Supporting Information

Site-specific protein ubiquitylation
using an engineered, chimeric E1 activating enzyme
and E2 SUMO conjugating enzyme Ubc9

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Table S1. Mutations observed in evolved chimeric E1 variants.\textsuperscript{a}

| Round 4 NNNK | Residue | Chimeric E1 clones |
|-------------|---------|--------------------|
|             | 1.1     | 1.2                |
|             | 1.3     | 1.4                |
|             | 1.5     | 1.6                |
|             | 2.1     | 2.2                |
|             | 2.3     | 2.4                |
|             | 2.5     | 2.6                |
|             | 2.7     | 2.8                |
|             | 2.9     | 2.10               |
|             | 2.11    | 2.12               |
|             | 2.13    | 2.14               |
|             | 2.15    | 2.16               |
|             |         | 3.1                |
|             |         | 3.2                |
|             |         | 3.3                |
|             |         | 3.4                |
|             |         | 4.1                |
|             |         | 4.2                |
|             |         | 4.3                |
|             |         | 4.4                |
|             |         | 4.5                |

| N566 |      |        |        |
| T571 |      |        |        |
| S577 |      |        |        |
| S579 | N     | N      | N      |
| T583 | S     |        |        |
| N599 |      |        |        |
| N607 | R     |        |        |
| N609 |      |        |        |
| D610 |      |        |        |
| N622 | D     | V      |        |
| N623 |      |        |        |
| T628 |      |        |        |
| R631 | E     |        |        |
| F632 |      |        |        |
| E634 | V     |        |        |
| T636 | A     |        |        |
| L637 |      |        |        |
| A640 | S     | T      |        |
| V647 |      |        |        |
| L655 |      |        |        |
| Q658 |      |        |        |
| N661 | L     |        |        |
| H672 |      | L      | L      |
| Q677 |      |        |        |
| S679 |      |        |        |
| N680 | S     |        |        |
| N681 |      |        |        |
| L685 | Q     |        |        |
| N688 | Y     | Y      | Y      |
| N689 |      |        |        |
| A699 |      |        |        |
| F700 |      |        |        |
| C704 |      |        |        |
| H708 |      |        |        |
| N709 |      |        |        |
| R713 |      |        |        |
| S715 |      |        |        |
| H717 |      |        |        |
| S719 |      |        |        |
| F720 |      |        |        |
| A724 |      |        |        |
| S726 |      |        |        |
| L728 |      |        |        |
| Y885 |      |        |        |
| I887 |      |        |        |
| I888 |      |        |        |
| T889 |      |        |        |
| E890 |      |        |        |
| Y891 |      |        |        |
| T892 |      |        |        |
| A893 |      |        |        |
| Y894 |      |        |        |
| V895 |      |        |        |
| S896 |      |        |        |

\textsuperscript{a}Residues that appear to be enriched are highlighted with gray fill. Star (★) marks residues subjected to saturation mutagenesis in the round 4.
Figure S1. β-Lactamase (BLA) exporting assay. ssTorA is a signaling peptide in the twin-arginine translocation (Tat) pathway.

Figure S2. Purification of ChE1 v4.5. Size exclusion chromatogram of purified ChE1 v4.5. The protein was eluted in 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM BME on Superdex 200 Increase 10/300 GL.
Figure S3. Michaelis-Menten curves for E2–Ub thioester formation. Reactions were carried out at 15 μL scale at 37 °C and pH 7.5, and quenched by 2x SDS-PAGE non-reducing sample buffer. The reactions contained 0.5 mM DTT, 0.5 U/mL IPP, 5 mM ATP, 10 μM Ub, and E1 and various concentration of E2. E1 concentrations and reaction times were as follows: (a) 10 nM ChE1 v4.5 and 3 min, (b) 500 nM ChE1 v0.2 and 15 min, (c) 5 nM Uba1 and 3 min, (d) 3 nM Uba1 and 3 min. Error bars are ± s.d. n = 3 independent experiments.

Figure S4. In-gel GFP fluorescence and Coomassie-stained SDS-PAGE of transfer of K63 hexaUb to GFP-I (Fig. 3g). Samples were not boiled to prevent smeared bands of polyUb, resulting in multiple bands and different mobility of GFP variants. The band marked with asterisk is likely to be mostly Ubc9–hexaUb.
Expression and in cellulo reaction

**Figure a**

Expression and in cellulo reaction

1. **Lysis**
2. **Amylose bound**
3. **1st Ni bound**
4. **Amylose FT**
5. **2nd Ni FT**
6. **Purified**

**Figure b**

**Tag K6**

| Mass (kDa) | Calc. | Obs. |
|------------|-------|------|
| 25-35      | 30565.7 | 30565.0 |
| 49         | 37694.6 | 37693.5 |

**Figure c**

**HRas K170**

| Mass (kDa) | Calc. | Obs. |
|------------|-------|------|
| 25-38      | 30565.7 | 30565.0 |
| 49         | 30727.0 |      |
Figure S5. Monoubiquitylation in *E. coli*. (a) Purification of monoubiquitylated substrates by successive Ni-NTA and amylose affinity chromatography. After the second Ni-NTA chromatography, the proteins were further purified by ion-exchange chromatography. *BD* and *FT* stand for bound fraction and flow through, respectively. (b, c) Coomassie-stained SDS-PAGE and anti-Ub western blot of the purification steps of GFP-I (b) and HRas (c) including arginine mutants as a negative control. (d, e) Deconvoluted ESI-MS of GFP-I–Ub (d) and HRas–Ub (e). HRas–Ub is partly glycosylated (+162 Da).
2. **General Information**

2.1. **Safety statement**

No unexpected or unusually high safety hazards were encountered.

2.2. **Synthetic methods and commercial materials**

Chemical reagents were purchased from Sigma Aldrich (Buchs, Switzerland), Acros Organics (Geel, Belgium), Fluorochem (Glossop, UK) and TCI Europe (Zwijndrecht, Belgium) and used without further purification. K63-linked hexaUb was obtained from Bio-Techne AG (Minneapolis, MN, USA). Milli-Q water was obtained from a Millipore purification system. Restriction endonucleases, Q5 R⃝ High-Fidelity DNA polymerases, Taq polymerase, T4 ligase and PCR reagents were purchased from New England BioLabs (Ipswich, MA, USA). DNase I and inorganic pyrophosphatase (200 U/mL) were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Lysozyme (22500 U/mg) was obtained from Axon Lab AG (Baden, Switzerland).

Gibson assembly master mix was prepared as reported. pRK793 was a gift from David Waugh (Addgene plasmid #8827) and was used to express the Tobacco etch virus (TEV) protease variant His6-TEVS219V-Arg7 as a maltose binding protein fusion as reported. DNA purification and gel extraction kits were purchased from Zymo Research (Irvine, CA, USA). Ampicillin sodium salt and kanamycin sulfate were obtained from AppliChem GmbH (Hilden, Germany), chloramphenicol form Hänseler AG (Herisau, Switzerland), carbenicillin from Apollo Scientific Ltd (Stockport, UK), and streptomycin from Sigma Aldrich. Ni-NTA agarose resin and amylose resin were obtained from Qiagen GmbH (Hilden, Germany) and New England BioLabs, respectively. Dialysis tubing and devices (SnakeSkinTM dialysis tubing, or Slide-A-Lyzer™ Mini dialysis devices for samples smaller than 2 mL, both with 3.5 kDa MWCO) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Amicon® Ultra centrifugal filters were purchased from Merck (Darmstadt, Germany), VivaSpin 500 centrifugal concentrators from Sartorius Stedim Lab (Stonehouse, UK), and PD Mini-/MidiTrap desalting columns from Cytiva. All buffers were prepared using Milli-Q water, pH adjusted for the temperature at which the buffer was used, and sterile-filtered (0.2 μm membrane filter). Oligonucleotide synthesis and sequencing were carried out by Microsynth AG (Balgach, Switzerland). Synthetic genes were purchased from Twist Bioscience (San Francisco, CA, USA).

2.3. **Gel electrophoresis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Mini-PROTEAN Tetra Cell system (Bio-Rad) connected to a PowerPac Basic (Bio-Rad) programmable power supply. Reducing samples were treated with an equal volume of sample buffer (Laemmli 2x Concentrate, Sigma Aldrich), placed in a heat block at 95 °C for 5 min and stored at –20 °C until separation. Non-reducing samples were treated with an equal volume of an in-house prepared buffer containing 100 mM Tris-HCl pH 6.8, 2% SDS, 4 M urea, 20% glycerol and 0.004% w/v bromophenol blue (2 x concentrated). PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo Fisher) was applied to at least one well of each gel (3 or 5 μL). Samples were separated on 8-16% gradient Mini-PROTEAN TGX Precast gels (Bio-Rad) for 30 min at 200 V. Gels were imaged on a Bio-Rad ChemiDoc MP Imaging System (Stain-
Free imaging, GFP fluorescence, Coomassie staining, and chemiluminescence. Images were cropped for illustration purposes. After in-gel fluorescence imaging, gels were stained with Coomassie (0.1% Coomassie Brilliant Blue R, 40% MeOH, 10% acetic acid) with gentle agitation for 1 h, followed by destaining (40% MeOH, 10% acetic acid). Densitometry analysis was performed using Bio-Rad Image Lab.

Agarose gel electrophoresis was carried out on a Sub-cell GT agarose gel electrophoresis system (Bio-Rad) connected to a PowerPac Basic (Bio-Rad) programmable power supply. Agarose gels were prepared using Tris-acetate-EDTA buffer and stained using GelRed (Biotium).

2.4. Western blotting

Samples were separated by SDS-PAGE and transferred to PVDF membranes using a Trans-Blot Turbo Transfer System (both BioRad). Membranes were blocked for 1 h with 5% skim milk in PBS with 1% Tween 20 (PBST), washed with PBST (3 x 5 min), and incubated with an antibody in PBST, 1% BSA. Membranes were washed with PBST (3 x 5 min) and images were developed using Clarity Western ECL chemiluminescence substrate (BioRad). Conditions for antibody incubation were as follows: Pierce High Sensitivity Streptavidin–HRP (Thermo Fisher Scientific) at a dilution of 1:5000 at rt for 1 h; anti-Ub (Cell Signaling) at a dilution of 1:6000 at 4 °C for 24 h; anti-rabbit IgG–HRP (Cell Signaling) at a dilution of 1:2000 at rt for 1.5 h.

2.5. Fast Protein Liquid Chromatography (FPLC)

Protein chromatography was performed on an ÄKTA pure chromatography system (Cytiva) at 4 °C. Cation exchange chromatography was performed Mono S 5/50 GL and HiTrap Capto S, anion exchange chromatography was performed using Mono Q 5/50 GL and HiTrap Capto Q, and size exclusion chromatography was performed using HiPrep 26/10 Desalting, Superdex 75 Increase 10/300 GL, Superdex 200 Increase 10/300 GL and HiLoad 16/600 Superdex 200. All columns were purchased from Cytiva. Protein elution was monitored at 280 and 260 nm.

2.6. Protein quantification and UV-Vis spectroscopy

Protein concentration and OD$\text{600}$ measurements were carried out on a NanoDrop 2000c UV-Vis spectrophotometer. Protein concentrations were determined by the absorption at 280 nm using extinction coefficients calculated by ProtParam<sup>48</sup> based on the amino acid sequence.

2.7. Characterization

High-resolution mass spectra were obtained by Functional Genomics Center Zurich on a Waters Synapt G2 QTOF.
3. Methods

3.1. Constructs

Full sequences of all newly prepared constructs are available in the supplementary Excel file. All constructs were verified by DNA Sanger sequencing.

pET-ChE1 v0.1. The sequence coding for N909-R1018 in pET-Uba1 (gift from Titia Sixma, Addgene #63571, UniProt P22314-2) was replaced by the sequence coding for V443-D549 from pET-Sae2 (gift from Frauke Melchior, Addgene #53117) by Gibson assembly.

pET-ChE1 v0.2. The sequence coding for F988-1018R in pET-Uba1 was replaced by the sequence coding for V450-D549 from pET-Sae2 by Gibson assembly. Other ChE1 variants from the directed evolution was cloned into a cut vector of this construct at KpnI and BsrGI by either Gibson assembly or restriction digest/T4 ligase ligation.

pET-Uba1ΔUFD. The sequence coding for M1-Y907 from pET-Uba1 was cloned into a pET-28 vector having N-terminal His6 tag and a TEV-cleavable sequence.

pET-GFP-I. This construct has been previously reported. Briefly, GSGPRKVIKMESEEGS was inserted into the GFP sequence after D173 in a pET vector. LACE tag K6R mutant was obtained by inverse PCR.

pSALECT-Ub:Ubc9-BLA:ChE1. A synthetic polycistronic gene coding for ssTorA-Ub:Ubc9-BLA:ChE1 v0.2 was cloned into pSALECT-EcoBam (gift from Christopher Anderson, Addgene #59705). ssTorA-Ub gene has a TEV-cleavable His tag after ssTorA, and Ubc9-BLA gene has an HA tag after Ubc9. The 3 genes are separated by internal ribosome biding sites. Further, KpnI site in the HA tag was deleted, and KpnI and XbaI sites were introduced at ChE1 G562 and L867 respectively by silent mutation to give pSALECT-Ub:Ubc9-BLA:ChE1-KpnI/XbaI.

pUb-His6-Ube9:ChE1 v4.5. To pSUMO1 (gift from Primo Schaer, Addgene #52258), genes of codon-optimized Ub with an N-terminal TEV-cleavable His tag, codon-optimized Ube9 K14R and ChE1 v4.5 were cloned after each of the three operons. The TEV-cleavable sequence is (M)GSSHHHHHHHDYDIPTTEENLYFQ/GAMGS (first Met processed during expression).

pSub-MBP-GFP(2M-IKQE). To pMAL-C2 vector, GFP-I gene having a TEV-cleavable linker (NGIEENLYFQ/GSGGSP) was cloned by Gibson assembly. The LACE tag was inserted in place of the LACE tag GSGPRKVMESEEGS. LACE tag K6R mutant was obtained by making PCR fragments having the mutation and ligating them back to a cut vector at BamHI and XhoI sites by Gibson assembly.

pSub-Asp6-MBP-HRas. The sequence of GFP-I in pSub-MBP-GFP(2M-IKQE) was replaced by a sequence of codon-optimized HRas (residues 1-189, UniProt P01112-1) and Asp6 tag was inserted to give an N-terminal sequence of MKTDIDDDDEEGKL. K170 mutant was obtained by making PCR fragments having the mutation and ligating them back to a cut vector at BamHI and XhoI sites by Gibson assembly.
3.2. Recombinant proteins

Ubiquitin was expressed in BL21-CodonPlus (DE3)-RIL cells and purified as previously reported. For other recombinant proteins, chemically competent BL21 (DE3) cells (GFP, Ub9, linear ubiquitins), or BL21-CodonPlus (DE3)-RIL cells (E1s, E2s except for Ub9) were heat-shock transformed with the plasmids and single colonies were used to inoculate overnight precultures in selective lysogeny broth (LB) Miller medium. Following 1:100 dilution with fresh selective LB Miller medium, cultures were grown in baffled shake flasks at 37 °C until an OD of approximately 0.6 was reached. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside at a final concentration of 0.25 mM. Expressions were carried out for 4 h at 30 °C (Ub9 and E2s), 4 h at 37 °C (Ub), overnight at 18 °C (GFP and E1s). Alternatively, the proteins were also expressed in autoinduction medium. In such cases, overnight precultures were diluted to 1:1000 and cells were grown overnight at 37 °C (GFP, E2, Ub, linear Ub), or 37 °C for 4 h and then 18 °C for 24 h (E1). Proteins were obtained in similar purity irrespective of expression media. After induction, cells were collected by centrifugation (4,500 x g, 30 min, 4 °C), resuspended in lysis buffer (50 mM HEPES pH 7.8, 350 mM NaCl, 20 mM imidazole) and stored at −80 °C until purification.

Uba1 and chimeric E1 variants. Cell suspensions were thawed at rt and placed on ice, supplemented with β-mercaptoethanol (BME, 5 mM), lysozyme (20 μg/mL), DNase I (0.1 mg/mL) and phenylmethysulfonflury fluoride (PMSF, 1 mM), and nutated at 4 °C for 30 min. Cells were lysed by sonication and the suspensions were cleared by centrifugation (16,000 x g, 45 min, 4 °C) and filtration (0.2 μm membrane filter). Supernatants were subjected to gravity Ni-NTA affinity purification using binding buffer (50 mM HEPES pH 7.8, 350 mM NaCl, 20 mM imidazole) and elution buffer (binding buffer containing 250 mM imidazole and 5 mM BME). Samples were either dialyzed or diluted to approximately 50 mM NaCl in 25 mM Tris pH 8.0 and then subjected to anion exchange chromatography.
(Mono Q 5/50 GL, 0.05 M to 1 M NaCl in 25 mM Tris pH 8.0), to obtain E1 variants in >80% purity by SDS-PAGE. Collected fractions were further purified by size exclusion chromatography (Superdex 200 Increase 10/300 GL or HiLoad 16/600 Superdex 200 depending on scale) in the reaction buffer (50 mM HEPES pH 7.5 at 37 °C, 50 mM KCl) supplemented with 1 mM TCEP and concentrated to ~10 μM by spin filtration (30 kDa MWCO). The samples were portioned into aliquots, flash-frozen in liquid N2 and stored at −80 °C for months without noticeable loss of activity. The purity of ChE1 v4.5 was verified by size exclusion chromatography (Superdex 200 Increase 10/300 GL) in an SEC buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM BME) (Fig. S2). Typical yield of ChE1 v4.5 was approximately 3 mg per liter cell culture.

**Ube2S-UBD, Cdc34, His6-Ubc9 variants.** The proteins were purified by Ni-NTA affinity purification as described for E1 variants and subjected to buffer exchange by diafiltration (10 kDa MWCO) in the reaction buffer (50 mM HEPES pH 7.5 at 37 °C, 50 mM KCl) supplemented with 1 mM DTT and concentrated to ~200 μM by spin filtration (10 kDa MWCO). The samples were portioned into aliquots, flash-frozen in liquid N2 and stored at −80 °C for months without noticeable loss of activity.

**Ubc13.** The protein was purified by GST affinity purification. After lysis and clarification as described for E1 variants, supernatant was applied to preequilibrated GST resin and the resin bed was washed with lysis buffer. The protein was eluted with an elution buffer (lysis buffer + 10 mM reduced glutathione), and dialyzed to 50 mM Tris pH 8.0 before the reconstitution of Ubc13/Mms2.

**Mms2.** The proteins were purified by Ni-NTA affinity purification as described for E1 variants and dialyzed to 50 mM Tris pH 8.0 before the reconstitution of Ubc13/Mms2.

**Ubc13/Mms2.** An equimolar mixture of Ubc13 and Mms2 was incubated at 4 °C for 1 h. The mixture was further purified by anion exchange chromatography (Mono Q 5/50 GL) using 40 mM Tris pH 8.0, 1 mM DTT. The protein was subjected to buffer exchange by diafiltration (10 kDa MWCO) into 50 mM HEPES pH 7.5 at 37 °C, 50 mM KCl, 1 mM DTT.

**AMSH*.** The protein was purified by Ni-NTA affinity purification as described for E1 variants, and further purified by size exclusion chromatography (HiLoad 16/600 Superdex 200) in 50 mM Tris pH 8.0, 200 mM NaCl and concentrated to ~60 μM by spin filtration (10 kDa MWCO). The sample was portioned into aliquots, flash-frozen in liquid N2 and stored at −80 °C for months without noticeable loss of activity.

**Sae1/Sae2.** The proteins were expressed separately, purified by Ni-NTA affinity purification as described for E1 variants, and further purified by size exclusion chromatography (HiLoad 16/600 Superdex 200) in 50 mM Tris pH 8.0, 200 mM NaCl. The proteins were concentrated to ~50 μM Sae1 and ~20 μM Sae2, and mixed in equimolar. The mixture was incubated at 4 °C for 4 h before purification by size exclusion chromatography (HiLoad 16/600 Superdex 200) in 50 mM HEPES pH 8.0, 50 mM KCl, 1 mM DTT and concentrated to ~10 μM. The sample was portioned into aliquots, flash-frozen in liquid N2 and stored at −80 °C for months without noticeable loss of activity.
**Ube2K.** The protein was purified by Ni-NTA affinity purification as described for E1 variants. Dialysis and His₆ tag cleavage were performed simultaneously in TEV cleavage buffer (50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM DTT) with TEV protease (1:30 w/w) at 4 °C overnight. The sample was applied to Ni-NTA resin and flow through was collected and diluted 20-fold with 25 mM Tris pH 8.0, 1 mM DTT. The protein was further purified by anion exchange chromatography (Mono Q 5/50 GL) using the same buffer, dialyzed against 50 mM HEPES pH 7.5, 50 mM KCl, 1 mM DTT, and concentrated to ~700 μM. The sample was portioned into aliquots, flash-frozen in liquid N₂ and stored at −80 °C for months without noticeable loss of activity.

**Ube2G1.** The protein was purified by Ni-NTA affinity purification as described for E1 variants. Dialysis and His₆ tag cleavage were performed simultaneously in TEV cleavage buffer (50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM DTT) and TEV protease (1:30 w/w) at 4 °C overnight. The sample was further purified by anion exchange chromatography (Mono Q 5/50 GL) with the same buffer, dialyzed against 50 mM HEPES pH 7.5, 50 mM KCl, 1 mM DTT, and concentrated to ~200 μM. The sample was portioned into aliquots, flash-frozen in liquid N₂ and stored at −80 °C for months without noticeable loss of activity.

**Ube9 (K14R).** The protein was purified by Ni-NTA affinity purification as described for E1 variants. Dialysis and His₆ tag cleavage were performed simultaneously in TEV cleavage buffer (50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM DTT) and TEV protease (1:20 w/w) at 4 °C overnight. Following buffer exchange using HiPrep 26/10 Desalting, the sample was further purified by cation exchange chromatography (Mono S 5/50 GL) with 25 mM HEPES pH 7.0, 1 mM DTT. The purified sample was exchanged to the reaction buffer (50 mM HEPES pH 7.5, 50 mM KCl, 1 mM DTT) and concentrated to ~200 μM by spin filtration (10 kDa MWCO). The sample was portioned into aliquots, flash-frozen in liquid N₂ and stored at −80 °C for months without noticeable loss of activity.

**SUMO2 (V10I).** Cells were lysed and spun down, and the supernatant was dialyzed against MilliQ-water with 1% AcOH, 30% MeCN. Precipitants were removed by centrifugation, and the supernatant was concentrated using spin filtration (10 kDa MWCO). The protein was further purified by size exclusion chromatography (HiLoad 16/600 Superdex 200) in an SEC buffer (50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT). Fractions were checked by SDS-PAGE, and fractions containing pure proteins were collected and concentrated to ~4 mM.

**Linear Ub.** Cells were lysed and spun down, and supernatants were precipitated with perchloric acid as described for Ub monomer.60 Supernatant was dialyzed against 50 mM NH₄OAc pH 4.5 overnight, and further purified by cation exchange chromatography (Mono S 5/50 GL) with a gradient of 0 to 0.5 M NaCl, 50 mM NH₄OAc pH 4.5. Purified sample was exchanged to reaction buffer (50 mM HEPES pH 7.5, 50 mM KCl, 1 mM DTT) and concentrated to ~300 μM by spin filtration (10 kDa MWCO).
3.3. Preparative Ub and SUMO chain formation

Based on previous reports, reactions were assembled in a Protein LoBind tube (Eppendorf) and carried out at 37 °C.

**K11 chain.** A reaction containing 0.5 μM Uba1, 30 μM Ube2S-UBD, 1 μM AMSH*, 2 mM Ub, 0.6 mM DTT, 10 mM ATP-Mg was incubated at 37 °C for 14 h. Another portion of the same amount of AMSH* was added to the reaction and the reaction was further incubated for 4 h. The reaction was diluted 20-fold into 50 mM NH₄OAc pH 4.5 prechilled at 4 °C, and purified by cation exchange chromatography (Mono S 5/50 GL) with a gradient of 0 to 0.5 M NaCl, 50 mM NH₄OAc pH 4.5 over 25 CV. Pooled fractions were concentrated by spin filtration (3 kDa MWCO) and further purified by size exclusion chromatography (Superdex 75 Increase 10/300 GL) in an SEC buffer (50 mM Tris pH 8.0, 150 mM NaCl). Pure fractions were collected and subjected to buffer exchange using the Amicon filters into the reaction buffer and concentrated to ~200 μM. The sample was portioned into aliquots, flash-frozen in liquid N₂ and stored at –80 °C.

**K48 chain.** A reaction containing 0.5 μM Uba1, 5 μM Cdc34, 1.5 mM Ub, 0.6 mM DTT, 10 mM ATP-Mg was incubated at 37 °C overnight (~26.5 h). The reaction was purified as described for K11 chain.

**K63 chain.** A reaction containing 0.5 μM Uba1, 5 μM Ubc13/Mms2, 1.5 mM Ub, 0.6 mM DTT, 10 mM ATP-Mg was incubated at 37 °C for 1.5 h. The reaction was purified as described for K11 chain.

**SUMO K11 chain.** A reaction containing 0.4 μM Sae1/Sae2, 40 μM Ubc9, 2 mM SUMO (V10I), 0.6 mM DTT, 10 mM ATP-Mg was incubated at 37 °C for 8 h. The reaction was purified by size exclusion chromatography (HiLoad 16/600 Superdex 200) in an SEC buffer (50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT). Pooled fractions were diluted 30-fold with 25 mM Tris pH 8.0 and further purified by anion exchange chromatography (Mono Q 5/50 GL) with a gradient of 0 to 0.5 M NaCl, 25 mM Tris pH 8.0. Pure fractions were dialyzed against the reaction buffer (50 mM HEPES pH 7.5, 50 mM KCl) supplemented with 1 mM DTT and concentrated to ~180 μM. The sample was portioned into aliquots, flash-frozen in liquid N₂ and stored at –80 °C.

3.4. β-Lactamase exporting assay (Fig. 2e)

Chemically competent DH5α cells were transformed with pSALECT plasmids and plated on LB plates supplemented with 25 μg/mL chloramphenicol. Single colonies were picked to inoculate 5 mL LB medium supplemented with 25 μg/mL chloramphenicol and 0.2% glucose and the cultures were incubated at 37 °C overnight (~19 h). The cultures were then normalized to OD 0.02 and 4 μL of each clone was spotted on LB plates supplemented with 25 μg/mL chloramphenicol and different concentrations of carbenicillin. Without supplementing 0.2% glucose in the overnight cultures, cells showed limited growth.

For screening purpose during the directed evolution, overnight cultures were diluted 100-fold with LB medium and spotted without normalization.
3.5. Directed evolution of chimeric E1 (Fig. 2e)

**General procedure.** Ligation products were purified by Zymo DNA clean and concentrator-5, and used to transform electrocompetent DH5α. The transformed cells were diluted 100-fold into LB medium supplemented with 0.2% glucose and 25 μg/mL chloramphenicol and grown at 37 °C overnight (~19 h) to reach OD 2.0–2.5. Cells were diluted 100-fold with LB medium and plated 100 μL each onto 10 cm selective LB plates containing 25 μg/mL chloramphenicol and appropriate concentration of ampicillin or carbenicillin and incubated at 30 °C for 2 days. Phenotype of the selected clones were checked again by spotting assay. The colonies were used to directly inoculate overnight cultures.

**Round 1 (epPCR).** An insert library of ChE1 v0.2 residues 570-873 was prepared by error-prone PCR (JBS error-prone kit, Jena Bioscience) using a template pSALECT-Ub:Ubc9-BLA:ChE1 and primers GPR-1. The insert library was gel-purified and ligated by Gibson assembly into a vector PCR-amplified using primers GPR-2 at 50 °C for 2 h. Insert 120 ng and vector 200 ng were used. Cells were selected on plates containing 32 or 64 μg/mL ampicillin.

In a separate experiment, an insert library and a cut vector were prepared using primers GPR-3 and GPR-4, respectively. Insert 100 ng and vector 160 ng were used for Gibson assembly. Cells were selected on plates containing 32 or 64 μg/mL ampicillin. The two experiments together, library size was 2 x 10⁵.

**Round 2 (epPCR).** An equimolar mixture of six clones from round 1 (ChE1 v1.1-1.6) as well as the original template ChE1 v0.2 were used as a template for error-prone PCR with primers GPR-5. pSALECT-Ub:Ubc9-BLA:ChE1-KpnI/XbaI was digested at KpnI and XbaI sites to obtain a cut vector. Insert 120 ng and vector 200 ng were used for Gibson assembly. Cells were selected on plates containing 150 μg carbenicillin. Library size was 2 x 10⁵.

**Round 3 (epPCR).** An equimolar mixture of 11 clones from round 2 (ChE1 v2.1-2.11) was used as a template for error-prone PCR with primers GPR-5. Insert 470 ng and vector 800 ng (same as round 2) were used for Gibson assembly. Cells were selected on plates containing 300-500 μg carbenicillin. Library size was 2 x 10⁷.

**Round 4 (saturation mutagenesis + gene shuffling).** For gene shuffling, the insert was PCR-amplified in five fragments (ChE1 residue number in parentheses): Fr1 (560-582), Fr2 (583-632), Fr3 (633-659), Fr4 (660-692), Fr5 (693-867). For the purpose of saturation mutagenesis, at residues 579, 688, 689, the reverse primers contained a degenerate MNN codon (reverse complement of NNK).

An equimolar mixture of four clones from round 3 (ChE1 v3.1-3.4) was used as a template for PCR amplification with Taq polymerase using primers GPR-6-10. The produced 5 fragments were mixed in equimolar and ligated by overlap extension PCR using Taq polymerase and primers GPR-6F and GPR-10R to give an insert library. Insert 510 ng and vector 800 ng (same as round 2) were used for Gibson assembly. Cells were selected on plates containing 300-500 μg carbenicillin. Library size was 9 x 10⁷.
Table S2. Primers for directed evolution.

| Name   | Forward (F, 5’-3’)                  | Reverse (R, 5’-3’)                  |
|--------|-------------------------------------|-------------------------------------|
| GPR-1  | gtgatccctctcctgcagagtcg             | gcacaaccttatacatgtggacacacacaaag   |
| GPR-2  | ccttggtgtctggacacttataaggaggacagtg | gcaaccttatacatgtggacacacacaaag     |
| GPR-3  | caggtggtgtctggaactgtataaggaggacagtg | gcaaccttatacatgtggacacacacaaag     |
| GPR-4  | ccttggtgtctggaactgtataaggaggacagtg | gcaaccttatacatgtggacacacacaaag     |
| GPR-5  | ctggagtcaggcaccaactg64              | gcaaccttatacatgtggacacacacaaag     |
| GPR-6  | aagccactgctggagtcagg                | ctcacccctctgcacaaactg64             |
| GPR-7  | tccagccaggaccacctg                  | ctcacccctctgcacaaactg64             |
| GPR-8  | cagaccccaagttgacagg                 | ctcacccctctgcacaaactg64             |
| GPR-9  | etgacccctcctggactgc                 | ctcacccctctgcacaaactg64             |
| GPR-10 | ctctgtgactgcagcagctg                | ctcacccctctgcacaaactg64             |

3.6. In vitro assays

**E2 thioester formation assay** *(Fig. 2d).* His<sub>6</sub>-Ubc9 Y134A was used to reduce potential steric clash between Ubc9 and Uba1 SCCH domain. Reactions containing 0.2 μM E1, 5 μM His<sub>6</sub>-Ubc9 Y134A, 5 μM biotin-Ub, 1 mM DTT, 5 mM ATP-Mg in reaction buffer (50 mM HEPES pH 7.5, 50 mM KCl) were incubated at 37 °C for 1 h and quenched with 2 x non-reducing SDS sample buffer.

**E2 thioester formation assay** *(Fig. 2g).* Microtubes containing 50 nM E1 in reaction buffer (50 mM HEPES pH 7.5, 50 mM KCl) were prewarmed at 37 °C in a PCR machine before the addition of 2 x master mix (final concentration: 0.5 mM DTT, 0.5 U/mL IPP, 5 mM ATP-Mg, 3 μM Ubc9 K14R, 30 μM Ub). Reactions were incubated at 37 °C for 4 min, and quenched with 2 x non-reducing SDS sample buffer. Conversion was estimated by densitometry analysis of Stain-Free imaging (5 min activation), using the same volume of 3 μM Ubc9 K14R as a standard.

**E2 thioester formation assay for determination of kinetic constants** *(Table 1).* Microtubes containing various concentration of E2 in reaction buffer (50 mM HEPES pH 7.5, 50 mM KCl) were prewarmed at 37 °C in a PCR machine before the addition of 2 x master mix (final concentration: 0.5 mM DTT, 0.5 U/mL IPP, 5 mM ATP-Mg, various concentration of E1, 10 μM Ub). The concentration of E1 and reaction time are shown in Table S3. Reactions were incubated at 37 °C, and quenched with 2 x non-reducing SDS sample buffer. Conversion was estimated by densitometry analysis of Stain-Free imaging (5 min activation), using a calibration curve obtained from 3 different concentrations of E2.

Table S3. Reaction conditions for E2 thioester assay.

| E1     | E2          | Reaction condition |
|--------|-------------|--------------------|
| ChE1 v4.5 | Ubc9 K14R  | 10 nM E1, 3 min    |
| ChE1 v0.2 | Ubc9 K14R  | 500 nM E1, 15 min  |
| Uba1   | Ube2K       | 5 nM E1, 3 min     |
| Uba1   | Ube2G1      | 3 nM E1, 3 min     |
**In vitro ubiquitylation** (Fig. 3). Reactions contained 15 μM substrate, 0.5 μM ChE1 v4.5, 30 μM His6-Ubc9 K14R, 75 μM mono- or oligo-Ub, 0.5 mM DTT and 5 mM ATP-Mg in 50 mM HEPES pH 7.5 and 50 mM KCl. For the transfer of K63 hexaUb (Fig. 3g), 7.5 μM substrate, 15 μM His6-Ubc9 K14R and 10 μM K63 hexaUb were used instead. Reactions were assembled on ice, carried out at 37 °C in a PCR machine and quenched with 2 x Laemmli buffer. Although it is known that boiling SDS-PAGE sample containing ubiquitin oligomers leads to formation of high molecular weight products, it was necessary to boil samples at 95 °C for 5 min to obtain a single band of GFP. For the transfer of K63 hexaUb (Fig. 3g), SDS-PAGE samples were not boiled.

### 3.7. Monoubiquitylation in E. coli (Fig. 4)

BL21-CodonPlus (DE3)-RIL cells were heat-shock cotransformed with both pSub (pSub-MBP-GFP(2M-IKQE) or pSub-Asp6-MBP-HRas) and pUb-His6-Ubc9:ChE1 v4.5 and plated on LB plates containing 100 μg/mL ampicillin and 25 μg/mL streptomycin. Single colonies were picked to inoculate overnight LB cultures supplemented with 100 μg/mL ampicillin, 25 μg/mL streptomycin and 25 μg/mL chloramphenicol. The overnight cultures were diluted 1000-fold into autoinduction media containing 100 μg/mL ampicillin and 25 μg/mL streptomycin and incubated at 30 °C for 20 h. Cells were collected by centrifugation (4,500 x g, 30 min, 4 °C), resuspended in lysis buffer (50 mM HEPES pH 8.0, 350 mM NaCl, 20 mM imidazole, 10% glycerol) and stored at –80 °C until purification. Cell suspensions were thawed at rt and placed on ice, supplemented with 5 mM BME, 20 μg/mL lysozyme, 0.1 mg/mL DNase I and nutated at 4 °C for 30 min. Cells were lysed by sonication and the suspensions were cleared by centrifugation (20,000 x g, 45 min, 4 °C) and filtration (0.2 μm membrane filter). Supernatants were subjected to gravity Ni-NTA affinity purification using binding buffer (50 mM HEPES pH 8.0, 350 mM NaCl, 20 mM imidazole, 10% glycerol), washed with binding buffer, wash buffer 1 (50 mM HEPES pH 8.0, 1 M NaCl, 20 mM imidazole), binding buffer again, and eluted with elution buffer (50 mM HEPES pH 8.0, 350 mM NaCl, 250 mM imidazole, 5 mM BME). Eluents were then applied to amylose resin preequilibrated with binding buffer (20 mM Tris pH 7.4, 200 mM NaCl, 1 mM EDTA, 5 mM BME) and washed with the same buffer. The resin was resuspended in TEV buffer (20 mM Tris pH 8.0, 200 mM NaCl, 0.5 mM EDTA, 5 mM BME) and TEV protease (1:5 w/w) was added. The suspension was nutated at 4 °C overnight (~20 h) before applied to an empty column to collect flow through. The resin was washed with 1 CV of amylose wash buffer and the eluent was combined with the flow through. Proteins bound on the resin was eluted with elution buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 10 mM maltose, 5 mM BME). The combined flow through and wash fraction was incubated with Ni-NTA resin at 4 °C for 0.5 h to remove TEV protease and supernatant was collected. The proteins were further purified by ion exchange chromatography: cation exchange chromatography (Mono S 5/50 GL, 50 mM NH4OAc pH 4.5) for GFP-I–Ub and anion exchange chromatography (Mono Q 5/50 GL, 50 mM Tris pH 8.0) for HRas–Ub. The proteins were diluted 20-fold with the respective buffer before loaded onto the columns. Pure fractions were collected and concentrated by spin filtration (10 kDa MWCO) to ~10 μM. Typical yields were found to be approximately 0.5 mg per liter culture for GFP-I–Ub and 2 mg per liter culture for HRas–Ub.
4. Supplementary References

46. Rabe, B. A.; Cepko, C. A Simple Enhancement for Gibson Isothermal Assembly. *bioRxiv* 2020, 2020.06.14.150979.

47. Kapust, R. B.; Tözsér, J.; Fox, J. D.; Anderson, D. E.; Cherry, S.; Copeland, T. D.; Waugh, D. S. Tobacco Etch Virus Protease: Mechanism of Autolysis and Rational Design of Stable Mutants with Wild-Type Catalytic Proficiency. *Protein Eng. Des. Sel.* 2001, 14, 993–1000.

48. Gasteiger, E.; Hoogland, C.; Gattiker, A.; Duvaud, S.; Wilkins, M. R.; Appel, R. D.; Bairoch, A. Protein Identification and Analysis Tools on the ExPASy Server. In *The Proteomics Protocols Handbook*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 2005; pp 571–607.

49. Pichler, A.; Knipscheer, P.; Oberhofer, E.; van Dijk, W. J.; Körner, R.; Olsen, J. V.; Jentsch, S.; Melchior, F.; Sixma, T. K. SUMO Modification of the Ubiquitin-Conjugating Enzyme E2-25K. *Nat. Struct. Mol. Biol.* 2005, 12, 264–269.

50. Hofmann, R.; Akimoto, G.; Wucherpfennig, T. G.; Zeymer, C.; Bode, J. W. Lysine Acylation Using Conjugating Enzymes for Site-Specific Modification and Ubiquitination of Recombinant Proteins. *Nature* 2007, 447, 1135–1138.

51. Werner, A.; Moutty, M.-C.; Möller, U.; Melchior, F. Performing In Vitro Sumoylation Reactions Using Recombinant Enzymes. In *SUMO Protocols*; Ulrich, H. D., Ed.; Humana Press: Totowa, NJ, 2009; pp 187–199.

52. Brzovic, P. S.; Lissounov, A.; Christensen, D. E.; Hoyt, D. W.; Klevit, R. E. A UbcH5/Ubiquitin Noncovalent Complex Is Required for Processive BRCA1-Directed Ubiquitination. *Mol. Cell* 2006, 21, 873–880.

53. Studier, F. W. Stable Expression Clones and Auto-Induction for Protein Production in E. Coli. In *Structural Genomics: General Applications*; Chen, Y. W., Ed.; Humana Press: Totowa, NJ, 2014; pp 17–32.

54. Pickart, C. M.; Raasi, S. Controlled Synthesis of Polyubiquitin Chains. In *Methods in Enzymology*; Academic Press, 2005; Vol. 399, pp 21–36.
62. Dong, K. C.; Helgason, E.; Yu, C.; Phu, L.; Arnott, D. P.; Bosanac, I.; Compaan, D. M.; Huang, O. W.; Fedorova, A. V.; Kirkpatrick, D. S. et al. Preparation of Distinct Ubiquitin Chain Reagents of High Purity and Yield. *Structure* **2011**, *19*, 1053–1063.

63. Tatham, M. H.; Jaffray, E.; Vaughan, O. A.; Desterro, J. M. P.; Botting, C. H.; Naismith, J. H.; Hay, R. T. Polymeric Chains of SUMO-2 and SUMO-3 Are Conjugated to Protein Substrates by SAE1/SAE2 and Ubc9. *J. Biol. Chem.* **2001**, *276*, 35368–35374.

64. Olsen, S. K.; Lima, C. D. Structure of a Ubiquitin E1-E2 Complex: Insights to E1-E2 Thioester Transfer. *Mol. Cell* **2013**, *49*, 884–896.