Supporting Information for

A Photocaged Cell-Permeable Ubiquitin Probe for Temporal Profiling of Deubiquitinating Enzymes

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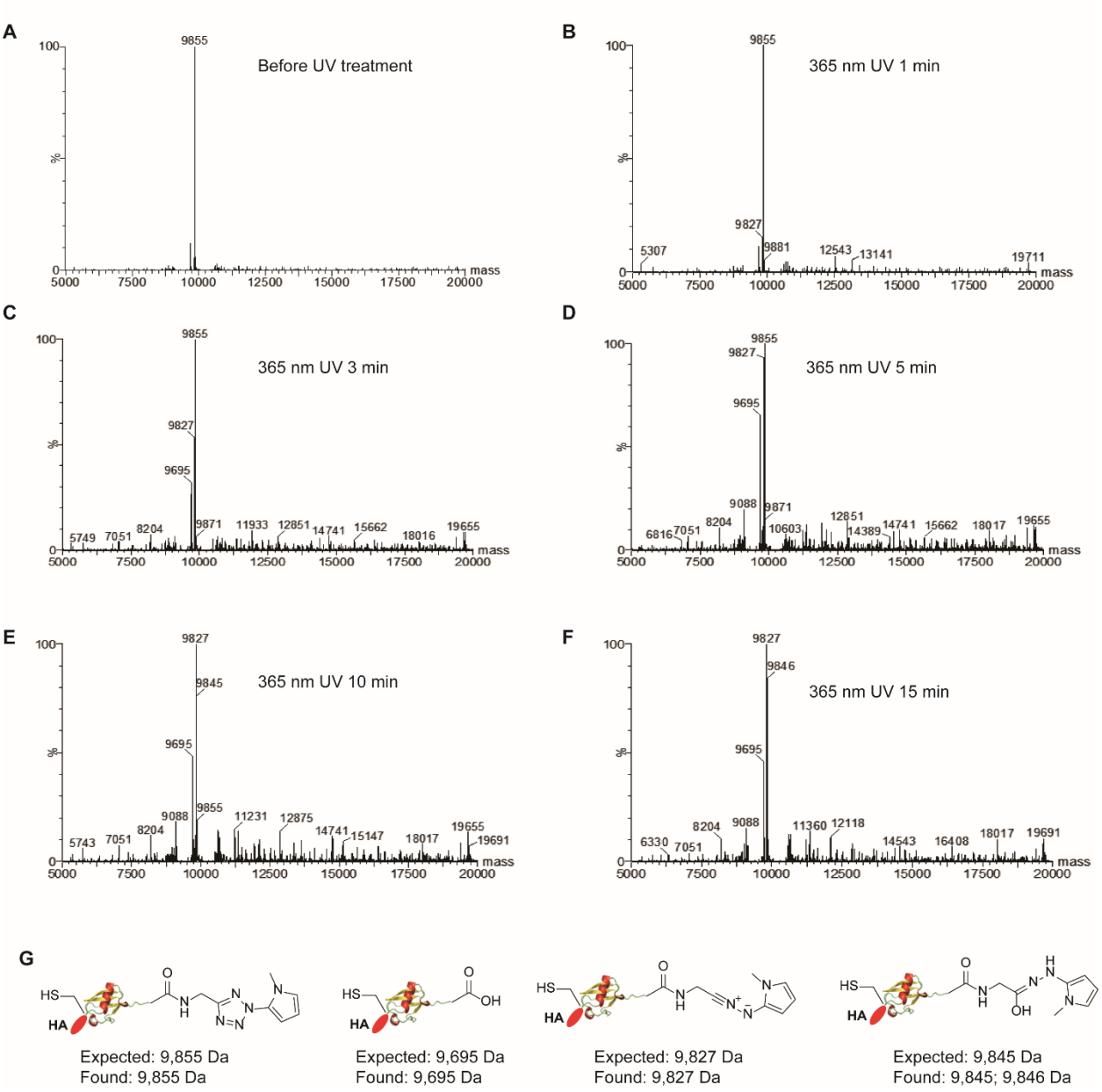


Figure S1. ESI-MS characterization of time dependent 365 nm UV irradiation of HA-Cys-Ub-TZ. A) ESI-MS characterization of 15 μM HA-Cys-Ub-TZ before UV irradiation; B-F) ESI-MS characterization of 15 μM HA-Cys-Ub-TZ UV irradiated at 365 nm for 1, 3, 5, 10, 15 mins, respectively; G) Assignment of the major peaks found in the ESI-MS spectrometry.

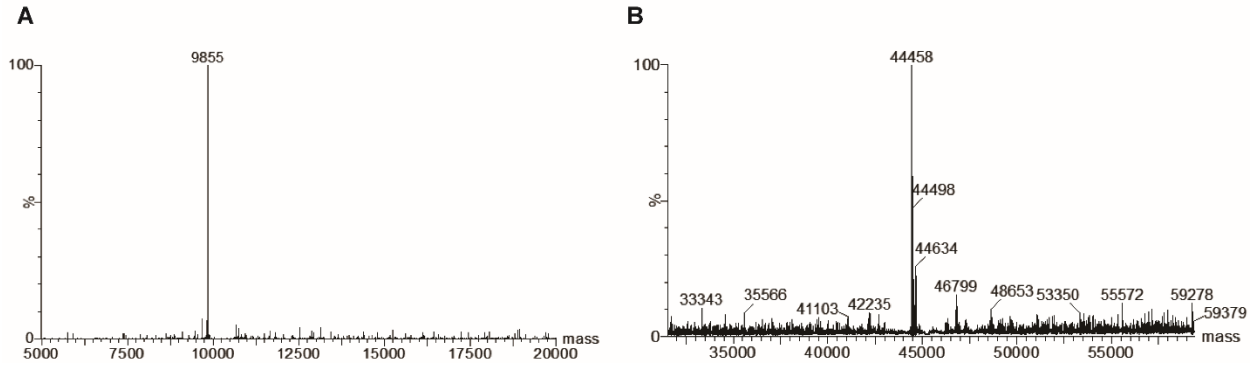


Figure S2. ESI-MS characterization of HA-Cys-Ub-TZ and HA-Cys-Ub-TZ incubated with USP2-CD without UV irradiation. A) ESI-MS characterization of HA-Cys-Ub-TZ; B) ESI-MS characterization of USP2-CD incubated with HA-Cys-Ub-TZ without UV irradiation.

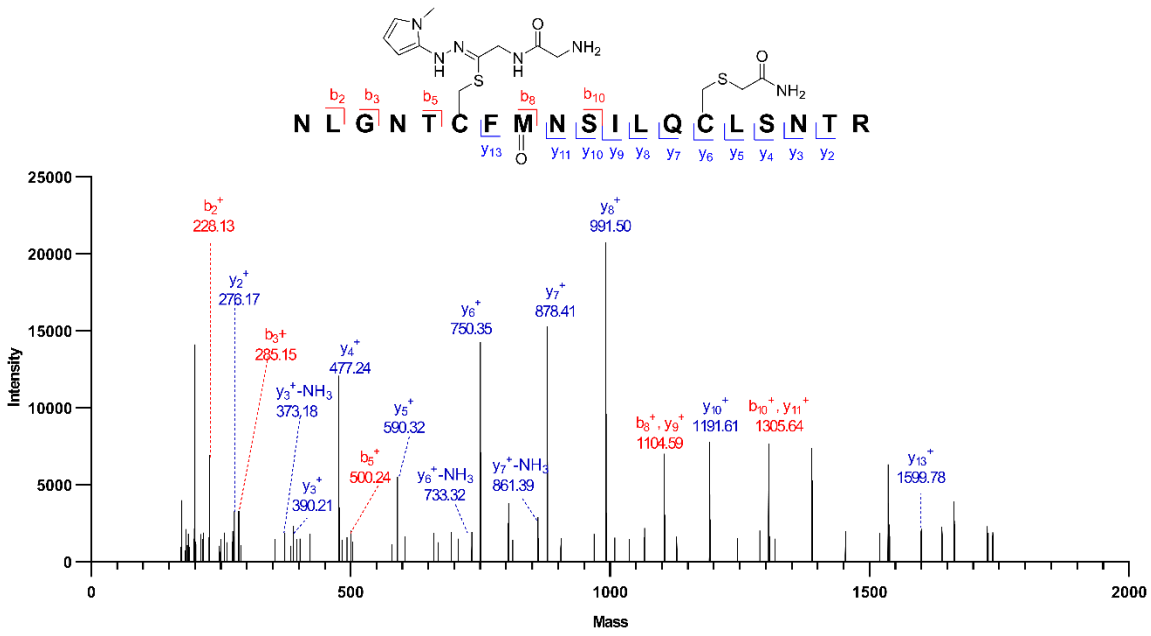


Figure S3. Nano-flow LC-MS/MS analysis of the labeled USP2-CD by HA-Cys-Ub-TZ probe after trypsin digestion. High-energy collision dissociation MS2 spectrum showing the identification of active site cysteine on USP2-CD modification by tetrazole probe peptide fragment.

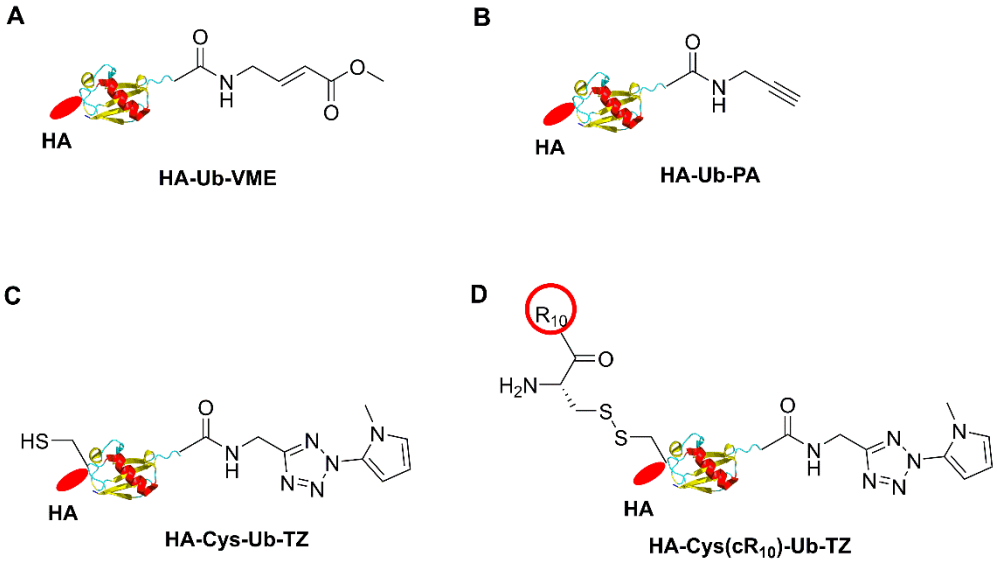


Figure S4. Structure of ubiquitin-based probes. A) Structure of probe HA-Ub-VME; B) Structure of probe HA-Ub-PA; C) Structure of probe HA-Cys-Ub-TZ; D) Structure of probe HA-Cys(cR_{10})-Ub-TZ.

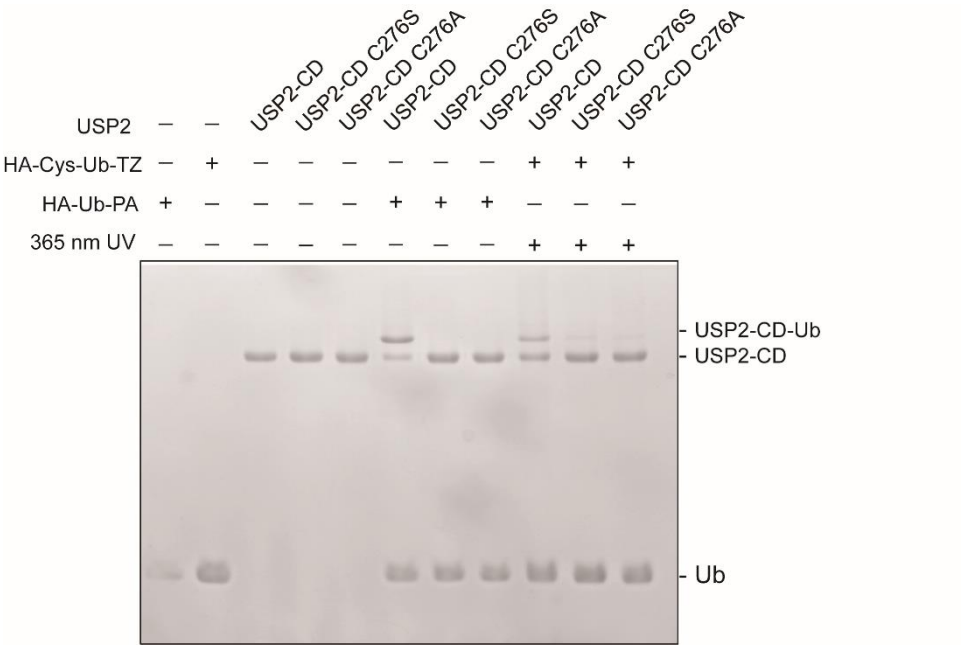


Figure S5. Wild-type USP2-CD and USP2-CD mutants labeling by 15 μM HA-Cys-Ub-TZ, analyzed using 20% SDS-PAGE gel and stained with Coomassie brilliant blue. HA-Ub-PA probe was used for a comparison.

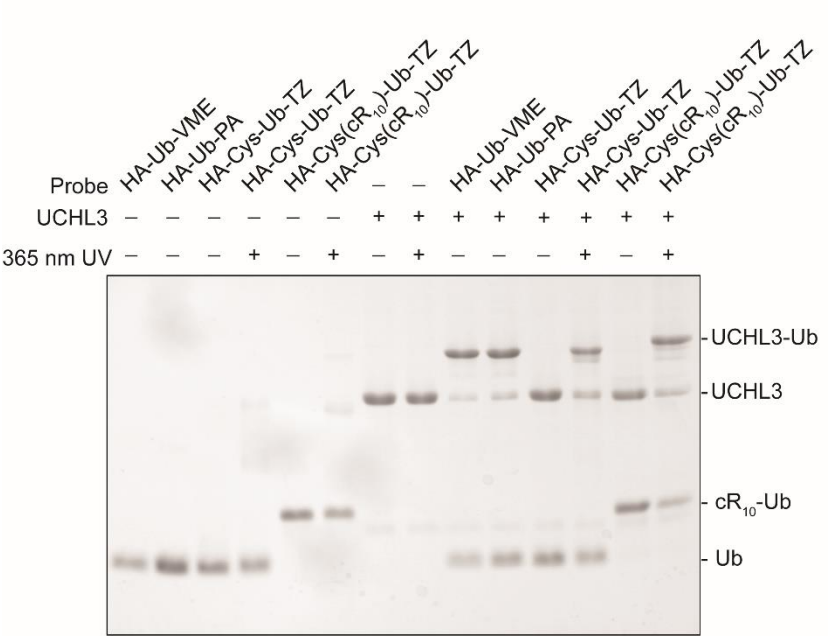


Figure S6. UCHL3 labeling by 15 μM Ub-TZ probes, analyzed using 20% SDS-PAGE gel and stained with Coomassie brilliant blue. HA-Ub-VME and HA-Ub-PA were used as a comparison.

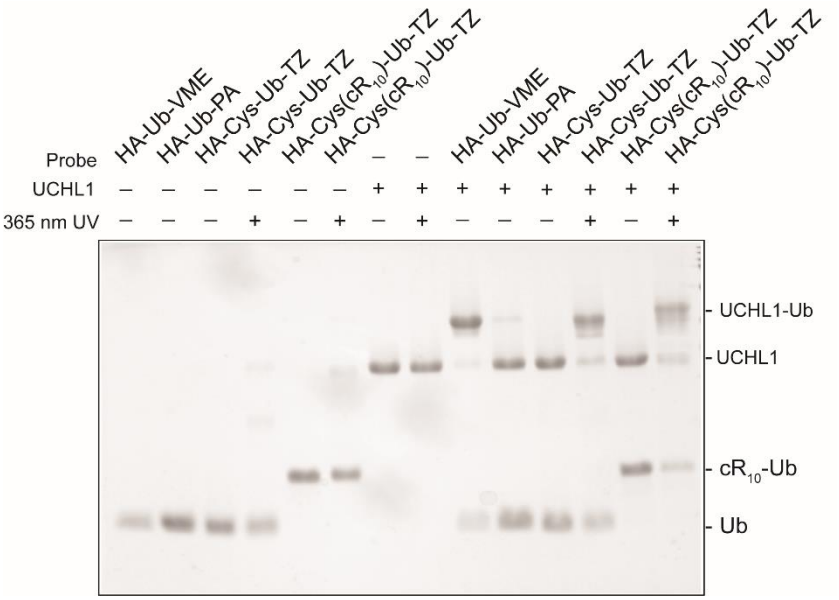


Figure S7. UCHL1 labeling by 15 μM Ub-TZ probes, analyzed using 20% SDS-PAGE gel and stained with Coomassie brilliant blue. HA-Ub-VME and HA-Ub-PA were used as a comparison.

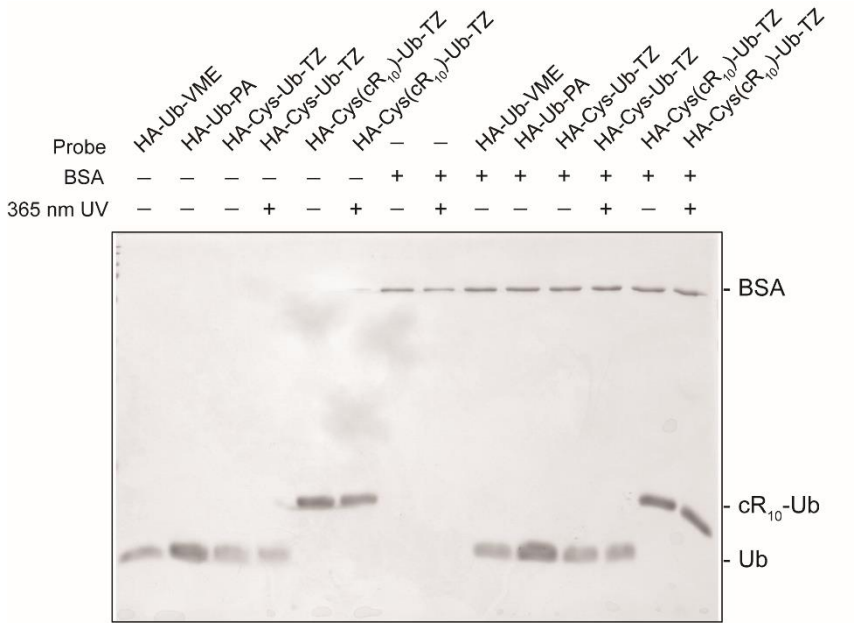


Figure S8. BSA labeling by 15 μM Ub-TZ probes, analyzed using 20% SDS-PAGE gel and stained with Coomassie brilliant blue. HA-Ub-PA and HA-Ub-VME were used as comparison.

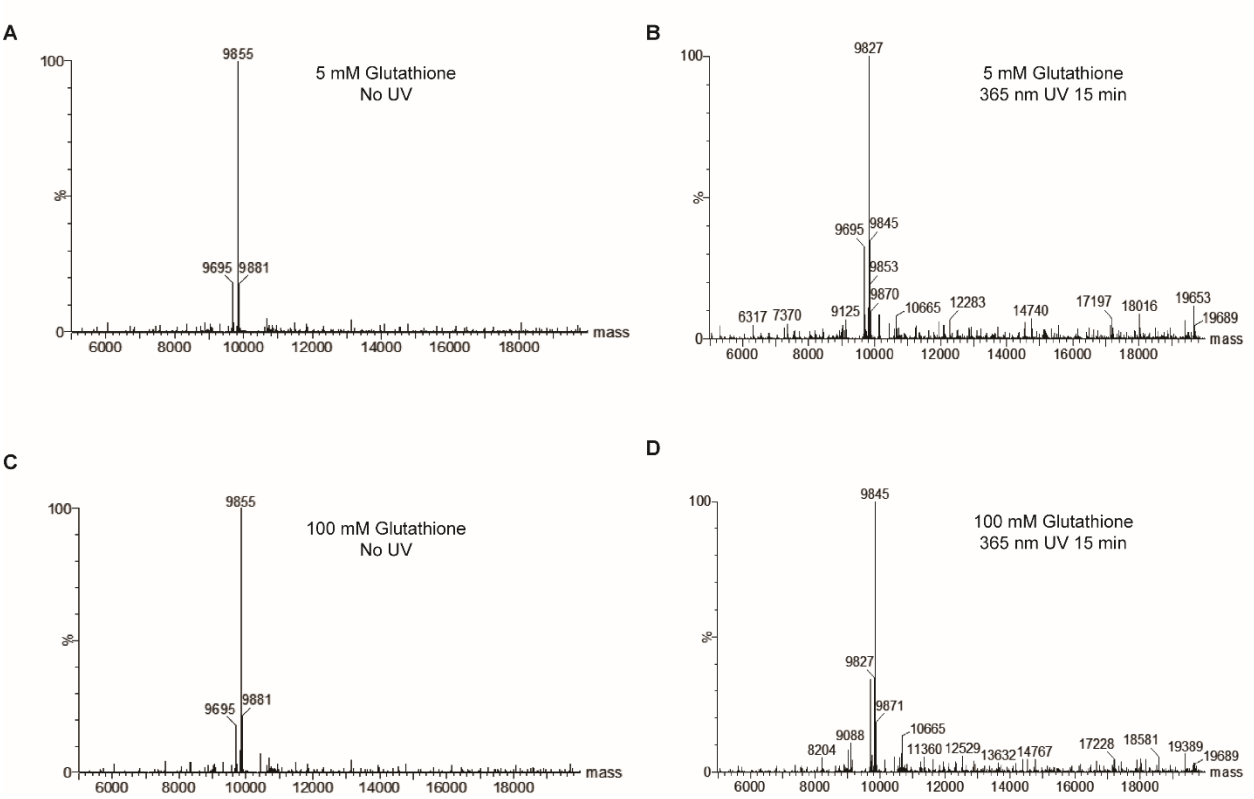


Figure S9. ESI-MS characterization of HA-Cys-Ub-TZ probe incubated with glutathione upon 365 nm UV irradiation. A) ESI-MS characterization of 15 μM HA-Cys-Ub-TZ incubated with 5 mM glutathione without UV irradiation; B) ESI-MS characterization of 15 μM HA-Cys-Ub-TZ incubated with 5 mM glutathione with 365 nm UV irradiation for 15 min; C) ESI-MS characterization of 15 μM HA-Cys-Ub-TZ incubated with 100 mM glutathione without UV irradiation; D) ESI-MS characterization of 15 μM HA-Cys-Ub-TZ incubated with 100 mM glutathione with 365 nm UV irradiation for 15 min. 9,827 Da corresponds to the nitrilimine intermediate HA-Cys-Ub-NM and 9,845 Da corresponds to the hydroxylation product of nitrilimine (HA-Cys-Ub-NM-OH).

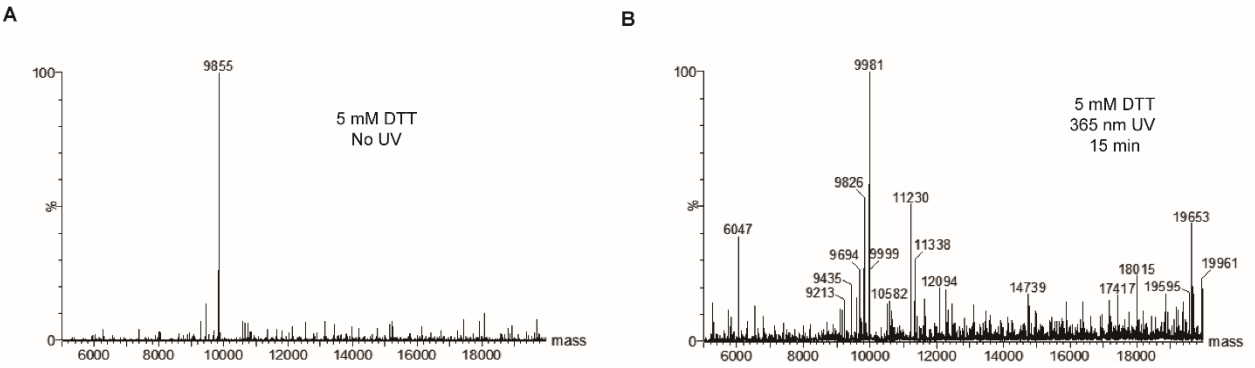


Figure S10. ESI-MS characterization of HA-Cys-Ub-TZ probe incubated with DTT upon 365 nm UV irradiation. A) ESI-MS characterization of 15 μM HA-Cys-Ub-TZ incubated with 5 mM DTT without UV irradiation; B) ESI-MS characterization of 15 μM HA-Cys-Ub-TZ incubated with 5 mM DTT with 365 nm UV irradiation for 15 min. The major peak 9,981 Da agrees with the expected mass of HA-Cys-Ub-NM-DTT.

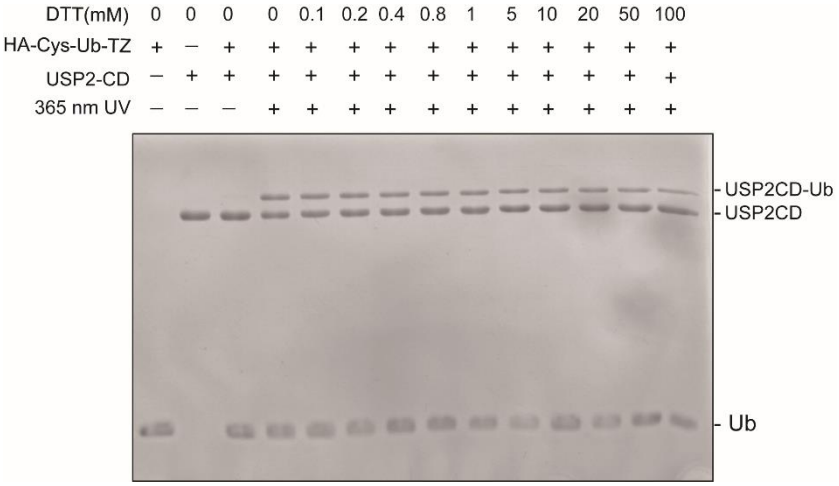


Figure S11. Wild type USP2-CD labeling by 15 μM HA-Cys-Ub-TZ in the presence of different concentrations of DTT. The labeling product was analyzed using 20% SDS-PAGE gel and stained with Coomassie brilliant blue.

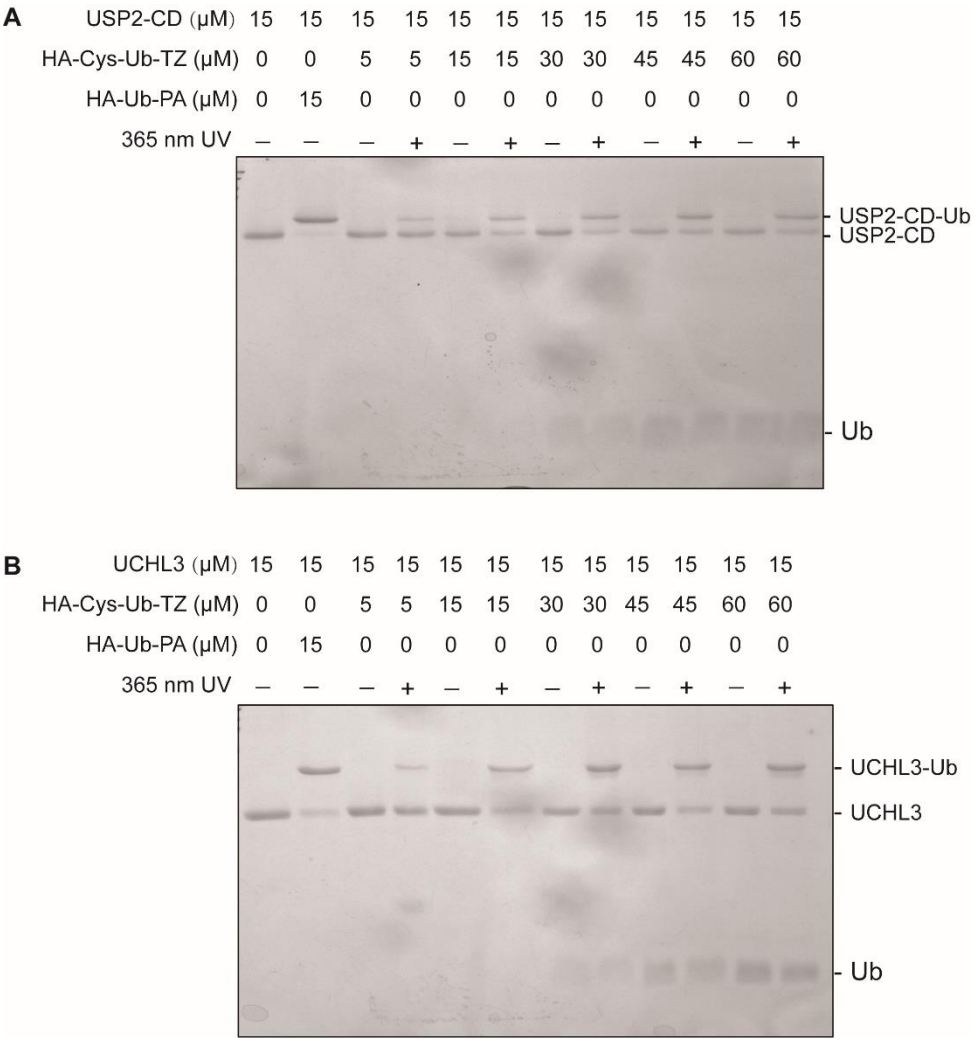


Figure S12. USP2-CD and UCHL3 labeling by different concentrations of HA-Cys-Ub-TZ. A) USP2-CD labeling by different concentration of HA-Cys-Ub-TZ; B) UCHL3 labeling by different concentration of HA-Cys-Ub-TZ. HA-Ub-PA was used as a control probe.

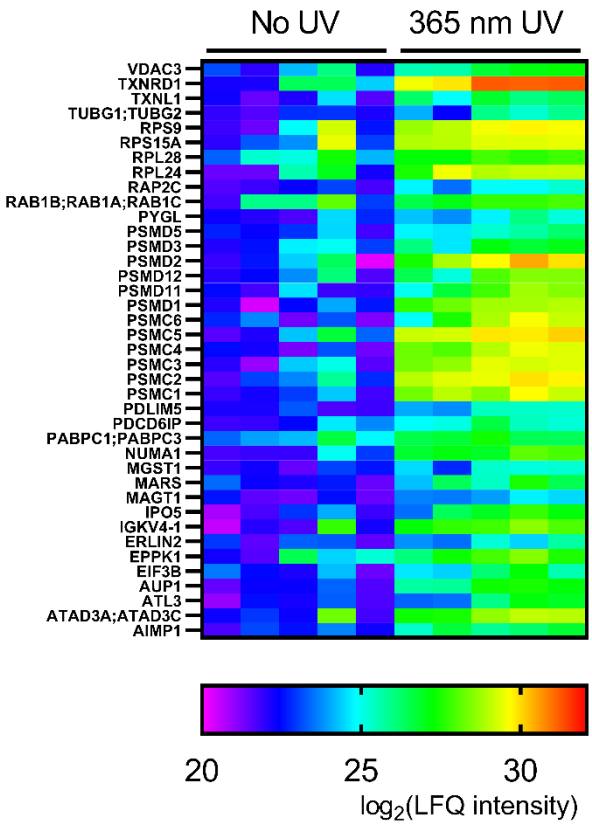


Figure S13: Heat map representing the LFQ intensity scores of significantly enriched protein groups other than DUBs by 15 μM HA-Cys(cR_{10})-Ub-TZ upon 365 nm UV irradiation. Red represents enrichment (higher LFQ intensity (log_2)), where lack of enrichment (lower LFQ intensity (log_2)) is shown in purple.

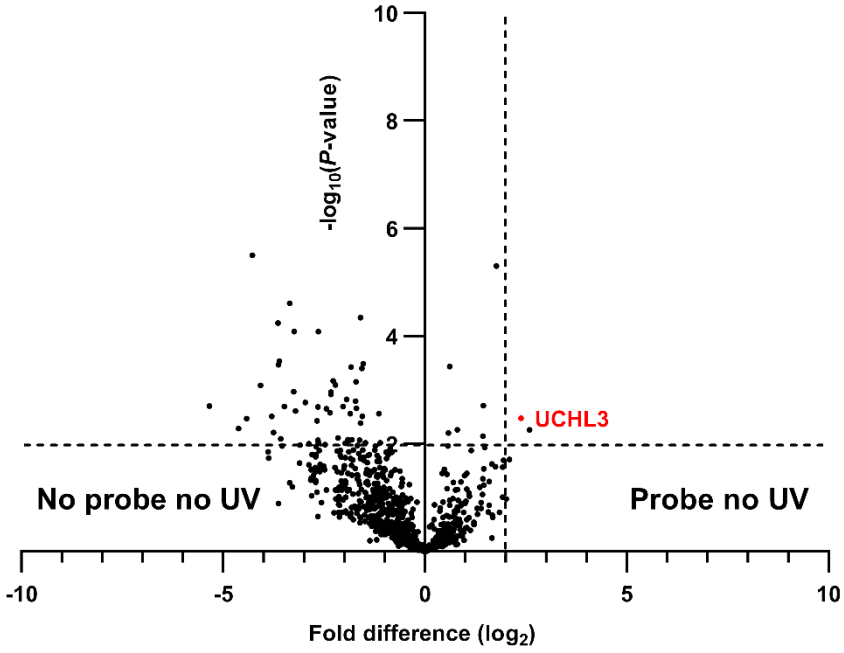


Figure S14: Volcano plot of protein groups pulled down in the absence of UV irradiation with 15 μM HA-Cys(cR_{10})-Ub-TZ treatment versus control pulldown without probe. Significantly enriched DUB is colored and labeled as red.

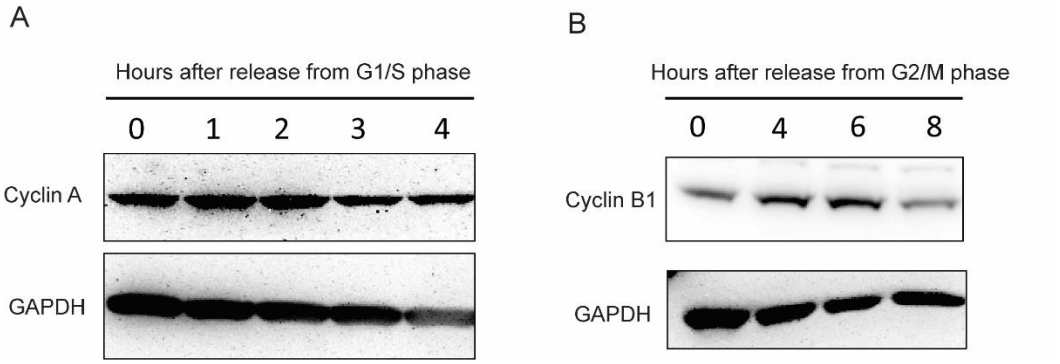


Figure S15: A) Expression levels of cyclin A and GAPDH in synchronized HeLa cells at indicated time points after release from G1/S phase block. B) Expression levels of cyclin B1 and GAPDH in synchronized HeLa cells at indicated time points after release from G2/M phase block.

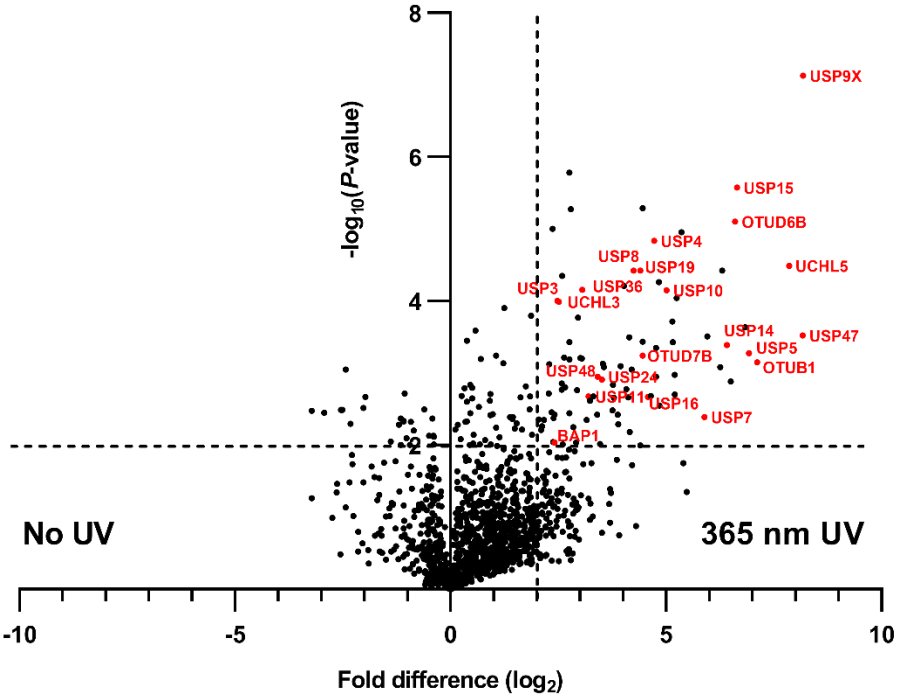


Figure S16: Volcano plot of pairwise comparison of protein groups pulled down in G1/S phase by 15 μM HA-Cys(cR_{10})-Ub-TZ with 365 nm UV irradiation relative to no UV irradiation. Significantly enriched DUBs are colored and labeled as red.

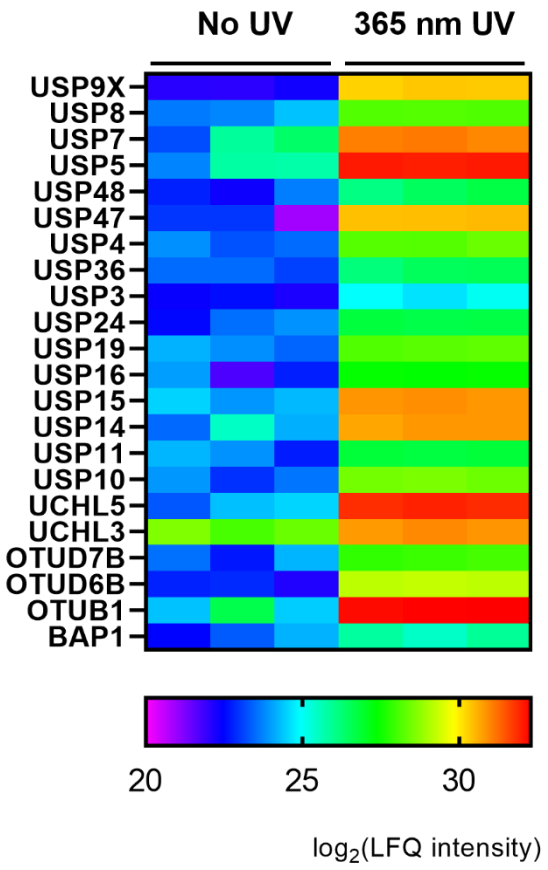


Figure S17: Heat map representing the LFQ intensity scores of G1/S phase DUBs significantly enriched by 15 μM HA-Cys(cR_{10})-Ub-TZ upon 365 nm UV irradiation. Red represents enrichment (higher LFQ intensity (log_2)), where lack of enrichment (lower LFQ intensity (log_2)) is shown in purple.

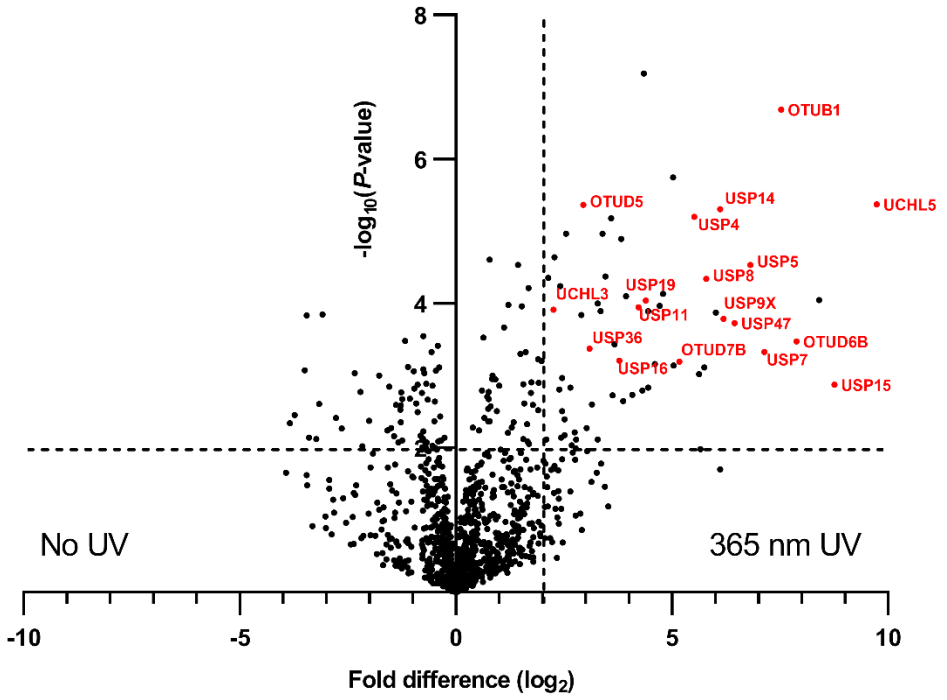


Figure S18: Volcano plot of pairwise comparison of protein groups pulled down in G2/M phase by HA-Cys(cR_{10})-Ub-TZ with 365 nm UV irradiation relative to no UV irradiation. Significantly enriched DUBs are colored and labeled as red.

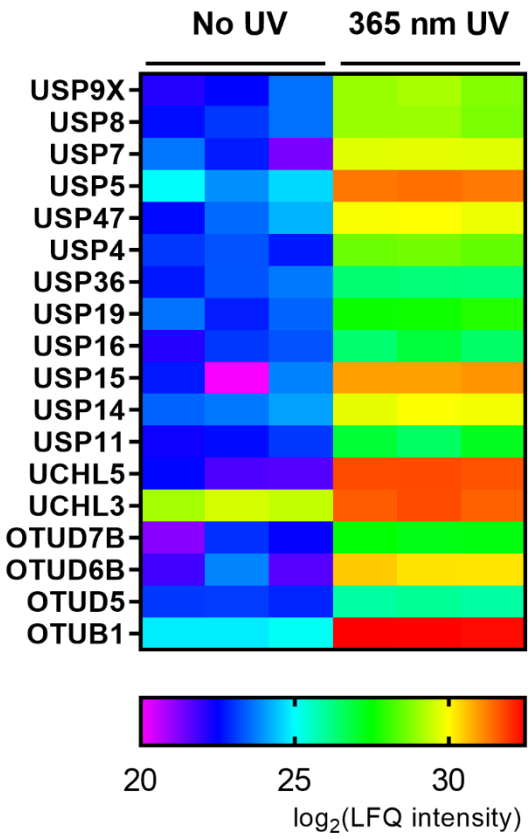


Figure S19: Heat map representing the LFQ intensity scores of G2/M phase DUBs significantly enriched by 15 μM HA-Cys(cR_{10})-Ub-TZ upon 365 nm UV irradiation. Red represents enrichment (higher LFQ intensity (log_2)), where lack of enrichment (lower LFQ intensity (log_2)) is shown in purple.

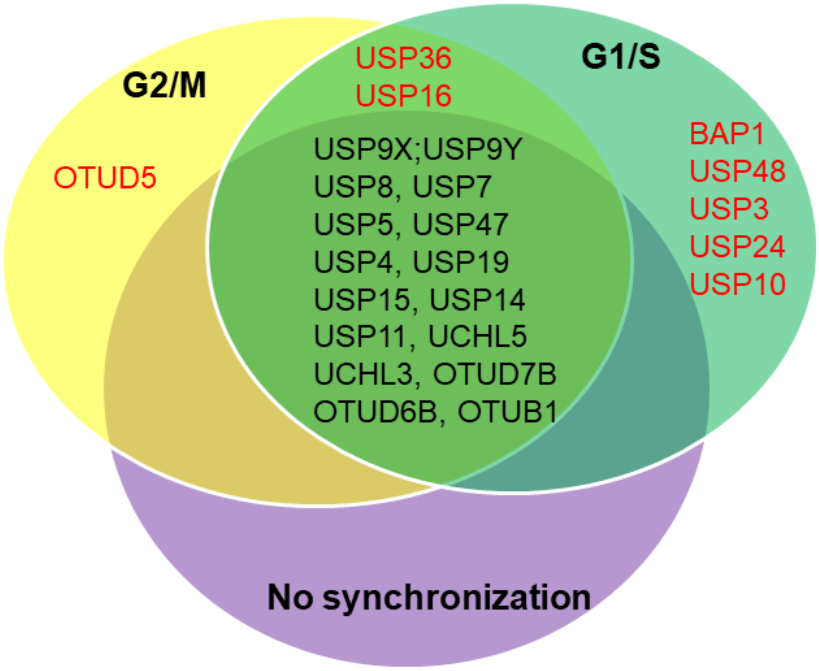


Figure S20: Intracellular proteome-wide DUB profiling by 15 μM HA-Cys(cR_{10})-Ub-TZ with 365 nm UV irradiation comparing DUBs captured in different phases of cell cycle of HeLa cells and unsynchronized cells.

Table S1. LFQ intensity scores for DUBs captured in HeLa cell using 15 μM HA-Cys(cR_{10})-Ub-TZ.

	No UV					365 nm UV				
Gene Name	1	2	3	4	5	1	2	3	4	5
USP9X;USP9Y	NaN	NaN	NaN	NaN	NaN	28.3315	28.7999	28.8674	28.0177	27.8655
USP8	NaN	NaN	NaN	NaN	NaN	27.5821	27.9135	28.0068	24.8527	24.4727
USP7	NaN	NaN	NaN	NaN	NaN	29.0685	29.0024	29.1896	28.6109	28.7265
USP5	20.2356	22.126	NaN	23.0551	NaN	31.3299	31.8014	31.5739	29.9474	30.1598
USP48	NaN	NaN	NaN	NaN	NaN	23.6241	25.0739	NaN	NaN	NaN
USP47	NaN	NaN	NaN	NaN	NaN	28.8619	29.2001	29.059	27.6968	28.7979
USP4	NaN	NaN	NaN	NaN	NaN	28.0277	28.2591	27.813	24.6895	25.326
USP36	NaN	NaN	NaN	NaN	NaN	24.413	26.8809	25.8991	NaN	NaN
USP32	NaN	NaN	NaN	NaN	NaN	22.6995	NaN	NaN	NaN	NaN
USP3	NaN	NaN	NaN	NaN	NaN	NaN	25.4547	NaN	NaN	NaN
USP24	NaN	NaN	NaN	NaN	NaN	24.5104	24.5575	24.4334	NaN	NaN
USP19	NaN	NaN	NaN	NaN	NaN	27.8889	27.9596	27.6817	26.1838	26.9584
USP16	NaN	NaN	NaN	NaN	NaN	26.3284	26.8476	26.107	NaN	25.837
USP15	NaN	NaN	NaN	NaN	NaN	30.5241	30.8309	30.71	29.1151	29.5596
USP14	NaN	NaN	NaN	21.3546	22.5708	30.189	30.3644	30.2598	29.1904	29.4062
USP11	NaN	NaN	NaN	NaN	NaN	27.545	27.5541	27.488	NaN	NaN
USP10	NaN	NaN	NaN	NaN	NaN	24.8332	25.2595	27.0565	24.2849	NaN
UCHL5	NaN	NaN	NaN	NaN	NaN	30.5535	30.5978	31.2686	31.3588	31.2413
UCHL3	25.0648	25.6011	25.7311	26.1971	24.7534	30.6053	30.6175	30.198	30.124	30.3168
OTUD7B	NaN	NaN	NaN	NaN	NaN	24.5043	24.1319	23.8216	27.3838	26.6543
OTUD6B	NaN	NaN	21.7102	NaN	NaN	25.1818	23.6787	29.1847	29.5897	29.3455
OTUD5	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	25.5665
OTUB1	23.204	NaN	24.8155	NaN	NaN	31.1374	31.2886	31.9004	32.07	31.8336

(NaN), protein groups not detected.

Table S2. LFQ intensity scores for proteins other than DUBs significantly enriched in HeLa cell using 15 μM HA-Cys(cR_{10})-Ub-TZ.

	No UV					365 nm UV				
Gene name	1	2	3	4	5	1	2	3	4	5
VDAC3	NaN	NaN	24.1911	26.0451	NaN	25.5378	25.6446	26.7551	27.1071	27.2153
TXNRD1	NaN	NaN	26.4228	26.4205	24.383	29.5149	29.8737	31.2127	31.1446	31.2132
TXNL1	NaN	NaN	NaN	NaN	NaN	26.239	24.8414	26.8243	25.9941	26.359
TUBG1;TUBG2	NaN	NaN	NaN	NaN	NaN	NaN	NaN	25.9719	25.2844	25.8985
RPS9	NaN	NaN	24.8518	29.2159	NaN	28.6813	29.0113	29.5475	29.7339	29.5761
RPS15A	NaN	NaN	NaN	29.4472	NaN	28.9104	28.9857	29.3549	29.3746	29.4466
RPL28	NaN	25.272	25.096	27.3829	24.1306	27.2031	27.2147	27.8742	27.6196	27.8021
RPL24	NaN	NaN	25.5913	26.9917	22.221	27.4561	29.5249	28.9248	29.0904	29.1317
RAP2C	NaN	NaN	NaN	NaN	NaN	24.7452	NaN	24.8806	24.8578	25.2572
RAB1s	NaN	NaN	NaN	28.2238	NaN	27.543	27.0064	28.5305	28.7422	28.5595
PYGL	NaN	NaN	NaN	24.4705	NaN	24.2626	NaN	24.7085	26.0089	25.1663
PSMD5	NaN	NaN	NaN	24.4639	NaN	24.7451	24.5933	25.2666	25.6942	26.2348
PSMD3	NaN	NaN	24.6898	24.9453	NaN	26.0478	24.6055	27.1184	26.7428	27.0417
PSMD2	NaN	NaN	24.3679	26.3623	NaN	27.4101	28.8331	29.6791	30.4561	29.9177
PSMD12	NaN	NaN	NaN	26.0903	NaN	26.4866	25.1397	27.9649	28.4647	28.4363
PSMD11	NaN	NaN	24.5586	NaN	NaN	24.9424	26.6761	27.824	28.751	28.4815
PSMD1	NaN	NaN	NaN	NaN	22.5961	27.6909	28.2707	28.7738	28.8057	28.9372
PSMC6	NaN	NaN	NaN	NaN	NaN	24.8715	27.4638	28.8751	29.5989	29.1026
PSMC5	NaN	NaN	24.2692	26.6947	23.3601	29.1465	29.2441	29.8786	29.8263	30.0863
PSMC4	NaN	22.2232	21.2669	23.1635	NaN	28.2573	27.9877	28.9581	29.5309	29.3455
PSMC3	NaN	NaN	NaN	25.0519	NaN	28.2949	28.5933	29.418	29.2574	29.3574
PSMC2	NaN	NaN	NaN	25.8201	NaN	28.9473	29.3472	29.4719	29.9304	29.7466
PSMC1	NaN	NaN	NaN	24.3122	NaN	28.2992	28.9927	28.48	29.63	29.1337
PDLIM5	NaN	NaN	NaN	NaN	NaN	24.0299	23.742	25.4015	25.3455	25.3096
PDCD6IP	NaN	NaN	NaN	24.6357	23.7052	24.8547	25.0669	26.5999	25.4366	25.1956
PABPC1;C3	23.3585	23.9333	24.1752	26.6471	24.7792	26.5504	26.7457	27.3759	26.4746	26.4159
NUMA1	NaN	NaN	NaN	24.9626	NaN	26.7693	27.0973	26.8749	28.1156	27.8927
MGST1	NaN	NaN	NaN	NaN	NaN	24.495	NaN	25.3089	25.1222	25.2498
MARS	23.4149	NaN	NaN	22.6214	NaN	24.2382	26.4202	25.3701	27.4579	26.5003
MAGT1	NaN	NaN	NaN	NaN	NaN	NaN	NaN	23.9187	24.703	24.4729
IPO5	NaN	NaN	22.8805	NaN	NaN	23.4608	26.397	26.9349	27.7489	27.1534
IGKV4-1	NaN	NaN	NaN	27.7336	NaN	27.1822	27.6874	27.8236	28.5357	27.9662
ERLIN2	NaN	NaN	NaN	NaN	NaN	NaN	23.4575	25.202	NaN	25.6952
EPPK1	NaN	NaN	26.492	NaN	25.2328	26.0604	27.3481	27.8788	28.4958	27.4981
EIF3B	NaN	NaN	NaN	24.2068	NaN	24.6114	24.3998	26.1814	27.2162	25.5444
AUP1	NaN	NaN	NaN	NaN	NaN	25.7209	25.7907	27.3528	27.4678	27.1832
ATL3	20.9944	NaN	NaN	23.2999	NaN	NaN	NaN	25.9572	27.1395	26.9036
ATAD3A;3C	NaN	NaN	22.3204	28.282	21.7979	27.3405	27.4363	28.6142	29.049	28.9578
AIMP1	NaN	NaN	NaN	NaN	NaN	25.3229	26.6795	25.8377	26.2572	26.7655

(NaN), protein groups not detected.

Table S3. Pairwise comparison of fold difference and *P*-value ($-\log_{10}$) for DUBs captured in HeLa cells at different time points following the release from G1/S phase using 15 μM HA-Cys(cR_{10})-Ub-TZ.

	1 h/0 h following the release from G1/S		4 h/0 h following the release from G1/S		4 h/1 h following the release from G1/S	
Gene name	fold difference	*P* value ($-\log_{10}$)	fold difference	*P* value ($-\log_{10}$)	fold difference	*P* value ($-\log_{10}$)
OTUB1	0.88	0.18	0.52	1.34	0.58	1.64
OTUD6B	0.95	0.07	0.54	1.23	0.57	1.75
OTUD7B	1.02	0.02	0.74	0.66	0.73	0.71
UCHL3	0.95	0.09	0.72	0.85	0.75	1.48
UCHL5	0.98	0.03	0.72	1.22	0.73	1.29
USP10	1.23	0.21	0.33	1.99	0.27	1.55
USP11	1.12	0.11	1.09	0.08	0.97	0.03
USP14	1.85	1.08	1.11	0.14	0.60	0.78
USP15	1.45	0.55	1.23	0.27	0.85	0.65
USP16	1.11	0.09	0.52	0.85	0.47	1.75
USP19	4.21	1.43	2.45	0.62	0.58	0.61
USP4	1.01	0.00	0.46	0.55	0.45	0.60
USP47	1.53	0.66	1.12	0.15	0.73	0.42
USP5	0.97	0.08	0.90	0.51	0.93	0.27
USP7	1.22	0.35	0.84	0.33	0.69	1.08
USP8	1.10	0.15	0.65	0.85	0.59	1.09
USP9X;USP9Y	1.81	0.70	0.96	0.04	0.53	1.22

Note: When protein groups were not detected by MaxQuant analysis (NaN), LFQ value was imputed by Perseus for calculation of fold-difference between pulldowns (see Supplemental Methods), which is the case for USP16 not being detected in 4 h.

Supplemental Methods

Plasmid Construction and Gene Cloning. The yeast Ub_{1-75} gene was mutated using QuikChange polymerase chain reaction (PCR) to generate the humanized Ub_{1-75} (S19P, D24E, and S28A) and used throughout this work. The generation of HA-Cys-Ub_{1-75} is described in previous publication.[1]

Protein Expression and Purification. Ubiquitin was expressed and purified as described below. HA-Cys-Ub_{1-75} plasmid was transformed into BL21(DE3) cells and cultured at 37 °C until OD_{600} reached 0.3-0.4. The incubation temperature was decreased to 16 °C until OD_{600} reached 0.4-0.6. Then, protein expression was induced with 1 mM IPTG. Cells were cultured for an additional 15 - 20 h at 16 °C after the induction and harvested by centrifugation at 6,000 rpm for 10 min at 4 °C. Cells expressing HA-Cys-Ub were sonicated in lysis buffer containing 20 mM Tris (pH 7.5), 200 mM NaCl, 5% glycerol and 1 mM EDTA, 1 mM PMSF, and centrifuged at 6,000 rpm. The cell free extract was incubated chitin resin (New England Biolabs, Ipswich, MA) overnight at 4 °C. After removal of unbound proteins via centrifugation (2,000 rpm at 4 °C), the resin was first washed with a high salt buffer containing 20 mM Tris (pH 7.5), 0.5 M NaCl, 5% glycerol, 1 mM EDTA, followed by a low salt buffer wash with 20 mM MES (pH 6.5), 100 mM NaCl. The washed resin was then incubated with cleavage buffer containing 20 mM MES (pH 6.5), 100 mM NaCl, 80 mM MESNA for 15 h at 4 °C. Protein was then eluted and concentrated using Amicon Ultra-15 Centrifugal Filter Units (Thermo Fisher Scientific, Waltham, MA). The purity of proteins was estimated to be 90% or greater by SDS-PAGE gel analysis with Coomassie Blue staining. Bradford assay was used to determine the concentration of the purified protein and protein sample was flash frozen and stored at -80 °C. USP2-CD, UCHL1 and UCHL3 were expressed and purified as previously described.[2]

Mammalian Cell Culture. HeLa cell line (ATCC CCL-2) was cultured in RPMI-1640 Medium (Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10% FBS. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

Immunoblotting Analysis of DUB Labeling by Ubiquitin Probes. HeLa cells were plated in Corning Costar Flat Bottom Cell Culture 6-well plates (Mediatech, Inc., Manassas, VA) at 50% confluence and allowed to adhere for 12 h in RPMI-1640 medium supplemented with 10% FBS at 37 °C and 5% CO_2. Cells were treated with 15 μM of HA-Cys(cR_{10})-Ub-TZ for 4 h at 37°C at 5% CO_2 in the same medium. Post probe treatment, 0.25% trypsin, 0.1% EDTA in HBSS buffer without calcium, magnesium, and sodium bicarbonate (Mediatech, Inc., Manassas, VA) was added to cells and allowed to incubate for 3 min at room temperature. After trypsin solution was removed, cells were washed three times with cold DPBS buffer (Dulbecco's Phosphate-Buffered Salt solution) (Mediatech, Inc., Manassas, VA). Cells were then harvested and washed with cold DPBS and used immediately for the following experiments or stored at -80°C.

Cell lysate for immunoblotting was generated according to previous publication.[1] 40 μg cell lysate was incubate with 15 μM probe at 37°C for 2h. After incubation samples were quenched using a 6X gel loading dye (35 mM Tris, 10% SDS, 30% glycerol and 9.3% DTT, pH 6.8) and resolved on a 12% reducing SDS-PAGE gel followed by transfer to a PDVF membrane (Thermo Fisher Scientific Inc., Waltham, MA). The membrane was immunoblotted with an anti-HA antibody. HRP-conjugated anti-mouse antibody was used as the secondary antibody. GAPDH protein level was utilized as a loading control and detected using an anti-GAPDH antibody. HRP-conjugated anti-rabbit antibody was used as the secondary antibody for GAPDH. ECL Western blotting substrate (Thermo Fisher Scientific Inc., Waltham, MA) was used to detect the immunoblotting signal.

Pulldown and Mass Spectrometry Analysis. For intracellular proteome-wide protein profiling using MS-based LFQ analysis, 200 μg cell lysate generated from the HA-Cys(cR_{10})-Ub-TZ treated HeLa cells as described above was incubated with 100 μL anti-HA magnetic beads in RIPA buffer supplemented with 1X Complete Protease Cocktail Inhibitor at room temperature for 2 h. Unbound proteins were removed by aspiration. The remaining anti-HA magnetic beads were washed 3 times with Tris-buffered saline, 0.1% Tween-20 (TBST). The enriched proteins by HA beads were eluted with 100 μL 50 mM NaOH. Eluate was neutralized to pH 8 using 100 μL 1M NH_4HCO_3 solution, and adjusted to have 0.1% SDS. Then the protein solution was reduced with 20 mM DTT at 60 °C water bath for 1 h. The sample was then alkylated with 40 mM iodoacetamide at room temperature for 30 min with protection from light. The alkylation reaction was quenched by adding 10 mM DTT. Then, 1.2 mL cold acetone was added to the mixture to precipitate proteins from the solution at -20 °C overnight. The mixture was centrifuged at 14,000 rpm for 10 min to collect protein pellet at the bottom of the tube. The protein pellet was then dissolved in 200 μL 100 mM NH_4HCO_3 and digested with 2 μg of trypsin at 37 °C for 18 h. Tryptic digestion was quenched by 0.1% formic acid. Centrifugation at 13,000 g at room temperature for 10 min was used to remove residual precipitation. Digested peptide sample was desalted with C18 zip tip and lyophilized prior to LC-MS/MS analysis.

Lyophilized trypsin digested samples were dissolved in 0.1% formic acid in ddH_2O prior to LC-MS/MS analysis using a nano UHPLC column (15 cm long, 75 μm inner diameter, 3 μm C18 resin, Thermo Fisher Scientific, Waltham, MA)) connected to Orbitrap Q-Exactive operated in a positive polarity mode and collision induced dissociation (Thermo Fisher Scentific, Waltham, MA) with a nano-electrospray ion source. A linear gradient of 5% - 60% acetonitrile in water with 0.1% formic acid for 150 minutes was used to separate peptides at a constant flow rate of 200 nL/min. The MS system operates in a data-dependent

mode with MS/MS scan of the six most abundant peaks from a full MS scan, which was acquired between 300 to 1800 m/z with a resolution of 60,000.

MaxQuant software version 1.4.0.6 and its Label-Free Quantification (LFQ) module were used to quantify enriched proteins as described previously.[1] When protein groups were not detected by MaxQuant analysis (NaN), an imputation by Perseus (version 1.6.1.3) was used to generate LFQ values based on normal distribution of valid LFQ values for calculation of fold-difference between pulldowns. Two-sample *t*-test was performed using the default parameters in Perseus. To generate the volcano plots, the $-\log_{10}$(*P*-value) and $\log_2$(fold difference of LFQ values between paired samples) were plotted in GraphPad Prism (version 8).

Double Thymidine and Nocodazole Synchronization of HeLa Cells. To synchronize cells to G1/S phase, Hela cells were seeded at approximately 40% confluency in RPMI-1640 Medium supplemented with 10% FBS. The Corning™ Costar™ Flat Bottom Cell Culture 6-well plates (Mediatech, Inc., Manassas, VA) were used. Cells were incubated at 37°C and 5% CO_2 for approximately 24 h. RPMI-1640 medium supplemented with 2.0 mM thymidine (MilliporeSigma Inc., Burlington, MA) was used to replace the cell culture medium and cells were incubated at 37 °C and 5% CO_2 for another 12 h. The medium was removed and cells were washed three times by DPBS buffer. Fresh RPMI-1640 medium was added to the cells and incubated for 12 h at 37°C and 5% CO_2. This thymidine-block procedure was repeated one more time. Following this double thymidine block procedure, cells in RPMI-1640 medium supplemented with 2.0 mM thymidine were treated with HA-Cys(cR_{10})-Ub-TZ probe as described above.

To synchronize cells to G2/M phase, Hela cells were seeded at approximately 60% confluency in RPMI-1640 medium using Corning™ Costar™ Flat Bottom Cell Culture 6-well plates (Mediatech, Inc., Manassas, VA). Cells were incubated at 37°C and 5% CO_2 for approximately 24 h. RPMI-1640 medium supplemented with 0.5 μg/mL nocodazole (MilliporeSigma Inc., Burlington, MA) was used to replace the cell culture medium and cells were incubated at 37 °C and 5% CO_2 for another 12 h. Cells in RPMI-1640 medium supplemented with 0.5 μg/mL nocodazole were then treated with HA-Cys(cR_{10})-Ub-TZ probe as described above.

Time Dependent Pulldown Following the Release of HeLa Cells from G1/S Phase. HeLa cells were synchronized to G1/S phase as described above. Then, HeLa cells were incubated with 15 μM HA-Cys(cR_{10})-Ub-TZ probe in RPMI1640 media containing 2 mM thymidine at 37 °C and 5% CO_2 for 4 h. Cells were then rinsed with a trypsin-EDTA solution followed by several cold DPBS wash to remove untransduced probe. For the 0 h time point treatment, the HeLa cells were irradiated immediately with 365 nm UV for 15 min in cold DPBS buffer. For the 1 and 4 h time point treatment, the HeLa cells were

incubated with fresh RPMI1640 media without thymidine for 1 h and 4 h respectively. Then the cells were rinsed with cold DPBS buffer and irradiated with 365 nm UV for 15 min in cold DPBS buffer. The HeLa cells were harvested and pulldown was carried out using anti-HA magnetic beads as described above.

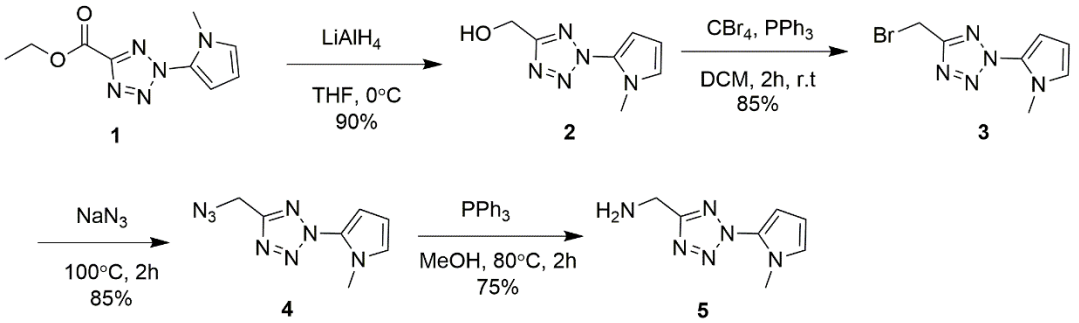
General Information.

Chemical reagents were purchased from Fisher, MilliporeSigma, Alfa and Acros. ^{1}H and ^{13}C NMR spectra were obtained with Bruker AV400 NMR Spectrometer with a CryoProbe. Chemical shifts are reported in δ (ppm) units and ^{13}C and ^{1}H signals from deuterated solvents were used as references. ACQUITY UPLC H-Class/SQD2 instrument equipped with an electrospray ionization (ESI) source was used to obtain mass spectra for small molecules. Xevo G2-S QTof instrument equipped with an electrospray ionization (ESI) source was used to obtain mass spectra for proteins. 60 GF254 (Merck) silica plate was used in thin layer chromatography (TLC) analysis. Small molecule column chromatography was conducted on silica gel (230-400 mesh). For the purification of ubiquitin probes, a Jupiter C18 column (10 ×250 mm, 10 micron, Phenomenex, Torrance, CA) was used at flow rate of 4 mL/min. The UV absorbance at 214 nm was used to monitor the purification process. Water (with 0.1% formic acid) and acetonitrile (with 0.1% formic acid) were used as solvent A and B respectively.

GAPDH antibody was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Anti-HA-tag and HRP-conjugated anti-mouse antibodies were purchased from MilliporeSigma (Bilerica, MA). HRP-conjugated anti-rabbit antibody was purchased from Abcam (Cambridge, MA). Anti-HA magnetic beads and trypsin protease (MS grade) were purchased from ThermoFisher Scientific (Waltham, MA).

Experimental Procedures and Characterization Data

Scheme S1. Synthesis of (2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazol-5-yl)methanamine (**5**)



(2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazol-5-yl)methanol (2): Ethyl 2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazole-5-carboxylate (**1**) was generated according to previous publication.[3] **1** (2.21 g, 10 mmol) was dissolved in 50 mL dried THF, then cooled to 0 ℃ with ice-bath. $LiAlH_4$ (0.61 g, 16 mmol) was added in small portions during approximately 20 minutes. The mixture was stirred for another 20 minutes until the reaction was completed, as monitored by TLC. 1.5 mL water was added dropwise into the mixture to quench the reaction followed by filtration. The filtrate solution was dried with Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel column chromatography with Hex:EtOAc (1:1) to provide (2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazol-5-yl)methanol (1.61 g, 9 mmol, 90%) as a colorless oil. ^{1}H NMR (CDCl3, 400 MHz) δ 6.70 (t, *J*=2.4 Hz 1H), 6.55 (m, 1H), 6.22 (m, 1H), 5.04 (s, 2H), 3.68 (s, 3H); ^{13}C NMR (CDCl3, 100 MHz): δ 165.38, 123.22, 107.64, 105.73, 56.15, 34.75. MS (ESI, positive) m/z calculated for $C_7H_9N_5O$ $[M+H]^+$: 180.09, found: 180.09.

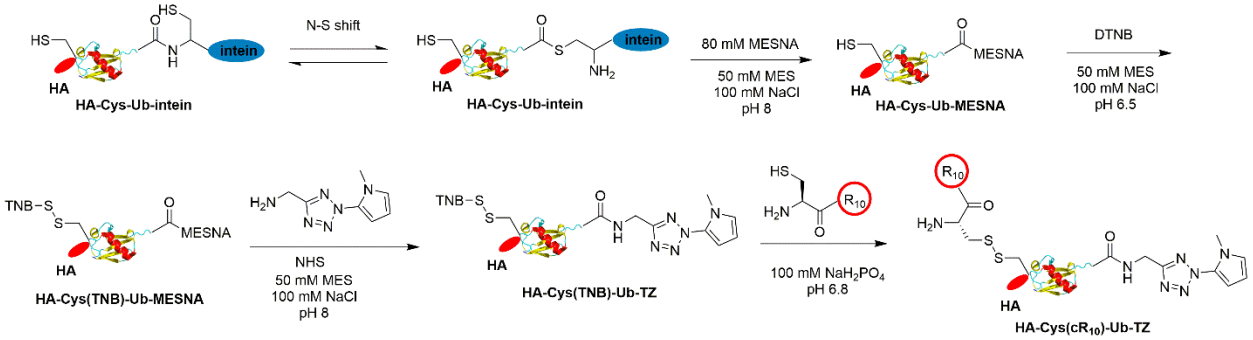
5-(bromomethyl)-2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazole (3): To a solution of (2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazol-5-yl)methanol (**2**) (1.00 g, 5.59 mmol) and PPh_3 (1.61 g, 6.15 mmol) in 50 mL CH_2Cl_2, CBr_4 (2.78 g, 8.39 mmol) was added under stirring. Stirring was continued for 4 h, then the solvent was evaporated and the crude residue was purified by silica gel column chromatography with Hex:EtOAc (3:1) to obtain 5-(bromomethyl)-2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazole (1.15 g, 4.75 mmol, 85%) as a colorless oil. ^{1} H NMR (CDCl3, 400 MHz) δ 6.71 (t, *J*=2.4 Hz 1H), 6.57 (m, 1H), 6.22 (m, 1H), 4.70 (s, 2H), 3.70 (s, 3H); ^{13}C NMR (CDCl3, 100 MHz): δ 162.94, 123.41, 107.71, 105.79, 34.89, 18.38. MS (ESI, positive) m/z calculated for $C_7H_9N_5O$ $[M+H]^+$: 242.00, found: 242.02.

5-(azidomethyl)-2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazole (4): 5-(bromomethyl)-2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazole (**3**) (1.00 g, 4.13 mmol) and sodium azide (0.54 g, 8.26 mmol) were dissolved in 20 mL dry DMF and stirred at 100 °C for 2 h. After cooling to room temperature, the suspension was diluted with water (150 mL). The aqueous layer was extracted with diethyl ether (3 x 30 mL) and the combined organic layers were washed with water (30 mL) and brine (2 x 30 mL), dried with anhydrous Na_2SO_4 and the solvent was evaporated and the crude residue was purified by silica gel column chromatography with

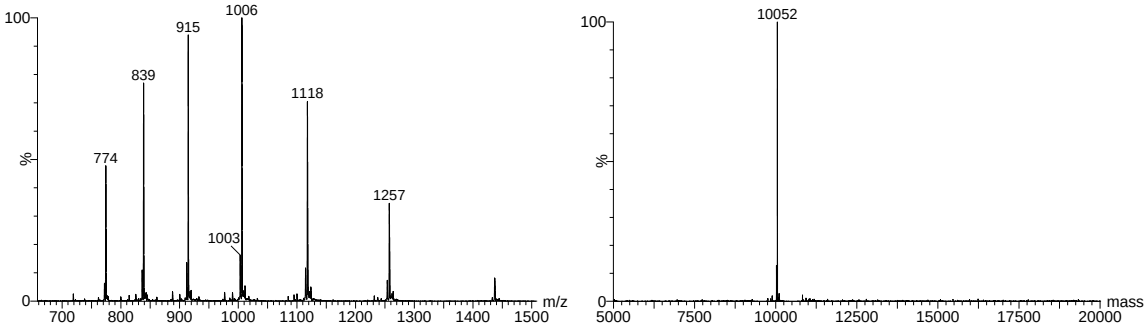
Hex:EtOAc (3:1) to obtain 5-(azidomethyl)-2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazole (0.72 g, 3.51 mmol, 85%) as a colorless oil. ^{1}H NMR (CDCl3, 400 MHz) δ 6.71 (t, *J*=2.4 Hz 1H), 6.58 (m, 1H), 6.23 (m, 1H), 4.69 (s, 2H), 3.70 (s, 3H); ^{13}C NMR (CDCl3, 100 MHz): δ 161.60, 123.43, 107.71, 105.82, 44.69, 34.87. MS (ESI, positive) m/z calculated for $C_7H_7N_9$ $[M+H]^+$:205.10, found: 205.08.

(2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazol-5-yl)methanamine (5): To a solution of 5-(azidomethyl)-2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazole (**4**) (0.50 g, 2.45 mmol) in MeOH (25 mL) triphenylphosphine (0.96 g, 3.68 mmol) was added. The resulting mixture was stirred at 80 °C for 1h. After evaporation of the solvent, the crude residue was purified by silica gel column chromatography with DCM:MeOH:Et_3N (20:1:1) to obtain (2-(1-methyl-1H-pyrrol-2-yl)-2H-tetrazol-5-yl)methanamine (0.33 g, 1.84 mmol, 75%) as a colorless oil. ^{1}H NMR (CDCl3, 400 MHz) δ 6.68 (t, *J*=2.4 Hz 1H), 6.51 (m, 1H), 6.20 (m, 1H), 4.22 (s, 2H), 3.65 (s, 3H), 2.02 (s, 2H); ^{13}C NMR (CDCl3, 100 MHz): δ 167.26, 123.07, 123.04, 107.57, 105.57, 37.44, 34.66. MS (ESI, positive) m/z calculated for $C_7H_7N_9$ $[M+H]^+$:179.10, found: 179.17.

Scheme S2. Generation of HA-Cys(cR$_{10}$)-Ub-TZ

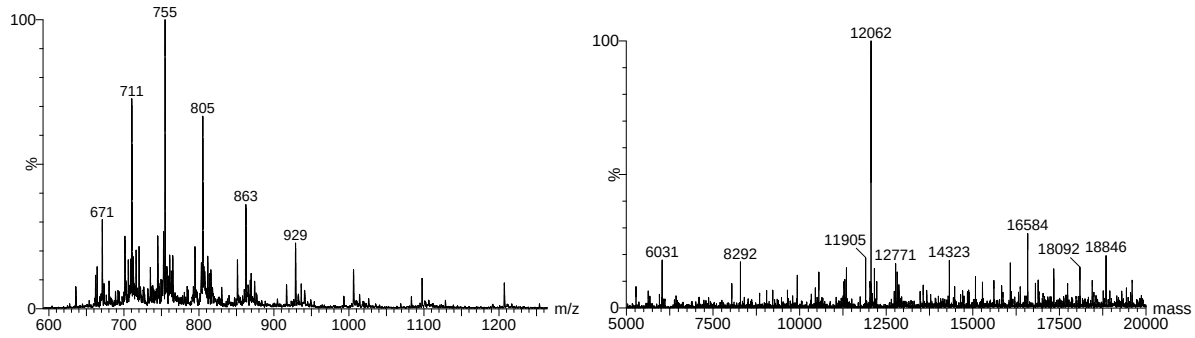


Generation of HA-Cys(TNB)-Ub–TZ. HA-Cys(TNB)-Ub–MESNA was generated as described in a previous publication.[1] To a solution of HA-Cys(TNB)-Ub-MESNA (10 mg/mL, 1 mL) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5), NHS (500 µL, 2 M solution in ddH_2O, 1 mmol, 1000 eq.) and **5** (500 µL, 2 M in DMSO solution, 1 mmol, 1000 eq.) were added sequentially. The final pH of the reaction solution was approximately 8. The mixture was immediately vortexed and reacted at room temperature for 12 h. The reaction mixture was then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5). The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Cys(TNB)-Ub-TZ was determined by ESI-MS. (Expected mass: 10,052 Da, found mass: 10,052 Da, mass spectrum shown below).



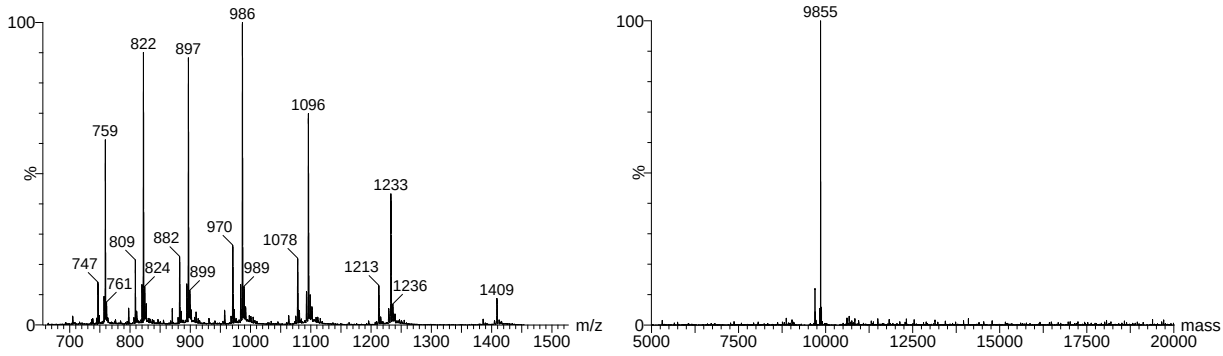
Generation of HA-Cys(cR$_{10}$)-Ub-TZ. cR$_{10}$ peptide (100 mg/mL, 0.14 mL, 6.4 µmoL, 20 eq.) was added to a 50 mM NaH_2PO_4 buffer (pH 6.8) solution containing HA-Cys(TNB)-Ub-TZ (1 mg/mL, 3.2 mL, 0.32 µmol). The mixture was vortexed and incubated at room temperature for 2 h. The reaction mixture was then diluted with 1 mL 6 M Guanidine-HCl and concentrated to a total volume of 1.0 mL. A C18 HPLC column was used to purify the product. Solvent A is consisted of water with 0.1% formic acid, Solvent B is consisted of acetonitrile with 0.1% formic acid. Gradient in "time → % B": 0 min → 5%, 10 min → 30%, 70 min → 40%, 79 min→ 95%. The product was eluted out at approximately 28% buffer B. Fractions containing

product were pooled and SpeedVac concentrated overnight. The pure product was dissolved in 6 M Guanidine-HCl (pH 6.0), then buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) for four times. The molecular weight of HA-Cys(cR_{10})-Ub-TZ was determined by ESI-MS. (Expected mass: 12,063 Da, found mass: 12,062 Da, mass spectrum shown below).



Generation of HA-Cys-Ub-TZ.

To a solution of HA-Cys(TNB)-Ub-TZ (500 μL, 1 mg/mL) in MES buffer (20 mM MES, 100 mM NaCl, pH 6.5), DTT (1 M, 0.1 mL) was added. The mixture was immediately vortexed and incubated at room temperature for 1 h. Following the reaction, the solution was buffer exchanged into MES buffer (20 mM MES, 100 mM NaCl, pH 6.5) for four times. The final concentration of the product was determined by Bradford assay. The molecular weight of HA-Cys-Ub-TZ was determined by ESI-MS. (Expected mass: 9,855 Da, found mass: 9,855 Da, mass spectrum shown below).

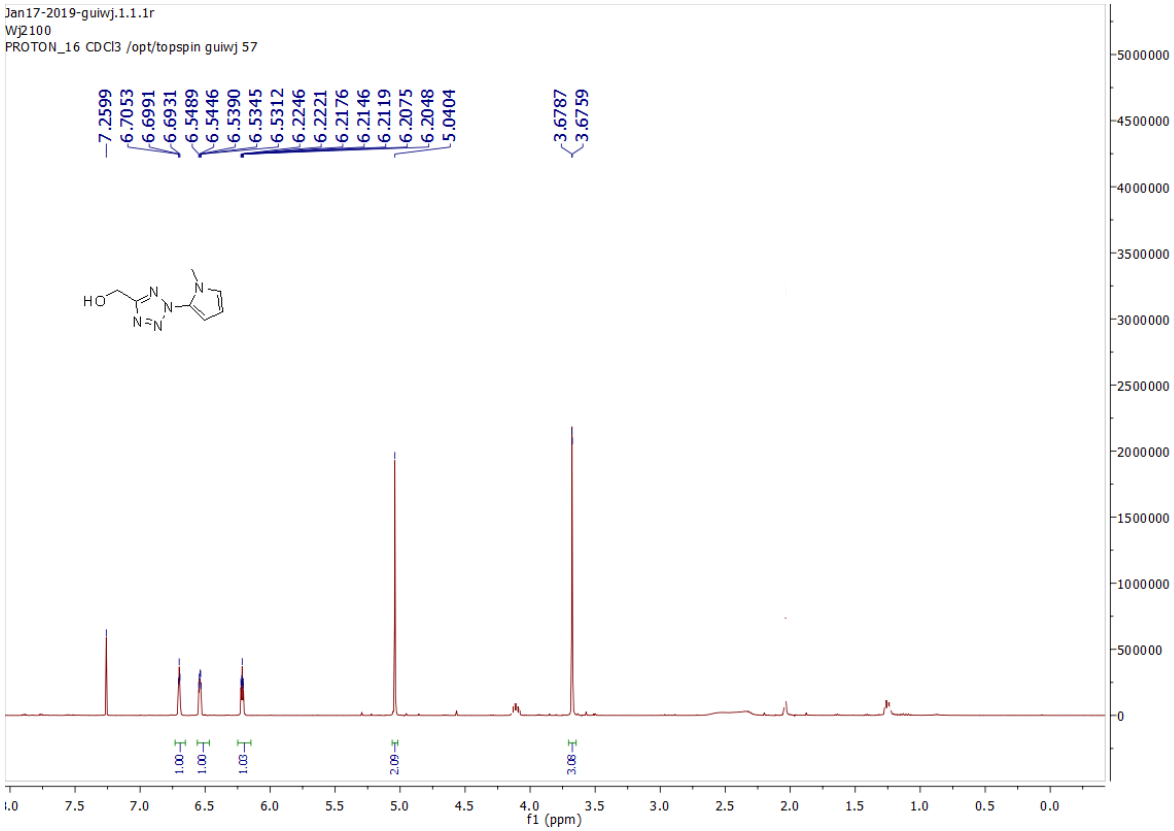


Reference

[1] W. Gui, C. A. Ott, K. Yang, J. S. Chung, S. Shen, Z. Zhuang, *J Am Chem Soc* **2018**, *140*, 12424-12433.

[2] C. A. Ott, B. Baljinnyam, A. V. Zakharov, A. Jadhav, A. Simeonov, Z. Zhuang, *ACS Chem Biol* **2017**, *12*, 2399-2407.

[3] Y. Tian, M. P. Jacinto, Y. Zeng, Z. Yu, J. Qu, W. R. Liu, Q. Lin, *J Am Chem Soc* **2017**, *139*, 6078-6081.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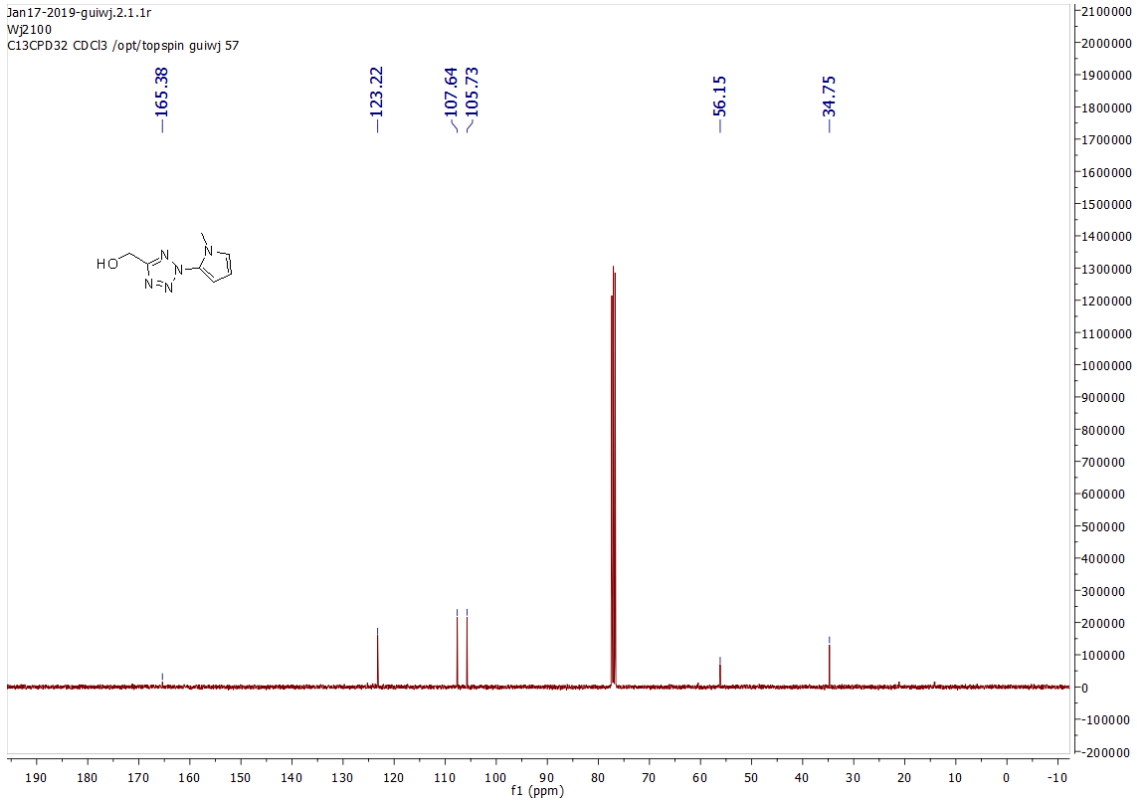
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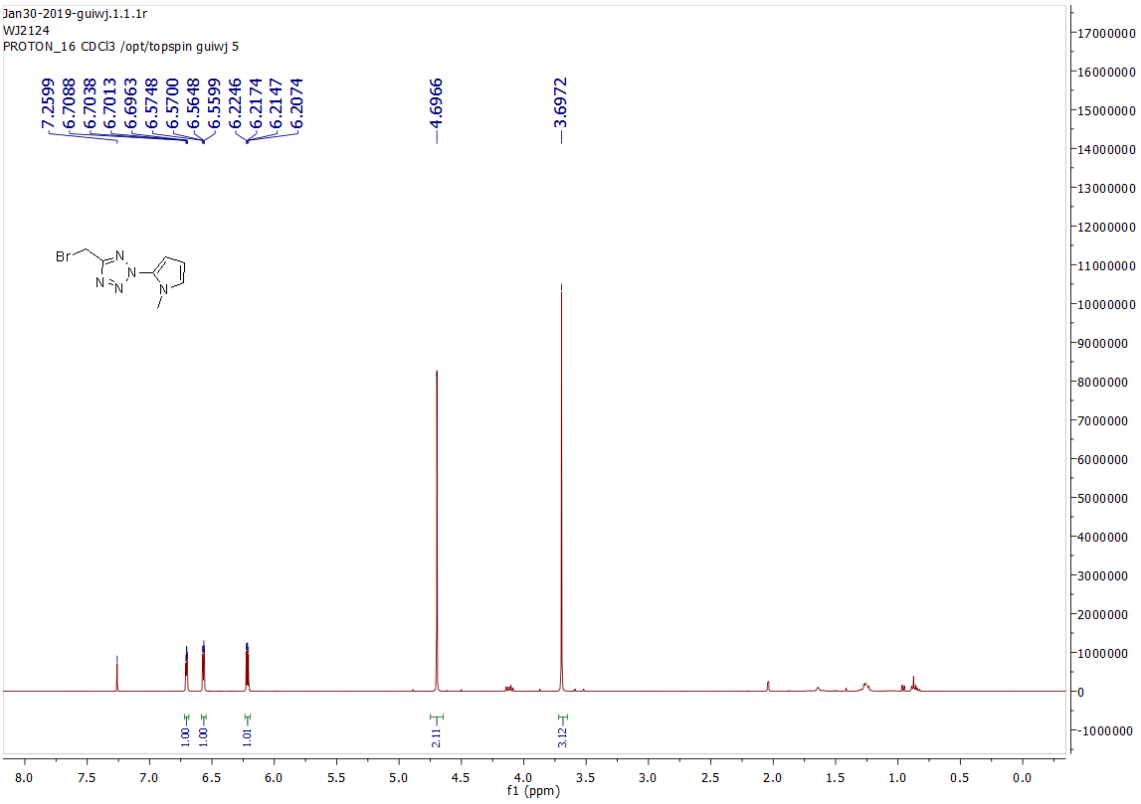
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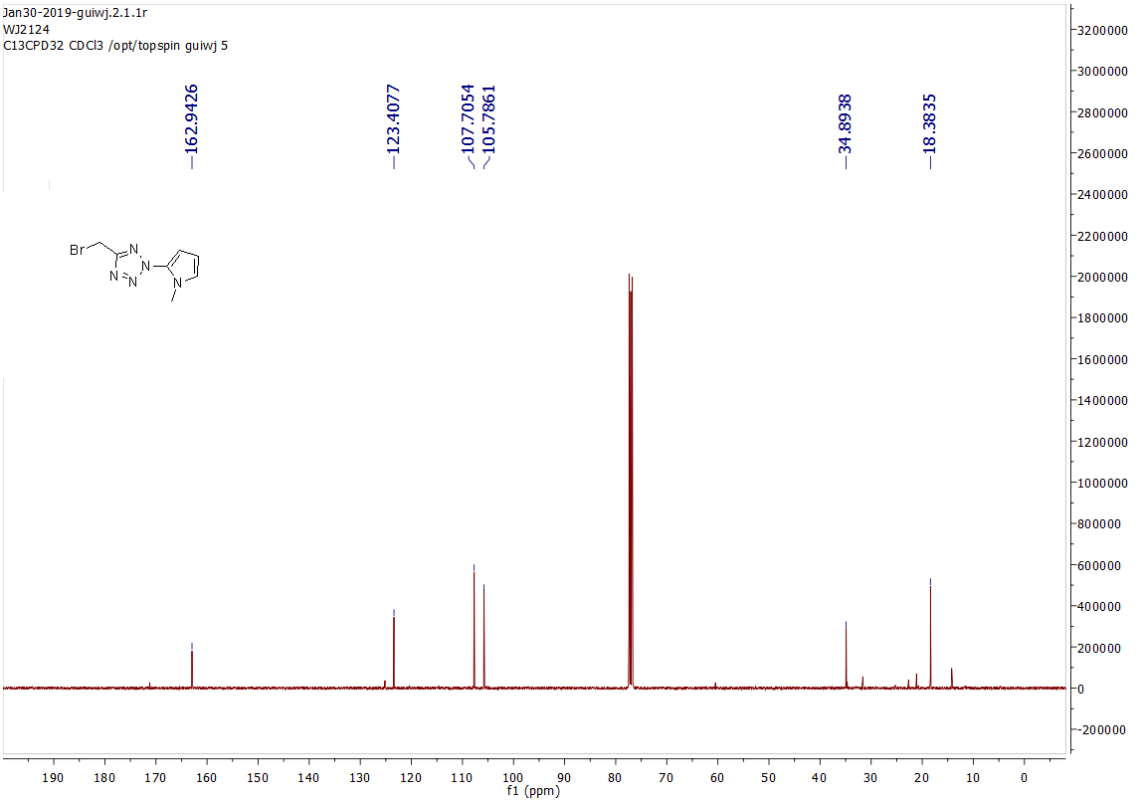
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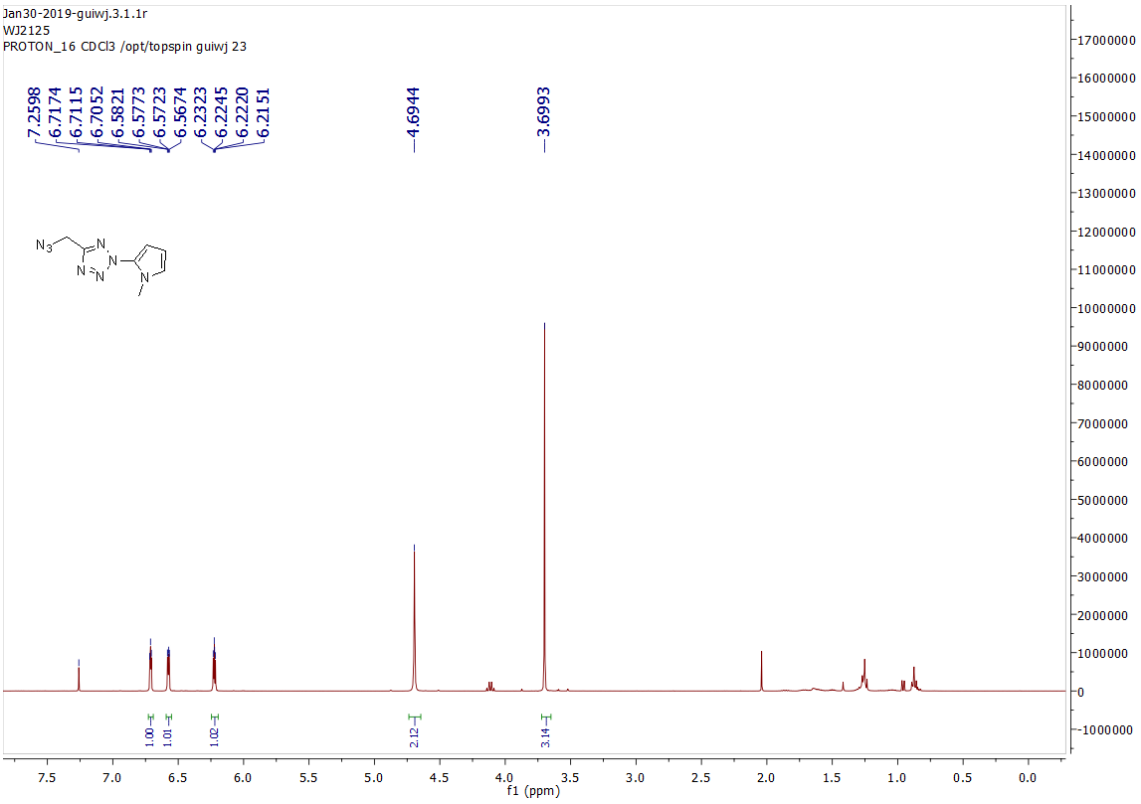
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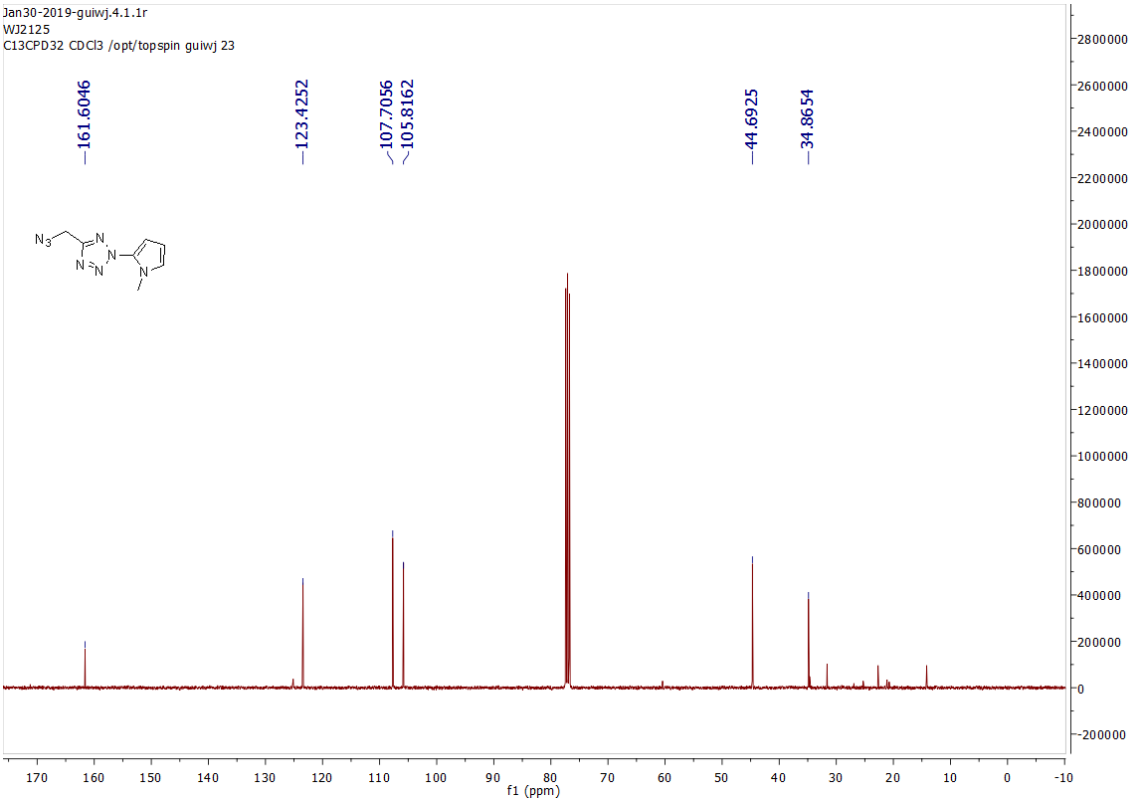
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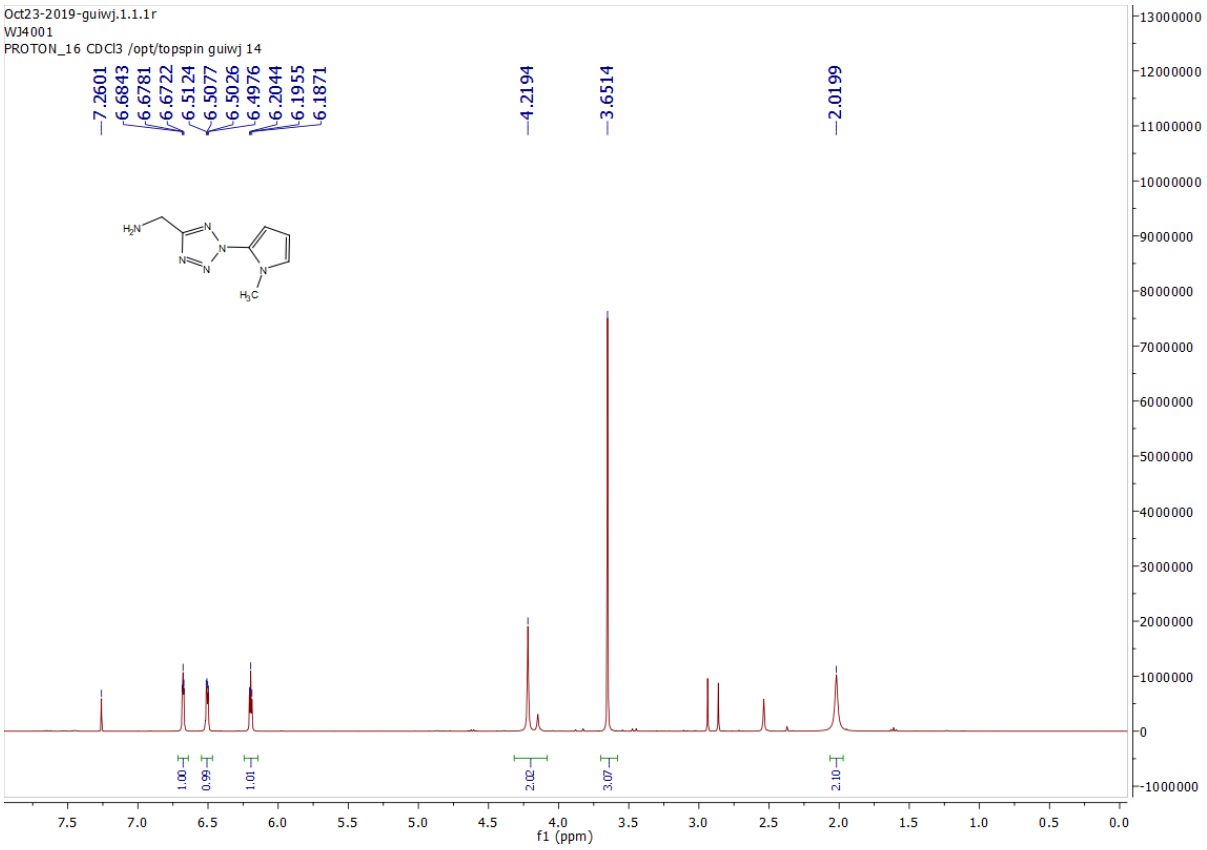
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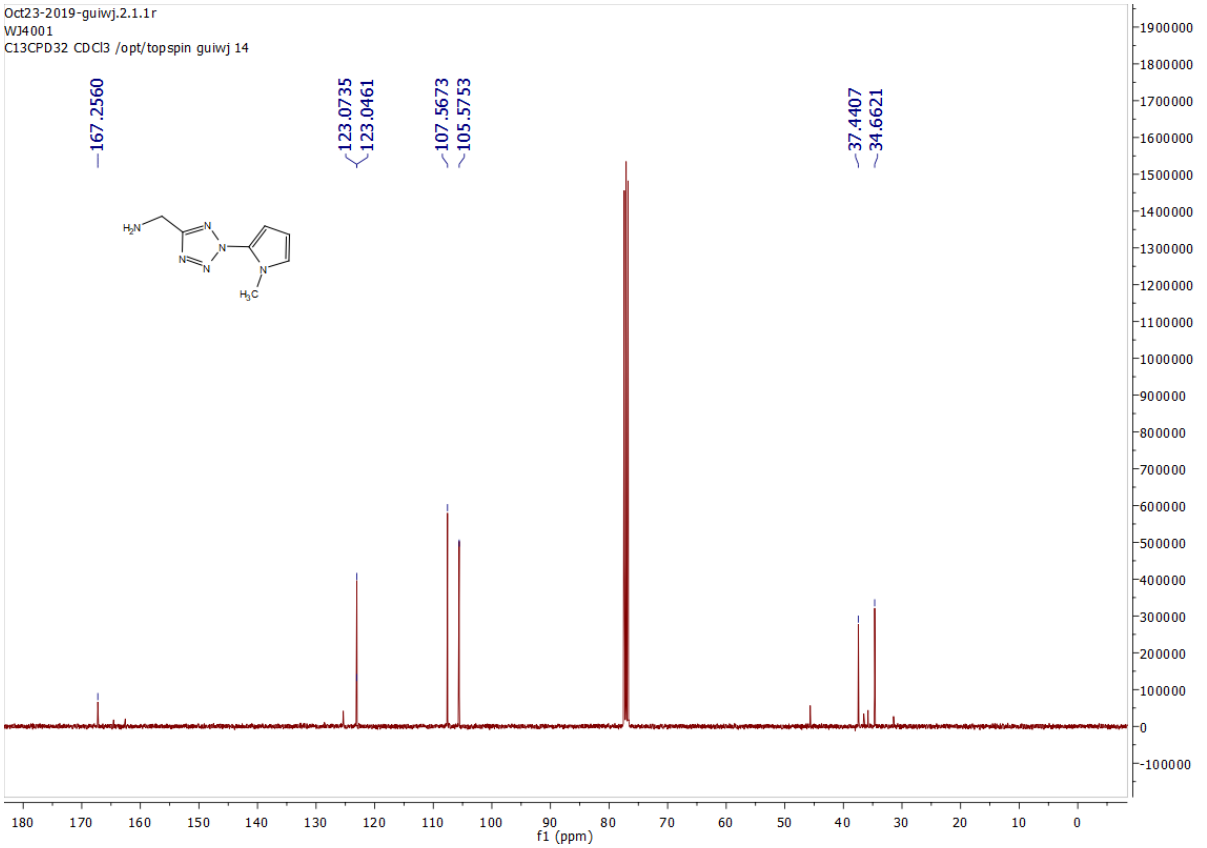
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