

Supporting Information

Dual-Labeling of Ubiquitin Protein by Chemoselective Reactions for Sensing UCH-L3

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Including the Experimental Section and Figures S1-S9, Schemes S1-S2.

Experimental

General Procedures and Materials

All chemicals were purchased from Aldrich, Sigma and Acros and used without further purification if not specified. 9-fluornylmethoxycarbonyl (Fmoc) amino acids and peptide synthesis reagents were purchased from GL Biochem. Ni-NTA was purchased from Bio-Red. SUMO-protease was prepared as described¹. The ¹H and ¹³C NMR spectra were measured on a JEOL 400MHz magnetic resonance spectrometer. ESI-MS was performed on an Agilent 1200 and series chromatography system equipped with a LCQ ESI mass spectrometer. Analytical gel filtration chromatography was performed on AKTA purifier 10 instrument equipped with desalting column HiPrep™ 26/10 and anion exchange column Mono Q™ 5/50 GL. The peptide was purified by Shimadzu 2010 HPLC and characterized by Waters 600. All SDS-PAGE analysis and fluorescence SDS-PAGE analysis in Fig. 2 were performed on Gel Doc™ XR+ imaging System. The circular dichroism was detected by Chirascan plus in 20 mM PB buffer (pH=7.4) at room temperature. The fluorescent SDS-PAGE analyses about the cleavage of Coumarin-Ub-FAM **7** by UCH-L3 were performed on Typhoon Trio+. Fluorescence spectra measurements were performed on Hitachi High-Technologies Corporation F-4500. All fluorescence measurements were monitored at 37°C in HEPES buffer (pH=8.0). All signals were corrected by subtracting the corresponding buffer signal.

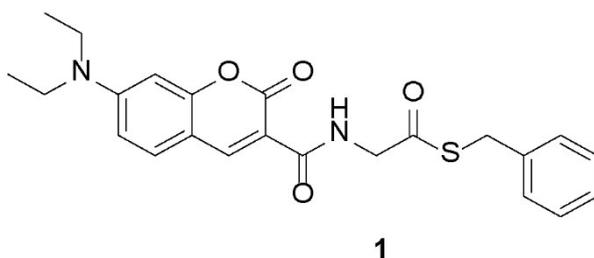
Synthesis of Coumarin thioester **1**

We prepared the compound **1** as reported².

¹H NMR (400 MHz, DMSO) δ ppm: 1.14 (t, J=6.18 Hz, 6 H), 3.44 - 3.55 (m, 4 H), 4.10 (s, 2 H), 4.29 (d, J=4.58 Hz, 2 H), 6.63 (br. s., 1 H), 6.82 (d, J=9.16 Hz, 1 H), 7.20 - 7.33 (m, 5 H), 7.66 - 7.72 (m, 1 H), 8.68 (s, 1 H), 9.17 (t, J=5.04 Hz, 1 H).

¹³C NMR (400 MHz, DMSO) δ ppm: δ 198.25 163.55 162.07 157.99 153.26 148.92 138.18 132.38 129.33 129.04 127.67 110.80 108.86 108.18 96.40 49.75 44.91 32.25 12.85.

ESI-MS *m/z* for coumarin thioester **1**: [M+H]⁺ calcd 425.2, found 425.8.

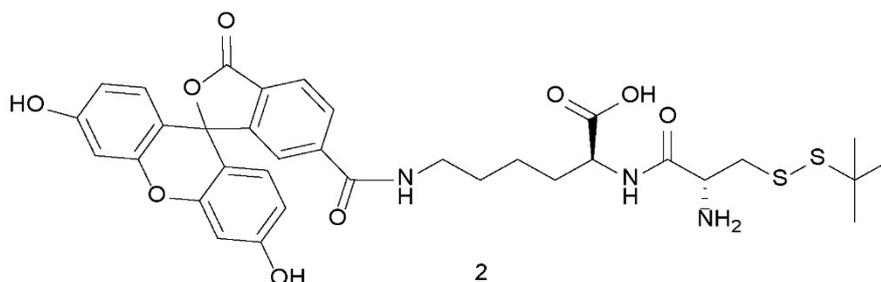


Synthesis of NH₂-Cys-Lys (FAM)-COOH 2

The NH₂-Cys-Lys (FAM)-COOH 2 was prepared by standard Fmoc method.

ESI-MS *m/z* for NH₂-Cys-Lys (FAM)-COOH 2: [M+H]⁺ Calcd.696.2, found 696.5.

Analytical HPLC condition: C18 reversed-phase column. 25% B-75% B in 30 min, A: 0.06% aqueous TFA, B: 80% acetonitrile; flow rate: 0.8 ml/min; monitored at 215 nm. *t_R*: 15 min.



Expression and purification of SUMO-Ub thioester 4

The Ub76-intein-CBD gene was amplified by PCR using forward primer (5'-CGGGATCCATGCATCACCATCACCATCACG-3') and reverse primer (5'-CCGCTCGAGTCATTGAAGCTGCCACAAG-3'). The amplified Ub76-intein-CBD gene was inserted into the pET28a SMT3 vector by BamH1 and Xho1 sites. Then, the SUMO's C terminal serine was mutated to cysteine by using forward primer (5'-ATGCAGATCTTCGTGAAAACCCTTAC-3') and reverse primer (5'-GCATCCACCAATCTGTTCTCTG-3'). The generated plasmid was transformed into *E. coli* BL21 (DE3) codon plus cells and the culture was grown at 37°C until the absorbance at 600 nm reached 0.6-0.8. IPTG was added to a final concentration of 0.5 mM and induction was performed at 16°C for 20 h. Cells were harvested by centrifugation and washed once in wash buffer (pH 7.5, 50 mM Hepes, 500 mM NaCl). The bacterial pellet was resuspended in lysis buffer (50 mM Hepes, pH 7.5, 500 mM NaCl). Then the fresh portion of 0.5 mM PMSF and Triton X-100(1% (v/v) final

concentration) were added. The bacteria cells were lysed by ultrasonification (40 min, on ice). The 40 ml chitin beads were equilibrated with lysis buffer containing 1% (v/v) Triton X-100 with 2 L of fresh LB medium handled. The mixture was incubated with the equilibrated chitin beads for 2 h at 4°C. Then the chitin beads were washed by 5-fold column volume lysis buffer. The SUMO-ubiquitin thioester **4** was generated by using 500 mM MESNA to cyclic washing the chitin beads for 24 h at 4°C. After that, the MESNA solution containing SUMO-Ub thioester **4** was concentrated to an appropriate concentration and changed to low desalting buffer by ultrafiltration device (10K)³. The SUMO-Ub thioester **4** was shock frozen and stored at -80°C.

Ligation of NH₂-Cys-Lys (FAM)-COOH **2 with SUMO-Ub thioester **4** to produce SUMO-Ub-FAM **5****

SUMO-Ub thioester **4** (50 μM) was ligated with NH₂-Cys-Lys (FAM)-COOH **2** (final concentration 2 mM) in ligation buffer (50 mM Hepes, 500 mM NaCl, 200 mM TCEP, 120 mM MPAA, pH=7.5) for 5 h at 16°C. After NH₂-Cys-Lys (FAM)-COOH **2** was attached to SUMO-Ub thioester **4**, unreacted small molecules were removed using desalting column HiPrep™ 26/10 pre-equilibrated with the buffer (20 mM Hepes, 10 mM NaCl, pH=7.5).

Cleavage of SUMO tag from SUMO-Ub-FAM **5 by SUMO protease Ulp to produce Ub-FAM **6****

SUMO-Ub-FAM **5** (30 μM) was treated with SUMO protease Ulp (50 nM) in cleavage buffer (20 mM Hepes, 10 mM NaCl, pH=7.5) at 16°C overnight. Then, the SUMO tag and Ulp were removed in the buffer (20 mM Hepes, 10 mM NaCl, pH=7.5) using Ni-NTA.

Ligation of Coumarin thioester **1 with Ub-FAM **6** to produce Coumarin-Ub-FAM **7****

Ub-FAM **6** (20 μM) was ligated with Coumarin thioester **6** (final concentration 2 mM) in ligation buffer (50 mM Hepes, 500 mM NaCl, 200 mM TCEP, 250 mM MPAA, pH=7.0) for 5 h at 37°C.

Purification of Coumarin-Ub-FAM **7**

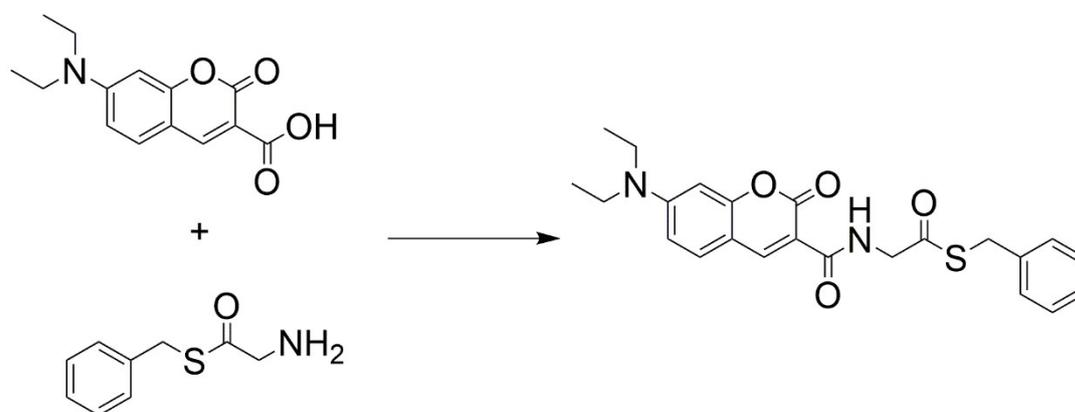
After Coumarin thioester **1** was attached to Ub-FAM **6**, unreacted small molecules were removed using desalting column HiPrep™ 26/10 pre-equilibrated with the buffer (20 mM Hepes, 10 mM NaCl, 1 mM DTT, pH=8.0). Then, anion exchange was performed on AKTA 10 equipped with column Mono Q™ 5/50 GL in the low concentration buffer (20 mM Hepes, 10 mM NaCl, 1 mM DTT, pH=8.0) and high concentration buffer (50 mM Hepes, 1 M NaCl, 1 mM DTT, pH=8.0).

CD spectroscopy measurements

CD spectra was detected by Chirascan plus from 260 to 190 nm in a 0.1 cm path length cell at room temperature. The Coumarin-Ub-FAM **7** was dissolved in 20 mM PB buffer (pH=7.4) with a final concentration of 20 μ M. The results are the average of 5 recorded scans and plotted as mean residue ellipticity $[\theta]$ (degrees $\text{cm}^2 \text{dmol}^{-1}$).

Monitoring the cleavage of Coumarin-Ub-FAM **7** by UCH-L3 in the presence or absence of RA-9 using Hitachi High-Technologies Corporation F-4500

The Coumarin-Ub-FAM **7** was dissolved in Hepes buffer (20 mM Hepes, 50 mM NaCl, 1 mM DTT, pH=8.0) at a final concentration of 20 μ M. Excitation was 400 nm and emission was recorded from 420 nm to 620 nm.



Scheme S1 Synthesis of coumarin thioester **1**.

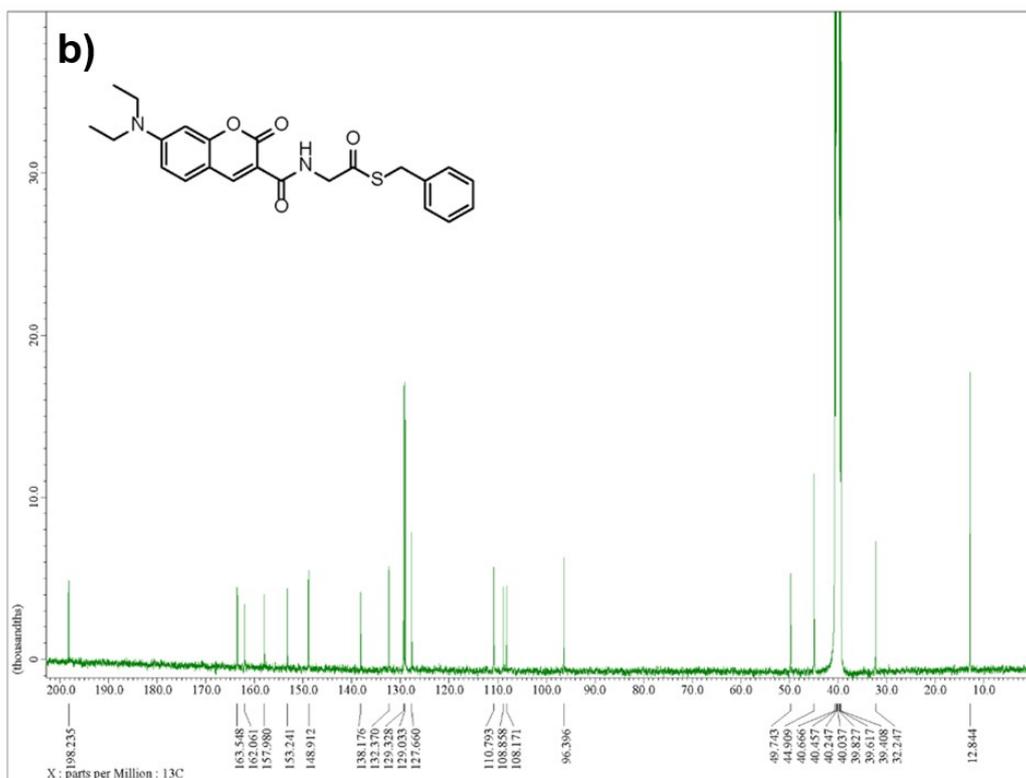
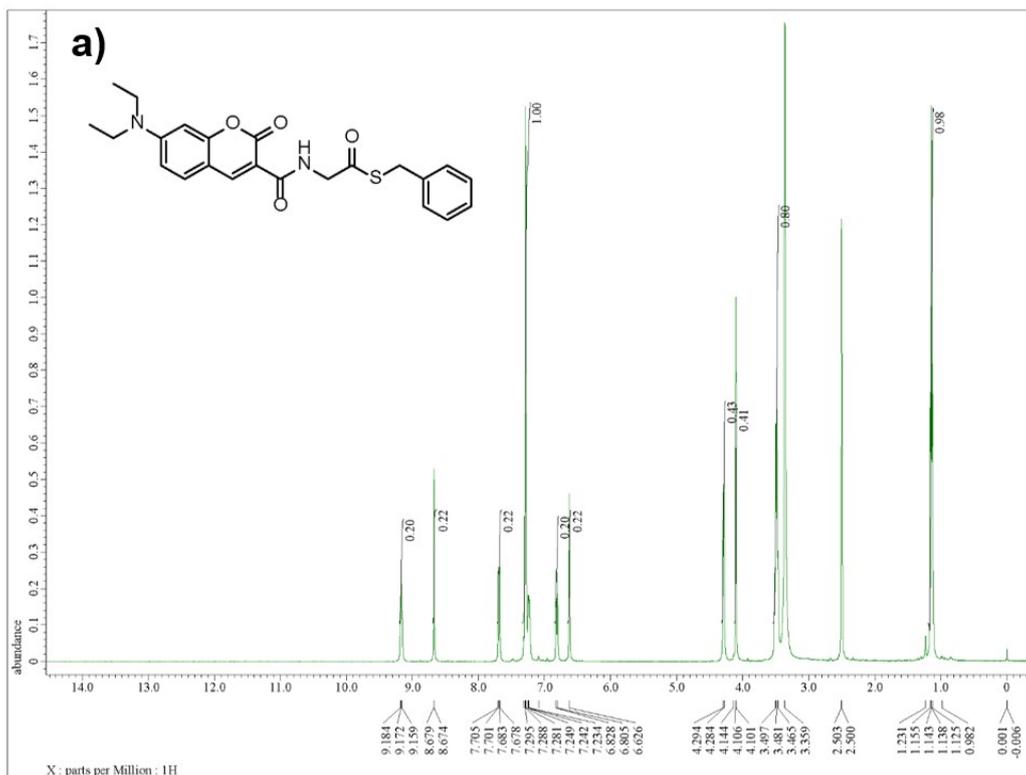


Fig. S1 NMR spectra of Coumarin thioester **1**. a): ^1H NMR; b): ^{13}C NMR.

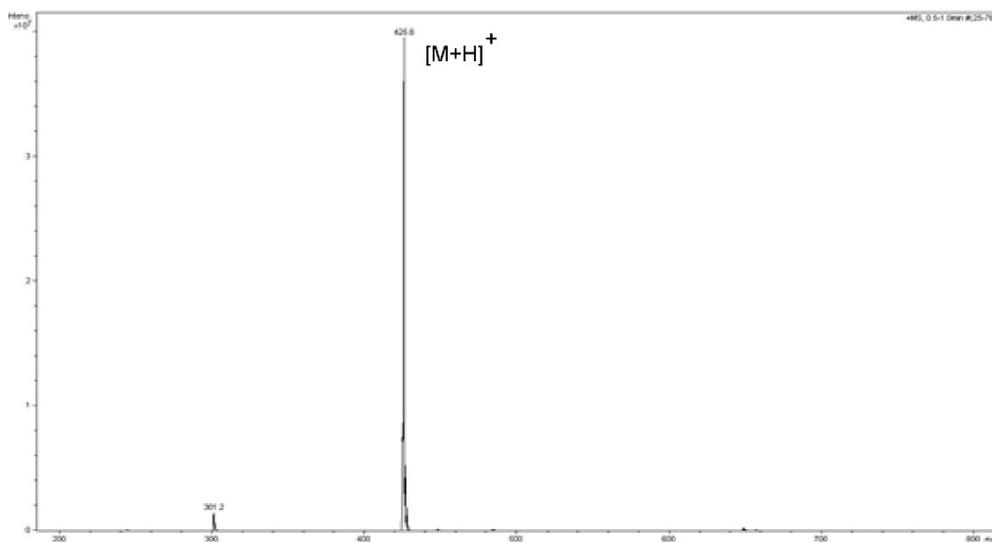
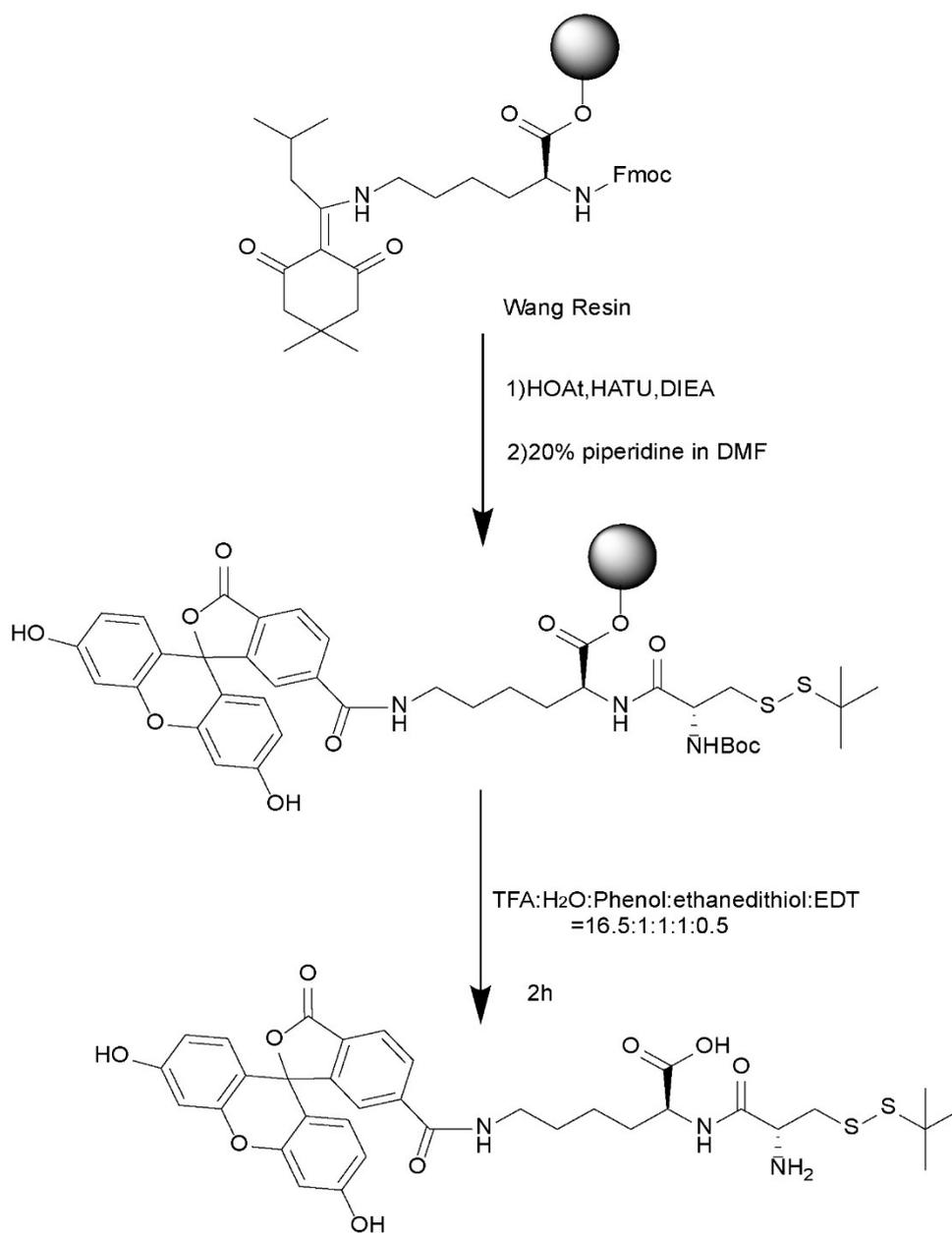


Fig. S2 ESI-MS of coumarin thioester **1** ESI-MS m/z for **1** $[M+H]^+$ Calcd.425.2, found 425.8.



Scheme S2 Synthesis of NH₂-Cys-Lys (FAM)-COOH **2**.

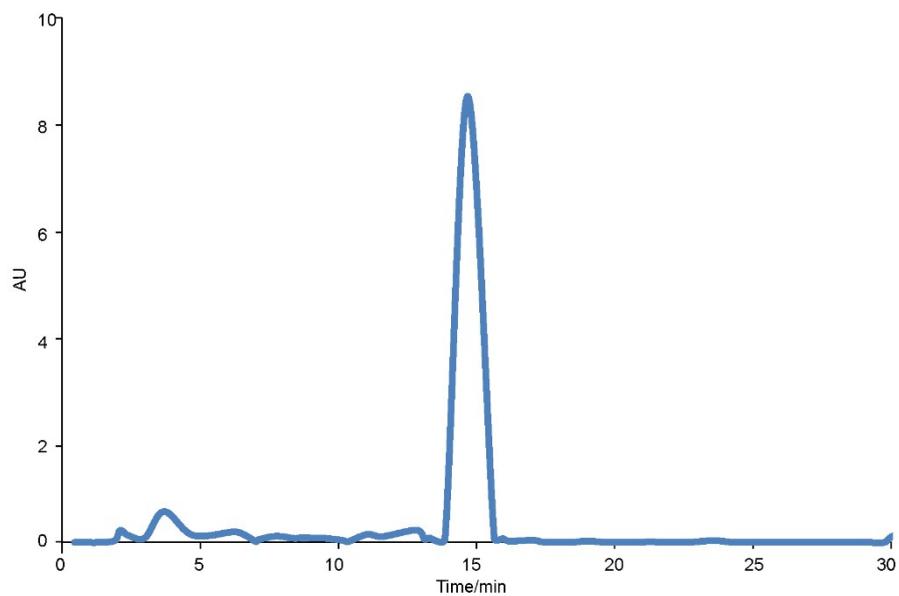


Fig. S3 Analytical HPLC plot of $\text{NH}_2\text{-Cys-Lys (FAM)-COOH 2}$. Monitored at 215 nm.
 Conditions: C18 reversed-phase column. 25%B to 65% B in 30 min. A: 0.06% aqueous TFA; B: 80% acetonitrile; flow rate: 0.8 ml/min.

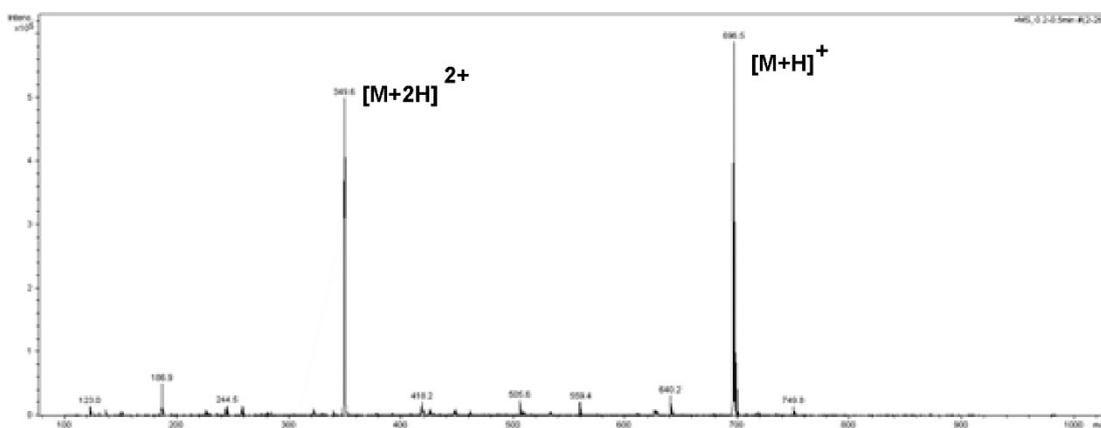


Fig. S4 ESI-MS of $\text{NH}_2\text{-Cys-Lys (FAM)-COOH 2}$. ESI-MS m/z for $2 [M+H]^+$
 Calcd.696.2, found 696.5.

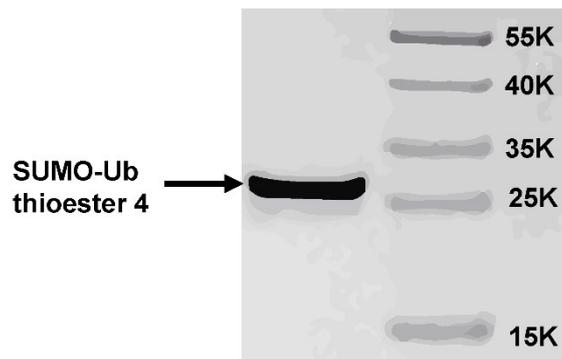


Fig. S5 SDS-PAGE analysis of SUMO-Ub thioester 4.

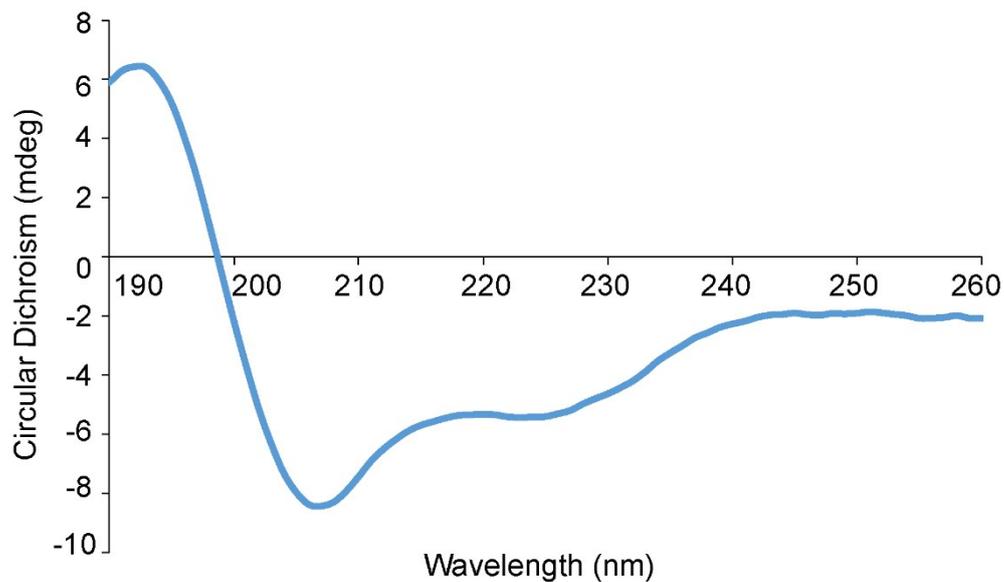


Fig. S6 The CD spectrum of Coumarin-Ub-FAM 7.

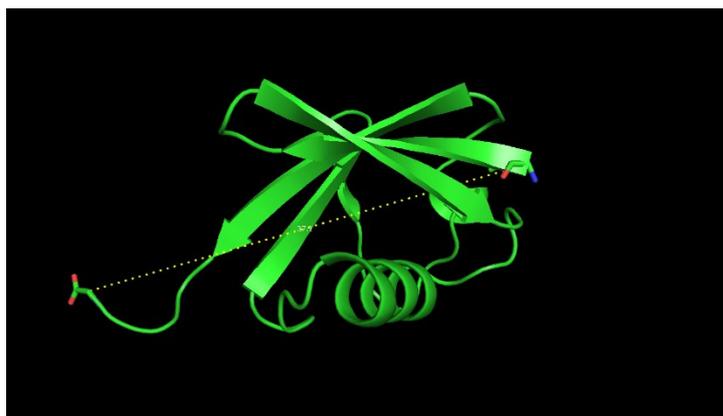


Fig. S7 The distance (~3.7 nm) from the C-terminus to N-terminus of Ub. The model of the Ub structure (PDB code: 1UBQ) was generated by PyMOL software.

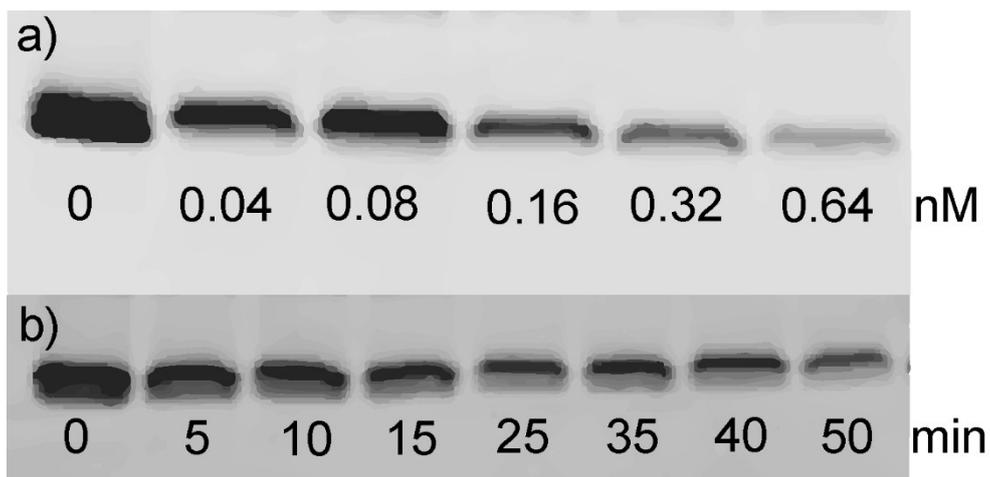


Fig. S8 Fluorescence images of SDS-PAGE results about cleavage of Coumarin-Ub-FAM 7 by UCH-L3. a) 20 μ M of Coumarin-Ub-FAM 7 was cleaved by different concentration of UCH-L3 for 120 min. Excitation wavelength: 488 nm. b) 20 μ M of Coumarin-Ub-FAM 7 was cleaved by 0.32 nM UCH-L3 for different time. Excitation wavelength: 488 nm.

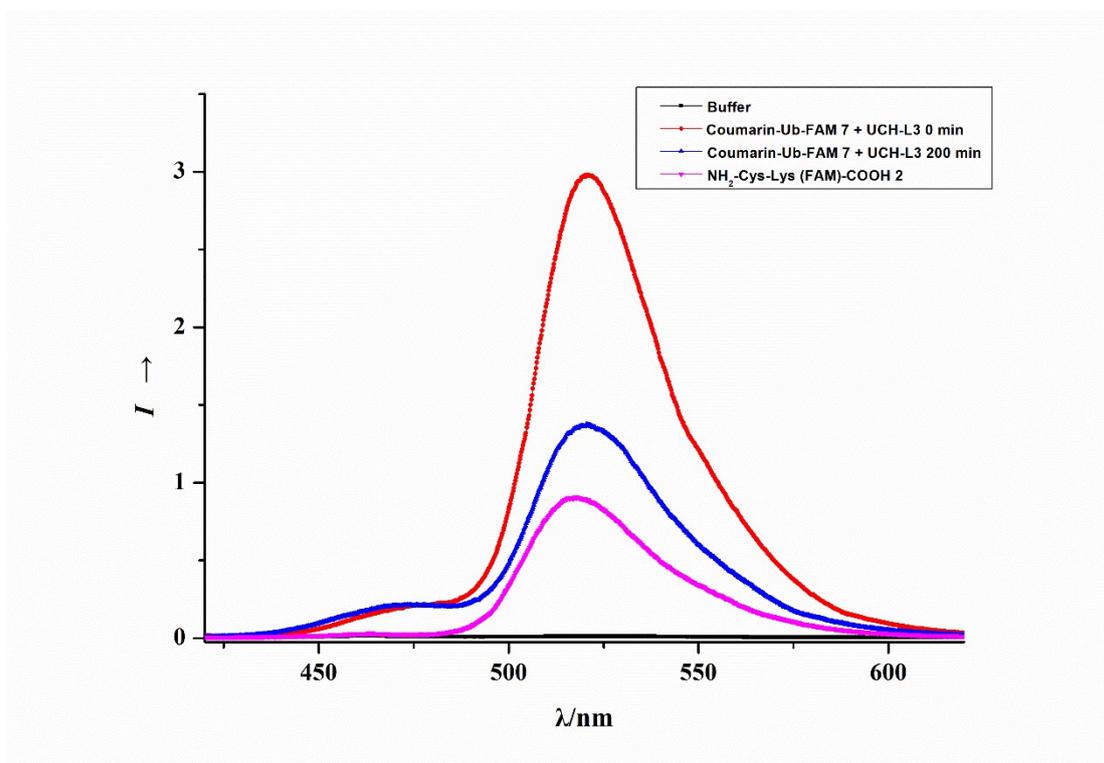


Fig. S9 Fluorescence emission spectra of NH₂-Cys-Lys (FAM)-COOH 2 (20 μ M) and Coumarin-Ub-FAM 7 (20 μ M). Excitation wavelength: 400 nm.

References

1. S. Zheng, J. Wang, Y. Feng, J. Wang and K. Ye, *Plos One*, 2012, **7**, e45437.

2. D. S. Yeo, R. Srinivasan, M. Uttamchandani, G. Y. Chen, Q. Zhu and S. Q. Yao, *Chem. Commun.*, 2003, 2870-2871.
3. Y. X. Chen, S. Koch, K. Uhlenbrock, K. Weise, D. Das, L. Gremer, L. Brunsveld, A. Wittinghofer, R. Winter, G. Triola and H. Waldmann, *Angew. Chem.In. Ed.*, 2010, **49**, 6090-6095.