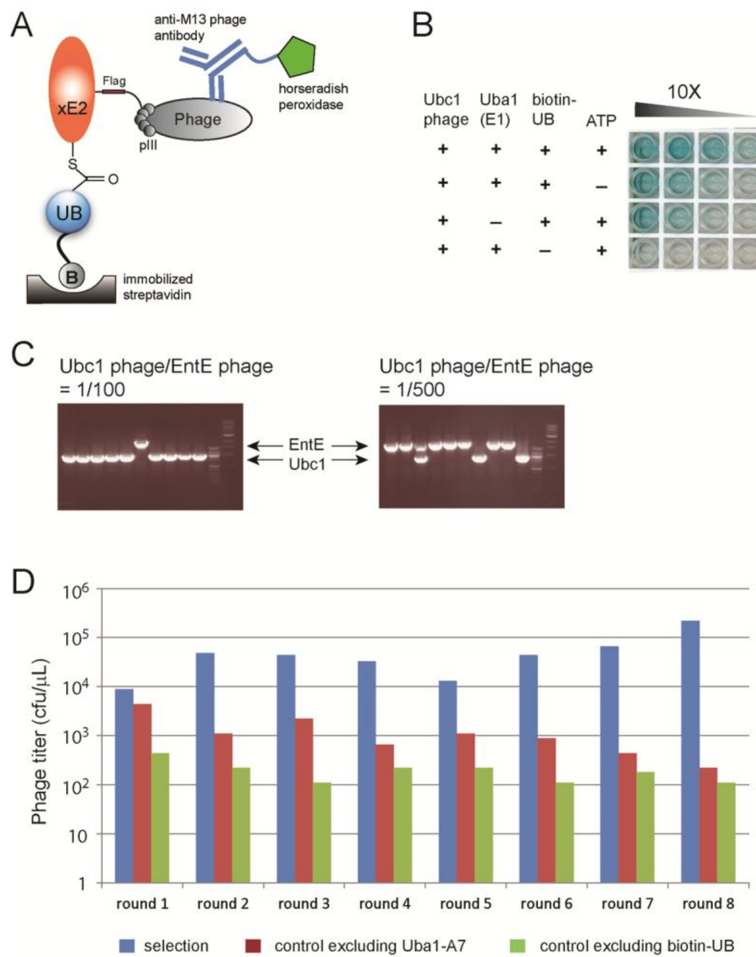

Figure S6. Phage selection of Ubc1 mutants that are reactive with A7. (A) Scheme of the ELISA assay to detect UB transfer to Ubc1 displayed on phage. After the UB transfer reaction, biotin-UB conjugated Ubc1 (E2) phage were bound to the streptavidin plate and detected with an anti-M13 phage antibody linked to a horseradish peroxidase. (B) Results of the ELISA assay. UB transfer reaction mixtures were diluted 10-fold across the plate. In control reactions, Uba1, biotin-UB, or ATP was excluded from the reaction. (C) Colony PCR of the model selection with 1/100 or 1/500 diluted Ubc1 phage in mixtures with the EntE phage. After one round of selection, 9 out of 10 clones from 1/100 diluted mixture were Ubc1 phage while 3 out of 10 clones from 1/500 diluted mixture were Ubc1 phage. (D) Phage titer for the selection of the Ubc1 library. Phage selection of the Ubc1 library was performed in parallel to the controls in which either A7 or biotin-UB was excluded from the UB transfer reaction. Numbers of the

eluted phage from the streptavidin plate for the selection and control reactions are plotted to a logarithmic scale. cfu, colony forming unit.

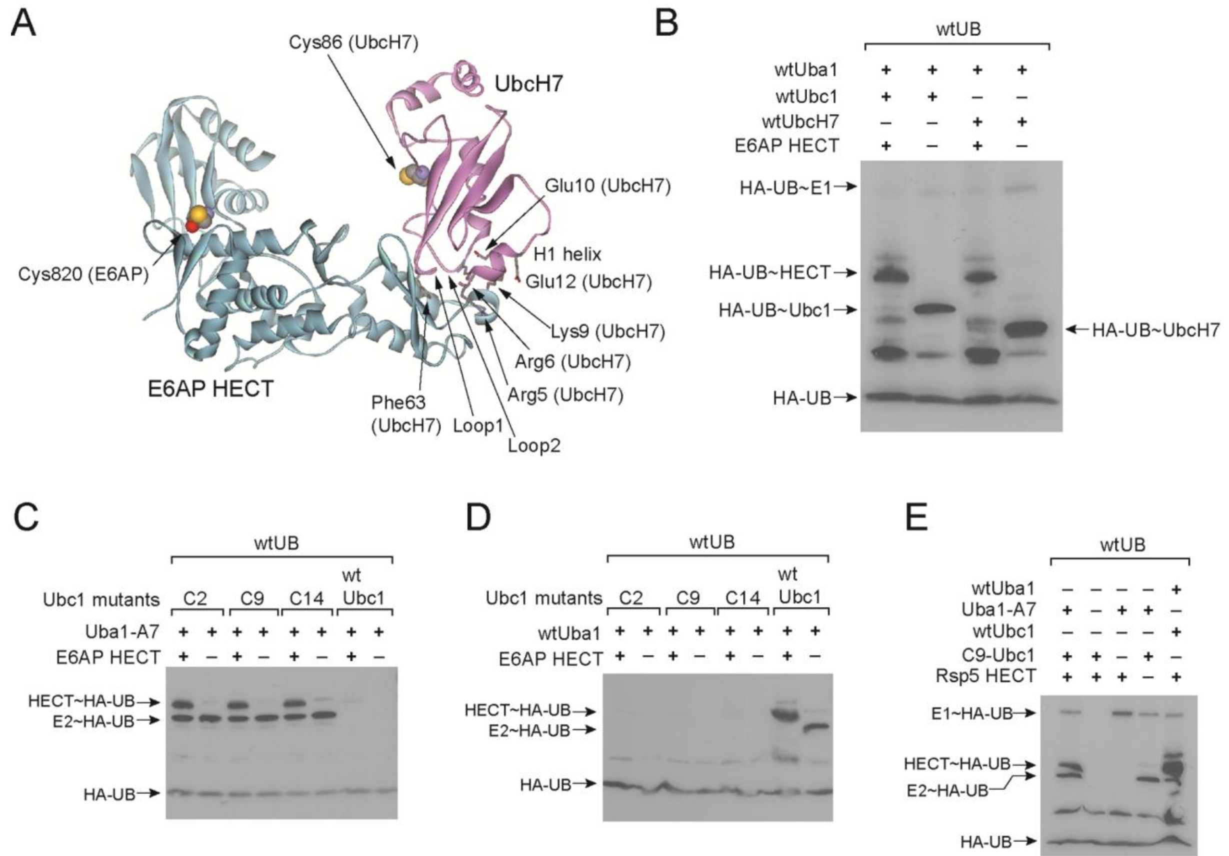

Figure S7. Reactivity of mutant E1-E2 pairs with the HECT domains of E6AP and Rsp5. (A) Crystal structure of UbcH7 in complex with the HECT domain of E6AP (PDB ID 1C4Z) (Huang, et al., 1999). UbcH7 is in purple and the E6AP HECT is in blue. Also shown are residues in the H1 helix of UbcH7 that are mutated in the corresponding positions in Ubc1 for the construction of the xE1-xE2 pair. Cys86 of Ubc1 and Cys820 of the HECT are also shown and they are the sites of UB attachment in the UB~E2 and UB~HECT conjugates. (B) UB transfer from wtUbc1 and UbcH7 to the HECT domain of E6AP. (C) UB transfer to the HECT domain of E6AP from xE1 (UFD) (A7) and the Ubc1 mutants from phage selection. (D) UB transfer to the HECT domain of E6AP from wtUba1 and Ubc1 mutants. (E) UB transfer to the HECT domain of Rsp5 from xE1 (UFD) (A7) and C9-Ubc1.

Table S1. Sequences of the primers used in this study.

Primer	Sequence
Bo 2	5’-AGTGATCAGCGCGCCGAGCTCTCTAGGGCTAAGAGAATTATG-3’
Bo 3	5’-CTGGTAGTCGGTACCACTAGTCTTCAACAATTCCTCGATG-3’
Bo 7	5'-CTAAAGGTAATACTCGAGTCATCATTCCAAG -3'
Bo 8	5'-CTCTTCTTCTAGAAACCCACCAGAAAAGTCTATCC -3'
Bo 9	5'-CTCTTCTTCTCGAGACCCACCACGAAAGTCTATCCCATTG -3'
Bo 10	5'-CTCTTCTTCTAGAAACCCACCACGAAAGTCTATCCCATTG -3'
Bo 12	5'-GAAATGAGAAGACTAC -3'
Bo 13	5’-GTACTTGACACAACCG-3’
Bo 22	5’-CTGGTATGGACTAGTCTTGTCATCGTCGTCCTTGTAGTCGGTACCCTTCAACAATTCCTCGATG-3’
Bo 23	5’-AGTTGTCAGCATATGGTGTTGGATTCTCTTGAATTTATTGCTAGTAAACTGGCGACTAGTAGCTTGG GTATGCAGATC-3’
Bo 35	5’-TGAAGAATTGCGGCCGCTTAACCACCAGCGGCTGCGACAAGATGTAAGGTCGACTCC -3’
Bo 38	5’-GTACACAATGGGATAGACTTTCGTGGTGGGTTTCTAGAAGAAGAGTATG-3’
Bo 39	5’-AAGTCTATCCCATTGTGTACACTACGTTCTTTCCCAAAC -3’
Bo 40	5’-GTTGGCATTTGGATCCGG -3’
Bo 41	5’-GTTAGAATCTGGTACCCTAGGTACTAAAGGTAATACTCGAGTCATCATTCCAAGATTG -3’
Bo 46	5’-GAACGTAGTGTACACAATGGGATAGACTTTCGTGGTGGGCGTCTAGAAGAAGAGTATGATTC -3’
Bo 47	5’-GAACGTAGTGTACACAATGGGATAGACTTTCGTGGTGGGCGTCTTAAAGAAGAGTATGATTC -3’
Bo 54	5’-AGTGATCAGCGCGCCGAGCTCATGGCGGCCAGCAGGAG-3’
Bo 55	5’-CTGGTAGTCACTAGTGGTACCGTCCACAGGTCGCTTTTC-3’
Bo 73	5’-CGGAGCTCGAATTCTCATAGATGAATGGTAATGAAAGGAACTTTAACCTTCTCTCCTTCCTTGTCAT CTGCGCAAATTTTGAGAATCATTGTAGATAC-3’
Bo 74	5’-CTAGAAGCTTCTAATGCGGCCGCTTAACCMNNMNNMNNMNNMNNGACAAGATGTAAGGTCG -3’
Bo 80	5’-ATGTCTTGTGAGCTCATGTCTAGGGCTGCGGCAATTATGGCAGCAATCGCAGCTGTGAAGGATGAT CCTGC-3’
Bo 81	5’-ATGTCTTGTGAGCTCATGTCTAGGGCTNNKNNKATTATGNNKNNKATCNNKGCTGTGAAGGATGAT CCTGC-3’
Bo 100	5’-GCTCGAGTGCGGCCGCTTAACCACCTCTTAGCTCTAAGACAAGATGTAAGGTCGAC-3’
Bo 104	5’-AGCTAGGCTCATATGGAGCTCATGTCTAGGGC-3’
Bo 125	5’-AGTGATCAGGAGCTCATGTCTAGGGCTGATGATATTATGGATCAGATCCATGCTGTGAAGGATTGT GGGATGAAAAACTTC -3’
Bo 126	5’-AGTGATCAGGAGCTCATGTCTAGGGCTGATGAGATTATGGAGCAGATCCTGGCTGTGAAGGATTG TGGGATGAAAAACTTC -3’
Bo 150	5’-AGATATACATATGGAAAATGATGATAAAATTAC-3’
Bo 152	5’-GCTCGAGTGCGGCCGCTCAGGGGGAATAAGTTAGCAC-3’
Jun 13	5’-ACTTTATGCTTCCGGCTCGTATGT-3’
Jun 14	5’-AATCAAAATCACCGGAACCAGAGCC-3’
Jun 340	5’-AGTGATCAGCGCGCCTCTTACCCATACGACGTTCC -3’
Jun 341	5’-GCTTCTAATGCGGCCGCTTAACCACCTCTTAGTCTTAAG -3’
Jun 348	5’-AGTGATCAGCATATGTCTTACCCATACGACGTTCC -3’
Jun 349	5’- GCTTCTAATGGATCCTCAACCACCTCTTAGTCTTAA -3’
Jun 352	5’-AGTGATCAGCGCGCCGAGCTCGATCCCACTGATCTGAGC -3’
Jun 353	5’-CTTGTAGTCGGTACCACTAGTAGTATCTCGTTCACATTCAGAGC -3
Kar 1	5’-ATGAGCGCCATATGGCGCTGAAGAGGATTCAG-3’
Kar 2	5'-CTGGTAGTCCTCGAGCATTGCATATTTCTGAGTCC-3'
Kar 28	5’-CTTCCATCAGGTACCACTAGTCAGCATGCCAAATCC TTTGGC-3’
Kar 30	5’-TTAGTTCAAGGTACCACTAGTTTCTTGACCAAACCC TATGG-3’
Kar 31	5'-CTAGTGATCGAGCTCCAGTTGAATCCATATTTGAGA CTC-3'
Kar 32	5'-CTAGTGATCGAGCTCGACCAAAATGTTCCACAATAC AAGC-3'

Supplemental Experimental Procedures

Reagents.

Unless otherwise indicated, all reagents were obtained from commercial sources and used without further purification. All solutions and buffers for phage selection were sterilized by either autoclaving or filtration. Kits for isolating DNA plasmids were from Qiagen. Restriction endonucleases, M13KO7 helper phage and Ni-NTA agarose resin for protein purification were from New England Biolabs. Taq DNA polymerase was from Promega. Biotin-CoA was prepared following a literature procedure (Yin, et al., 2006; Yin, et al., 2004). HPLC purification of biotin-CoA was carried out on a POLARIS BioInert Gradient LC System (Varian, Walnut Creek, CA) with a reverse phase Nucleodur C-18 column of 250 mm in length, 21 mm i.d. and 10 mm particle size (Phenomenex, Torrance, California). Biotin-CoA was analysed by MALDI-TOF spectra acquired with a Voyager DE PRO MALDI mass spectrometer (PerSpective Biosystems, Framingham, MA). Oligonucleotides were supplied by Integrated DNA Technologies and their sequences are listed in Table S1.

Strains and vectors.

XL1-Blue cells were purchased from Agilent Technologies (Santa Clara, California, USA). pET-15b, pET-21b and pET-28a plasmids for protein expression were from Novagen. pJF3H and pComb3H plasmids were obtained from Professor Carlos F. Barbas at The Scripss Research Institute. UbcH5a and UbcH7 plasmids were provided by Professor Bernard Roizman of the University of Chicago. HA-UB plasmid was provided by Professor Suzanne D. Conzen of the University of Chicago. Plasmid of the E6AP HECT gene was provided by Professor Jon M. Huibregtse of the University of Texas at Austin. The Rsp5 plasmid was provided by Professor Linda Hicke of the Northwestern University.

Construction of the protein expression plasmids

The HA peptide tag was fused to the N-terminus of UB by amplifying the UB gene with primer Jun 348 that encodes the HA peptide and primer Jun349 by the polymerase chain reaction (PCR). The amplified fragment was digested with restriction enzymes NdeI and BamHI, and cloned into the pET-15b plasmid (Novagen) for protein expression. After phage selection, the genes of UB mutants UB1, UB2 and UB5 were amplified by PCR primers Jun348 and Jun14 from the corresponding pJF3H vector. The purified PCR products were digested by NdeI and NotI and cloned into pET-28a. The single UB mutant Arg72Glu (UB8) was constructed by amplifying the wtUB gene with PCR primers Jun348 and Bo100. The PCR fragment was cloned into pET-28a after double digestion with the NdeI and NotI restriction enzymes. UB9 and UB10 was constructed by introducing Arg42Asp and Arg42Glu mutations into UB8. To introduce Arg42Glu mutation in UB10, overlap PCR was performed by amplifying the front fragment of UB with primers Jun348 and Bo97, and the back fragment of UB with primers Bo95 and Bo100. After purification, two fragments were added into a 50 μl PCR reaction containing 33 μl H_2O, 10 μl Mg^{2+} free PCR buffer (Promega), 5 μl 25 mM $MgCl_2$, 1 μl dNTP mix (25 mM of each dNTP) and 0.5 μl Taq DNA polymerase (Promega). The PCR reactions were run with the following program: 95°C for 10 minutes, then 35 cycles of 94°C for 1 minute, 50°C for 45 seconds, 72°C for 1.5 minute and a final step of 72°C for 5 minutes. After the first two rounds of PCR cycles, primers Jun348 and Bo100 were added into reaction and the PCR cycles were repeated for 30 times to amplify the full length UB gene with the Arg42Glu mutation. Similarly the Arg42Asp mutation was introduced into UB8 to afford UB9. The ybbR peptide tag was fused to the N-terminus of UB by PCR amplifying the UB gene with primer Bo23 that encodes the ybbR peptide and primer Jun349. The amplified fragment was digested with restriction enzymes NdeI and BamHI and cloned into the pET-15b plasmid for protein expression. To clone UB gene into the pJF3H phagemid for phage display, wtUB gene was amplified with primers Jun340 and Jun341 followed by restriction digestion with BssHII and NotI and ligation into pJF3H. To construct PJF-SV5V, SV5V gene was amplified by primers Jun352 and Jun353. The purified PCR fragment was double digested by BssHII and KpnI and cloned into pJF3H.

Mutations were introduced into the Uba1 mutants A1-6 by PCR amplification of the wtUba1 gene in pET-28a plasmid with primers Bo7, Bo8, Bo9, Bo10, Bo12, Bo13, Bo38, Bo39, Bo40, Bo41, Bo46, and Bo47 (Table S1). The PCR fragment was digested by the restriction enzymes BamHI and EcoRI and ligated with the pET-28a plasmid digested at the same restriction sites. Triple mutant of Uba1 with Glu1004, Asp1014 and Glu1016 to Lys mutations was constructed by PCR amplification of the wild type Uba1 in pET-28a plasmid with primers Bo13 and Bo73. The PCR fragment was digested by the restriction enzymes BamHI and EcoRI and ligated with the pET-28a-Uba1 plasmid digested at the same restriction sites.

Ubc1 gene was amplified by primers Bo2 and Bo3 and cloned into the pET-21b plasmid after double digestion by the restriction enzymes SacI and SpeI. UbcH5a gene was amplified by primers Kar1 and Kar2. The purified PCR fragment was double digested by NdeI and XhoI and cloned into pET-21b. UbcH7 gene was amplified by primers Bo54 and Bo55. After double digestion by the restriction enzymes SacI and SpeI, the UbcH7 gene was cloned into pET-21b. After phage selection, the genes of Ubc1 mutants C1, C2, C9, C14 and C16 were amplified by PCR with primers Bo3 and Bo104 from the corresponding phagemid vector. The purified PCR products were digested by NdeI and SpeI and cloned into pET-21b. Hybrid UbcH7 in fusion with the H1 helix from the C2 mutant of Ubc1 was constructed by amplifying the UbcH7 gene with primers Bo55 and Bo125 that encodes the mutated H1 helix of the C2. The PCR product was digested with SacI and SpeI and cloned into pET-21b. Similarly the C9-UbcH7 hybrid was constructed by PCR amplifying the UbcH7 gene with Bo55 and Bo126 that encodes the H1 helix of C9. Again the PCR product was digested with SacI and SpeI and cloned into pET-21b.

The RING domain of Mdm2 was PCR amplified from the Mdm2 gene with primers Bo150 and Bo152. The PCR fragment was digested with NedI/NotI, and cloned into the pGEX-4T plasmid as a fusion with an N-terminal GST tag. The gene of the HECT domain of Rsp5 was amplified by primers Kar30 and Kar32. After double digestion by the restriction enzymes SacI and SpeI, Rsp5 HECT was

cloned into pET-21b. The gene of the HECT domain of E6AP was amplified by primers Kar28 and Kar31, double digested by the restriction enzymes SacI and SpeI, and cloned into pET-21b.

Protein expression and purification.

The pET expression plasmids were transformed into BL21(DE3)pLysS chemical competent cells (Invitrogen) and plated on the LB-agar plates with appropriate antibiotics. Protein expression and purification followed the protocol provided by the vendor of the pET expression system (Novagen) and the Ni-NTA agarose resin (Qiagen).

Construction of the UB library in the pJF3H phagemid

For the construction of the UB library with randomized C terminal residues, we first mutated the wtUB gene in the pJF3H plasmid so that the five C-terminal residues corresponding to the sequence ^{71}LRLRG^{75} were all replaced by Ala. To construct the Ala mutant of UB, we amplified the wtUB gene with the Bo35 and Jun13 primers. The PCR fragment was digested with EcoRI and NotI and ligated into pJF3H to afford pJF3H-5Ala-UB. We then used the Ala mutant of UB as the template for PCR amplification with primers Jun13 and Bo 74. Bo74 primer has five NNK codons (MNN for the reverse compliment sequence) replacing the codons for residues ^{71}LRLRG^{75} at the UB C-terminus. The use of the Ala mutant instead of wtUB as the PCR template avoided the amplification of wt sequences in the PCR reaction that might lead to over-representation of wtUB clones in the library. After the PCR reaction, the amplified PCR fragment was digested by EcoRI and NotI. A large scale ligation was then set up with 20 μg of double digested PCR product ligating with 20 μg of double digested vector at a molar ration 5/1. The ligation reaction was incubated for overnight at 16°C. After transforming the ligated DNA into electro competent SS320 cells (Tonikian, et al., 2007), a library of 1×10^8 clones was acquired. We sequenced 20 clones and found 18 clones had randomized sequences at the intended positions and 2 clones were the PCR template pJF3H-5Ala-UB.

Phage preparation of the UB library

The phage library of UB was prepared following a protocol previously reported (Sunbul, et al., 2009). Briefly, *E. coli* SS320 cells infected with M13KO7 helper phage were transformed with the UB library in the pJF3H phagemid vector. The cell culture was shaken at 37°C overnight in 100 mL 2×YT that was supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin. The next day, the cells were pelleted by centrifugation, and the phage particles in the supernatant were PEG precipitated and resuspended in TBS buffer (20 mM TrisHCl, 150 mM NaCl, pH 7.5). Phage titrations were performed with *E. coli* XL1-blue cells with standard procedures (Barbas, et al., 2000; Kay, et al., 1996). Phages displaying wtUB and SV5V were prepared following the same procedure for the model selection.

Model selection of phage displayed UB

The fusion protein of PCP-Uba1 was first labeled with biotin-CoA catalyzed by Sfp phosphopantetheinyl transferase. The labeling reaction followed a documented protocol (Yin, et al., 2006; Yin, et al., 2004). Typically 100 μL labeling reaction was set up with 5 μM PCP-Uba1, 2 μM biotin-CoA, 0.3 μM Sfp in a buffer containing 10 mM $MgCl_2$ in 50 mM HEPES (pH 7.5). The reaction was allowed to proceed for 1 hour at 30˚C, and then mixed with 100 μL 3% BSA. 100 μL of the reaction mixture was distributed to each well of a 96-well plate coated with streptavidin. Biotin labeled PCP-Uba1 was incubated with the streptavidin plate for 1 hour at room temperature to allow binding of the Uba1 enzyme to the plate. The plate was then washed three times with TBS buffer (20 mM TrisHCl, 150 mM NaCl, pH 7.5) to remove the unbound enzymes and other components of the biotin labeling reaction.

For phage ELISA assays, 1×10^{11} UB displayed phage in 100 μL reaction buffer (50 mM HEPES, pH7.5, 50 mM $MgCl_2$, 1 mM ATP, 1.5% BSA) were added to each well of the streptavidin plate coated with PCP-Uba1 to initiate the transfer reaction of phage displayed UB to Uba1. Serial 10-fold dilutions of phage were performed in the neighboring wells with the reaction buffer. The UB activation reaction was allowed to proceed for 1 hour at room temperature. After the reaction, the plate was washed with TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, 0.05% Triton X-100, pH 7.5) for 6 times. To detect phage binding to the plate, a solution of 0.1 μg/mL mouse anti-M13 antibody - HRP conjugate

(Thermo Fisher Scientific) in 1% BSA/TBS was added to the plate (100 μL/well). After 1 hour incubation, the plate was washed with TBS-T for 6 times and a 1:1 mixture of tetramethylbenzidine (TMB) and hydrogen peroxide solution (Thermo Fisher Scientific) was added according to the manufacturer's protocol (Pierce). The ELISA plate was incubated at room temperature for 5 min before it was imaged.

For model selection with a mixture of wtUB and SV5V phage, wtUB and SV5V displayed phages were mixed at a ratio of 1/500 or 1/5000 in the reaction buffer. 100 μL of the phage mixture containing about 1×10^{11} phage was added to each well coated with PCP-wtUba1 fusion. The reaction was allowed to proceed for 1 hour at room temperature. The supernatant was discarded and the plate was washed 30 times with TBS-T and 30 times with TBS, each time with 200 μL of solution per well. After washing, phage bound to the streptavidin surface were eluted by adding 100 μL 10 mM dithiothreitol (DTT) in TBS. The eluted phage were combined, added to 10 mL of log phase *E. coli* XL1-Blue cells and shaken at 37°C for 1 hour to infect the cells. The cells were then plated on LB agar plates supplemented with 2% (w/v) glucose and 100 μg/mL ampicillin. After overnight incubation at 37°C, colonies on the plates were analyzed by colony PCR.

Molecular modeling

In silico mutagenesis of the A1 mutant of Uba1 and UB1 with the altered ^{71}FEFFGG^{76} C-terminus was carried out with COOT (Emsley, et al., 2010). Most of the altered side chains could be modeled by choosing one of the standard rotamers; slight adjustment of the side chain dihedral angles were carried out for residues Arg591 and Arg594 of Uba1-A1 as well as the Phe74 residue of UB1. No adjustments were made to the main chain of either protein. The UB-Uba1 complex (PDB entry 3CMM) contains two protomers in the asymmetric unit and protomer 2 (chains B and D) were chosen due to conformational changes in the vicinity of Arg594 and the fact that alterations of main chain atoms would have been necessary to introduce these changes in protomer 1.

ATP-PPi exchange assays

The initial velocity of E1-catalyzed UB activation was followed by the ATP-PPi exchange assay. Typically 50 μL reaction was set up containing 5 μM HA-UB, 0.5 μM E1, 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, and 1 mM ATP. The reaction was initiated by the addition of 1 mM sodium [^{32}P]pyrophosphate (4.6 Ci/mol). The reaction was incubated at room temperature and quenched at various time points by addition of 0.5 mL of a suspension of activated charcoal (1.6% (w/v) charcoal, 0.1 M tetrasodium pyrophosphate, and 0.35 M perchloric acid). The charcoal was pelleted by centrifugation. The charcoal pellet was washed with 1 ml 2% trichloroacetic acid for three times. Finally the charcoal pellet was resuspended in 0.5 ml water and the charcoal suspension was added to 3.5 ml Ultima Gold LSC-cocktail (PerkinElmer). The radioactivity bound to charchoal was determined by liquid scintillation counting.

To characterize the kinetics of E1 catalyzed activation of wtUB or UB mutants, initial velocities of ATP-PPi exchange were determined in the presence of 0.05 μM wtUba1 or the A5 mutant of Uba1 with varying concentrations of UB or xUB (UB10) from 0.05 μM to 5.0 μM. To measure the kinetics of UB or xUB activation in cross reactive wtUB-A5 or xUB-wtUba1 pairs, 0.5 μM wtUba1 or A5 was used and the concentrations of UB and UB10 were varied from 10 μM to 50 μM. The kinetic data were fitted to the Michaelis-Menten equation with the data analysis software Origin.

Construction of the Ubc1 library in the pComb3H phagemid

Ubc1 gene was cloned into the pComb3H phagemid by amplifying Ubc1 with primers Bo2 and Bo22 followed by restriction digestion with SacI and SpeI and ligation into pComb3H. For the construction of the Ubc1 library with randomized H1 helix residues, we first mutated the Ubc1 gene in the pComb3H plasmid so that the five residues to be randomized, Lys5, Arg6, Lys9, Glu10 and Gln12, were replaced by Ala. To create the Ala mutant of Ubc1, we amplified the Ubc1 gene with the Bo22 and Bo80 primers. The PCR fragment was digested with SacI and SpeI and ligated into pComb3H to afford pComb3H-5Ala-Ubc1. We then used the Ala mutant of Ubc1 as the template for PCR amplification with primers Bo81 and Jun14. The Bo81 primer has an NNK codon replacing the codons for residues Lys5, Arg6, Lys9,

Glu10 and Gln12 in the H1 helix of Ubc1. The use of the Ala mutant instead of wtUbc1 as the PCR template avoided the amplification of wild type sequences that might lead to over-representation of wtUbc1 clones in the library. After the PCR reaction, the amplified fragment was digested by SacI and SpeI. A large scale overnight ligation was then set up at 16°C with 20 μg double digested PCR product ligating with 20 μg double digested vector at a molar ration 5/1. After transformation into electro competent SS320 cells, a library of 1×10^8 clones was constructed. We sequenced 20 clones and found 19 clones had randomized sequences at the intended positions and 1 clone was the template Ubc1 with Ala mutations. Phage preparation of the Ubc1 library followed the same procedure for the preparation of UB displayed phage.

ybbR-UB labeling with biotin-CoA and Sfp phosphopantetheinyl transferase

Sfp catalyzed ybbR-UB labeling with biotin-CoA followed a reported protocol (Yin, et al., 2006). 100 μL of labeling reaction was set up with 25 μM ybbR-UB, 30 μM Biotin-CoA, 0.5 μM Sfp in a buffer containing 10 mM $MgCl_2$ in 50 mM HEPES (pH 7.5). The reaction was allowed to proceed for 1 hour at 30˚C. After the labeling reaction, the attachment of biotin to ybbR-UB was confirmed by MAIDI-TOF spectra.

Model selection of phage displayed Ubc1

UB transfer reaction with phage displayed Ubc1 was set up in a total volumn of 100 μL with 1 μM wtUba1, 5 μM biotin-UB, 2.5 mM ATP, 50 mM $MgCl_2$, and 1×10^{11} phage with Ubc1 and EntE displayed phages mixed at a ratio of 1/100 or 1/500 in TBS buffer (pH 7.5). The reaction was allowed to proceed for 1 hour at room temperature before it was added to 400 μL 3 % BSA in TBS buffer (pH 7.5). 100 μL of phage solution was distributed to each well of a 96-well plate coated with streptavidin. The streptavidin plate was incubated at room temperature for 1 hour. The supernatant was discarded and the plate was washed 30 times with 0.05% (v/v) Tween 20, 0.05% (v/v) Triton X-100 in TBS and 30 times with TBS, each time with 200 μL of solution per well. After washing, phage bound to the streptavidin surface were eluted by adding 100 μL of 10 mM dithiothreitol (DTT) in TBS. Eluted phage were

combined, added to 10 mL of log phase *E. coli* XL1-Blue cells and shaken at 37°C for 1 hour to infect the cells. The cells were then plated on LB agar plates supplemented with 2% (w/v) glucose and 100 µg/mL ampicillin. After overnight incubation at 37°C, colonies on the plates were analyzed by colony PCR.

Colony PCR

The identities of the colonies were assayed by colony PCR with primers Jun13 and Jun14. To perform colony PCR, 40 µL reaction mixture was prepared, containing 29.4 µL H_2O, 4 µL 10× Mg free PCR buffer (Promega), 4 µL 25 mM $MgCl_2$, 1 µL 10 µM primer Jun13, 1 µL 10 µM primer Jun14, 0.32 µL dNTP mix (25 mM each dNTP) and 0.2 µL Taq DNA polymerase (Promega). Pipette tips were used to transfer colonies from the agar plates to the PCR reaction mixture. The PCR reactions were run with the following program: 95°C for 10 minutes, then 35 cycles of 94°C for 1 minute, 50°C for 45 seconds, 72°C for 1.5 minute and a final step of 72°C for 5 minutes. For analysis, 20 µL of the PCR reaction was loaded on a 1% agarose gel and the PCR products were separated by electrophoresis.

Phage ELISA

The UB transfer reaction to Ubc1 displayed on phage was also analysed by phage ELISA. The UB transfer reaction with pure Ubc1 phage was set up as described in the model selection with controls in which Uba1, biotin-UB or ATP was excluded from the reaction. A 96-well streptavidin coated plate was blocked with 3% BSA in TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, 0.05% Triton X-100, pH 7.5) at room temperature for 1 hour. Serial 10× dilutions of the phage were performed in the 3% BSA/TBS-T solution and phage were incubated in the plate wells for 1 hour at room temperature to allow binding. The plate was then washed 6 times with TBS-T (0.3 mL/well). A solution of 0.1 µg/mL mouse anti-M13 antibody - HRP conjugate (Thermo Fisher Scientific) in 1% BSA/TBS was added to the plate (100 µL/well). After 1 hour incubation, the plate was washed with TBS-T for 6 times and a 1:1 mixture of tetramethylbenzidine (TMB) and hydrogen peroxide solution (Thermo Fisher Scientific) were added

according to the manufacturer's protocols (Pierce). The ELISA plate was incubated at room temperature for 5 min before it was imaged.

UB transfer reaction for Western blot analysis

The conditions of the UB transfer reactions in Figure 2 and 3A are the following. Figure 4 and Figure 5A: 10 μM HA-UB (wtUB or mutant UB) and 1 μM E1 (wtUba1 or mutant Uba1) were incubated with 1 mM ATP, 10 mM $MgCl_2$, and 50 μM DTT in TBS buffer (pH 7.5) for 1 hour at room temperature before SDS-PAGE and Western blot analysis. Figure 3B, 10 μM HA-UB (wtUB or UB10 mutant), 1 μM E1 (wtUba1 or A5 mutant), 5 μM UbcH5a, 5 μM CHIP were incubated with 1 mM ATP, 10 mM $MgCl_2$, 50 μM DTT in TBS buffer (pH 7.5) for 1 hour at room temperature before SDS-PAGE and Western blot analysis. UB transfer to the GST-Mdm2 RING domain fusion was done with the same condition. For UB transfer to the HECT domains of Rsp5 and E6AP in Figure 3D and 3E, 5 μM HA-UB (wtUB or UB10 mutant), 0.5 μM E1 (wtUba1 or A5 mutant), 3 μM Ubc1 or Ubc, 3 μM HECT domain were incubated with 1 mM ATP, and 10 mM $MgCl_2$ in TBS buffer (pH 7.5) for 1 hour at room temperature before SDS-PAGE and Western blot analysis. To cleave the UB~HECT thioester conjugate, DTT was added to the reaction mixture to the final concentration of 150 mM with the gel loading dye and the sample was boiled for 10 minutes before SDS-PAGE electrophoresis.

The conditions of the UB transfer reactions in Figure 4, 5, and 6 are as follows. Figure 4: 10 μM HA-UB, 1μM wtUba1 or A7, 5μM E2 (Ubc1, UbcH5, UbcH7 or Ubc1 mutants) were incubated with 1 mM ATP and 10 mM $MgCl_2$ in TBS buffer for 1 hour at room temperature before SDS-PAGE. Figure 5: 10 μM HA-UB, 1 μM E1 (wtUba1 or A7), 1 μM E2 (wtUbc1, UbcH7 or hybrid UbcH7 and UbcH5a), 5 μM HECT of E6AP were incubated with 1 mM ATP, 10 mM $MgCl_2$, 50 μM DTT in TBS buffer (pH 7.5) for 1 hour at room temperature before SDS-PAGE. Figure 6: 10 μM HA-UB (wtUB or xUB), 1 μM E1 (wtUba1 or xE1), 1 μM E2 (wtUbc1 or xE2 C9-Ubc1) were incubated with 1 mM ATP, 10 mM $MgCl_2$, 50 μM DTT in TBS buffer (pH 7.5) for 1 hour at room temperature before SDS-PAGE.

The conditions of the UB transfer reactions in Figure S7 are as follows. Figure S7B: 10 μM HA-UB, 1 μM E1 (wtUba1), 1 μM E2 (wtUbc1 or wtUbcH7), 5 μM HECT of E6AP were incubated with 1 mM ATP, 10 mM $MgCl_2$, 50 μM DTT in TBS buffer (pH 7.5) for 1 hour at room temperature before SDS-PAGE. Figure S7C-E: 10 μM HA-UB, 1 μM E1 (wtUba1 or Uba1-A7), 1 μM E2 (wtUbc1 or Ubc1 mutants), 5 μM HECT (E6AP or Rsp5) were incubated with 1 mM ATP, 10 mM $MgCl_2$, and 50 μM DTT in TBS buffer (pH 7.5) for 1 hour at room temperature before SDS-PAGE.

Western blot analysis of the UB transfer reaction

20 μL HA-UB transfer reaction was loaded on a 4-15 % SDS-PAGE gel (Bio-Rad). After electrophoresis, the protein bands were electroblotted onto a piece of polyvinylidene fluoride membrane (Bio-Rad). The membrane was blocked with 3% BSA in TBS buffer (pH 7.5) for 1 hour followed by incubation with 3 % BSA in TBS buffer (pH 7.5) containing 1:500 diluted 200 μg/mL anti-HA antibody (Santa Cruz Biotechnology) and 1:10,000 diluted anti-mouse horseradish peroxidase conjugate (Pierce) for 1 hour, respectively. The membrane was then washed 5 times by TBS-T buffer (0.05% (v/v) Tween 20, 0.05% (v/v) Triton X-100 in TBS, pH 7.5) and 5 times by TBS buffer (pH 7.5) followed by detection with the ECL luminescent detection kit (GE Healthcare).

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