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1. Supporting results

1.1 Recombinant enzyme experiments

1.1.1 Time dependence of ambient light induced OTUB1 labelling

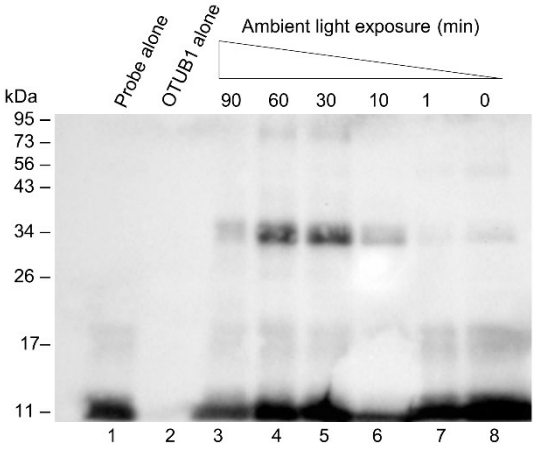


Figure S1. Time dependence of ambient light exposure for an Eosin Y mediated labelling of OTUB1 (2 µg) with probe **1** (3 µg) visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C before the addition of Eosin Y (5 µM). Samples were incubated for a further 30 min at 37 °C in ambient light.

1.2.2 Distance variation of lamp for white light (10 W) induced OTUB1 labelling

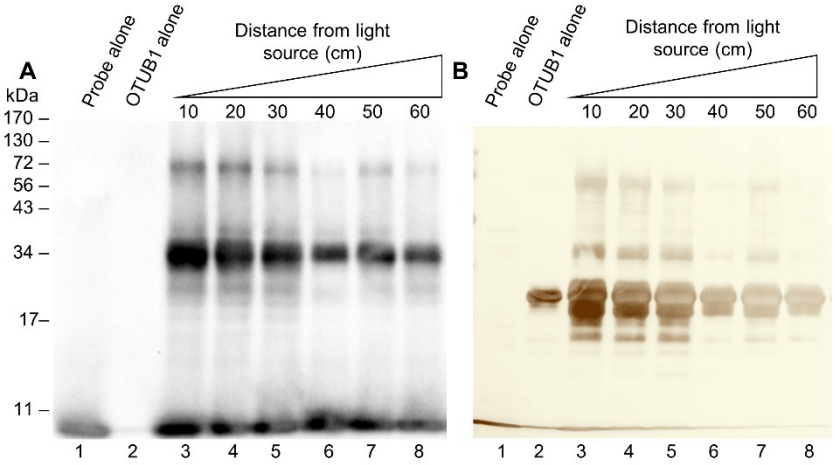


Figure S2. 10 W white light lamp distance variation for Eosin Y mediated OTUB1 (2 µg) labelling with probe **1** (3 µg) visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C before the addition of Eosin Y at the indicated concentration. Samples were incubated for a further 30 min at 37 °C in ambient light.

1.2 Optimisation of visible light mediated HEK 293T cell lysate labelling

1.2.1 Concentration dependence of ambient light induced OTUB1 labelling

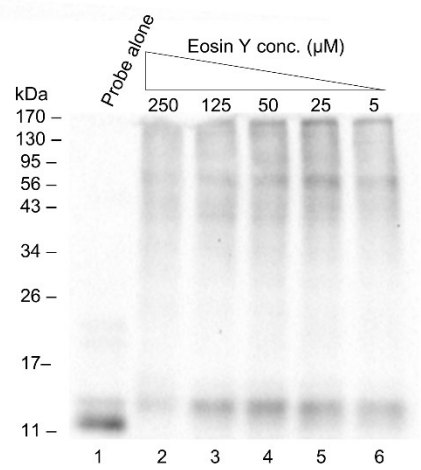


Figure S3. Time dependence of Eosin Y mediated labelling of HEK 293T (50 μg) with probe **1** (1 μg) visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C before the addition of Eosin Y at the indicated concentration. Samples were incubated for a further 30 min at 37 °C in ambient light.

1.2.2 Time dependence of ambient light induced OTUB1 labelling

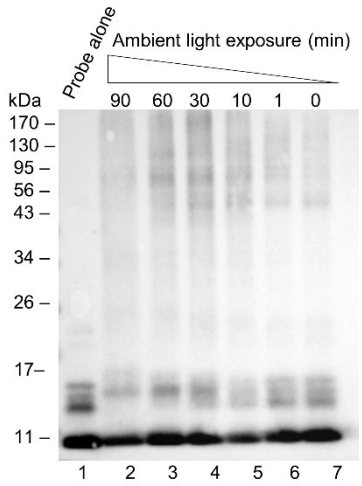


Figure S4. Time dependence of Eosin Y for a HEK 293T (50 μg) labelling with probe **1** (1 μg) visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C before the addition of Eosin Y (50 μM). Samples were incubated for a further 30 min at 37 °C in ambient light.

1.2.3 Distance variation of lamp for white light (10 W) induced OTUB1 labelling

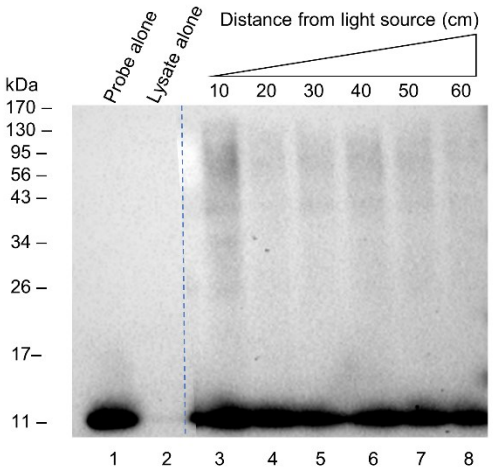


Figure S5. Eosin Y mediated labelling of HEK 293T lysate (50 µg) with probe **1** (1 µg) visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C before the addition of Eosin Y (50 µM) Samples were incubated for a further 10 min while exposed to white light (10 W) at the indicated distance.

1.2.4 Time dependence of white light (10 W) induced OTUB1 labelling

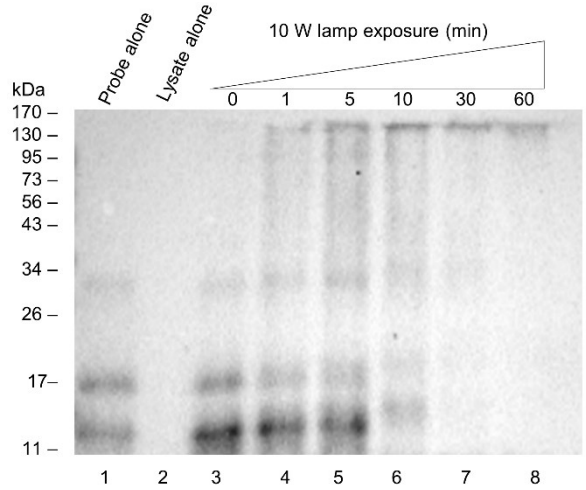


Figure S6. Time dependence of Eosin Y mediated labelling of HEK 293T lysate (50 µg) with probe **1** (1 µg) visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C before the addition of Eosin Y (50 µM) Samples were incubated for a further 10 min while exposed to white light (10 W) from 10 cm.

1.2.5 Comparative HEK 293T cell lysate labelling using optimised Eosin Y conditions relative to DPAP and MAP

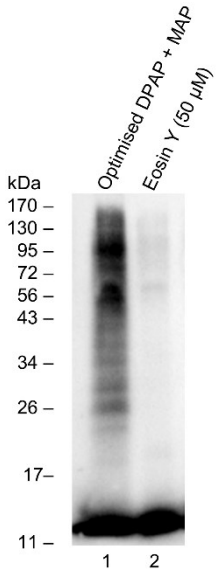


Figure S7. Comparative labelling analysis using Eosin Y as the initiator relative to DPAP and MAP for HEK 293T cell lysate (50 μg) with probe **1** (3 μg) visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C. Lanes 1 was degassed for 2 min using N2 before the addition of DPAP (0.25 mM) and MAP (0.25 mM). Lane 2 was not degassed and Eosin Y (50 μM) was added to the sample. A further incubation was carried out for 10 min at 37 °C while exposed to UV light (365 nm) for a 10 W white light lamp from 10 cm.

1.3 Fluorescent gel analysis

1.3.1 Eosin Y adduct formation in HEK 293T cell lysate experiments

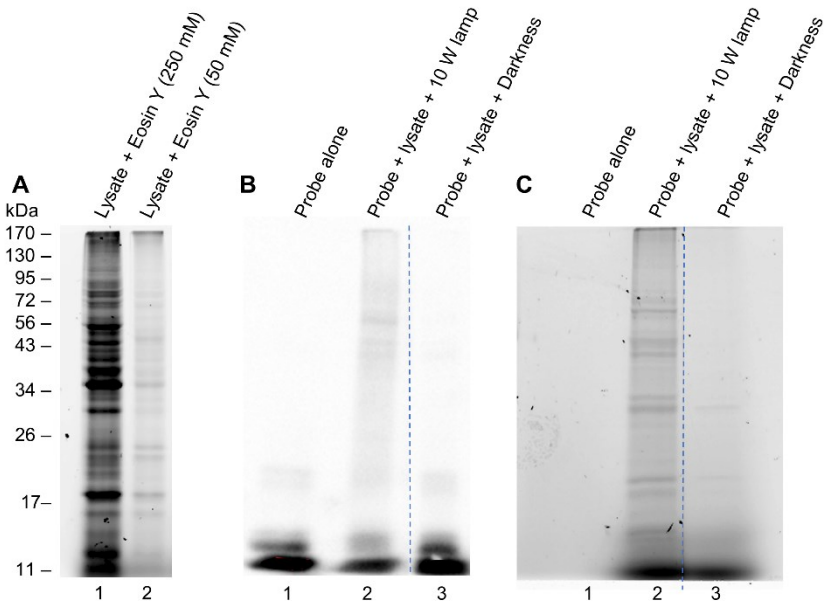


Figure S8. Formation of Eosin Y adducts in HEK 293T cell lysate following visible light excitation. Samples were visualised by (A) and (C) fluorescent imaging or (B) anti-HA western blot. Probe **1** (1 µg) was incubated with HEK 293T cell lysate (50 µg) at 37 °C with gentle shaking in homogenate buffer for 90 min. Eosin Y (50 µM or as indicated) was added and reactions were exposed to white light (10 W) from 10 cm for 10 min or kept in darkness for 10 min.

1.3.2 Eosin Y adduct formation recombinant enzyme experiments

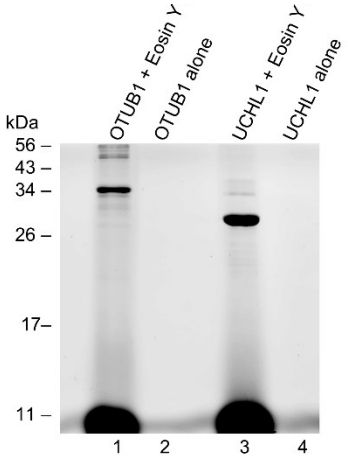


Figure S9. Formation of Eosin Y adducts with recombinant enzymes following visible light excitation. Visualised using fluorescent imaging. Probe **1** (3 µg) was incubated with OTUB1 (2 µg) or UCHL1 (2 µg) at 37 °C with gentle shaking in homogenate buffer for 90 min. Eosin Y (5 µM) was added and reactions were exposed to white light (10 W) from 50 cm.

2. General biological methods

2.1 SDS-PAGE

Proteins were separated on an acrylamide gel (resolving gel: 1.3 mL 1.5 M Tris-Cl pH 6.8, 1.5 mL 40% acrylamide/bis-acrylamide (29:1), 2 mL dH_2O, 50 µL 10% SDS, 50 µL 10% ammonium persulfate (APS), 5 µL Tetramethylethylenediamine (TEMED); stacking gel: 630 µL 0.5 M Tris-Cl pH 6.8, 300 µL acrylamide/bis-acrylamide (29:1), 1.3 mL dH_2O, 25 µL 10% SDS, 25 µL 10% APS, 2.5 µL TEMED). Samples were prepared for separation by adding 2X reducing sample buffer followed by heating at 95 °C for 5 min. The proteins were loaded along with Fisher's EZ-Run™ Pre-Stained Rec Protein Ladder. Separation was achieved at 150 V for 1-2 h in running buffer (25 mM Tris, 190 mM Gly, 1% SDS) and visualised either by western blotting or silver staining. All gels were imaged using Chemidoc XRS+ (Biorad, California USA) and Typhoon FLA9500 (GE Healthcare, Illinois USA).

2.2 Silver staining

Gels were treated with fixative (40% EtOH, 10% AcOH) at rt for 1 h or at 4 °C for 16 h. Gels were washed in 20% EtOH (2 x 10 min), then in dH_2O (2 x 10 min). Gels were sensitised in aq. $Na_2S_2O_3$ (0.02%) for 45 s and then immediately washed with dH_2O (2 x 1 min). Gels were incubated in a solution of $AgNO_3$ (12 mM) with formaldehyde (0.02%) at 4 °C for a minimum of 20 min and up to 2 h. Following this, gels were washed in dH_2O (2 x 30 s) and transferred to developer solution (3% K_2CO_3, 0.05% formaldehyde). Development was stopped using 5% AcOH.

2.3 Western Blotting

A nitrocellulose membrane (GE Healthcare, Illinois USA) was soaked in blotting transfer buffer (25 mM Tri, 190 mM Gly, 20% MeOH) along with the filter papers and sponges that will form the transfer sandwich. The sandwich was assembled, and proteins were transferred onto nitrocellulose membranes in blotting transfer buffer overnight at 15 V and 4 °C. The membrane was incubated in blocking buffer (5% skimmed milk powder in PBST) for 1 h at rt or 16 h at 4 °C prior to immunoblotting. The primary mouse monoclonal anti-HA antibody (Biolegend, California USA) was diluted 1:2,000 in blocking buffer and incubated with the membrane for 1 h at rt with

gentle shaking. The membrane was washed with PBST (2 x 5 min) and PBS (2 x 5 min). The secondary antibody, Peroxidase conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, Cambridgeshire UK), was diluted in blocking buffer 1:4,000, added to the membrane and incubated for 1 h at rt with gentle shaking. The membrane was washed with PBST (3 x 5 min), PBS (2 x 5 min) and dH_2O (1 x 5 min). Pierce ECL western blotting substrate (Thermofisher, Massachusetts USA) was used to visualise the chemiluminescence.

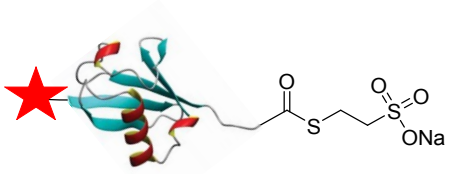
2.4 Expression and purification of OTUB1

The expression and purification of His tagged OTUB1 Cys91Ser mutant was carried out according to literature procedures.1, 2 BL21 (DE3) cells transfected with a pET28a-LIC vector containing an N-terminal His6 tagged OTUB1 was transferred from a glycerol stock into LB medium (8 mL) containing kanamycin (100 µg/mL) and grown for 18 h at 37 °C at 180 rpm. The cells were transferred into fresh LB medium (300 mL) containing kanamycin (100 µg/mL) and grown at 37 °C at 180 rpm until an OD600 of 0.6 to 0.9 was reached. IPTG was added at a final concentration of 0.4 mM and the bacteria were incubated at 18 °C for 16 h with vigorous shaking. The cells were centrifuged at 6,000 rpm for 15 min. The resulting pellet was re-suspended in homogenisation buffer (25 mL) containing PMSF (20 µM) and lysed via sonication. The lysate was centrifuged at 13,000 rpm for 45 min. Ni NTA agarose resin (Sigma-Aldrich, Missouri, USA) (1.5 mL, 1:1 suspension in 20 % EtOH) was centrifuged at 2,200 rpm for 5 min and the supernatant was discarded. dH2O (2 x 0.7 mL) was added to the beads which were gently inverted until the resin was fully resuspended. The resulting solution was centrifuged at 2,200 rpm and the supernatant was discarded. Ni wash buffer (1.4 mL, 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole) was added to the resin which was transferred to the clarified supernatant and incubated overnight at 4 °C with rolling. The resin was centrifuged at 2,200 rpm and the supernatant was discarded. Ni wash buffer (1 mL) was added to the resin which was fully resuspended by gentle inversion and centrifuged at 2,200 rpm for 5 min. This wash step was repeated four times. The supernatant was discarded after each washing step. Ni elution buffer A (4 x 0.7 mL; 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 150 mM imidazole) was added to the resin that was resuspended by gentle inversion. The solution was centrifuged at 2,200 rpm for 5 min and the supernatants from these washes were pooled in clean microcentrifuge tubes.

A final wash step was carried out with Ni elution buffer B (1 mL; 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 300 mM imidazole) at 2,200 rpm for 5 min. The supernatant was discarded, and the beads were stored in 20% EtOH. The pooled supernatants were concentrated in a 10 kDa MW cut-off Vivaspin 500 centrifugal concentrators in a centrifuge at 9,000 rpm to a final concentration of 50 µL. Ni wash buffer (450 µL) was added to the tubes and concentrated to 50 µL in a centrifuge at 9,000 rpm. This wash step was repeated, and the solution was resuspended in storage buffer (150 µL, 20 mM Tris-Cl pH 8.0, 1mM DTT, 10% glycerol, 50 mM NaCl). The concentration was measured by nanodrop (13.78 µg/µL in 150 µL).

2.5 Synthesis of HA-tagged activity-based monoubiquitin probes

2.5.1 Expression and purification of HA-Ub_{75}-MESNa

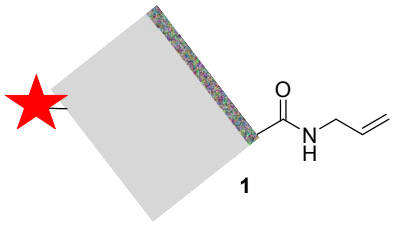


3

The expression and purification of HA-Ub_{75}-MESNa **3** was carried out according to literature procedures.[1, 2] BL21 (DE3) cells transfected with a pTYB2 plasmid encoding for a HA-tagged $ubiquitin_{75}$ fusion protein containing an intein domain and chitin-binding domain (HA-Ub_{75}-intein-CBD) were transferred from a glycerol stock into LB medium (8 mL) containing ampicillin (100 µg/mL) and grown for 18 h at 37 °C at 180 rpm. The cells were transferred into fresh LB medium (300 mL) containing ampicillin (100 µg/mL) and grown at 37 °C at 180 rpm until an OD_{600} of 0.6 to 0.9 was reached. IPTG was added at a final concentration of 0.4 mM and the bacteria were incubated at 18 °C for 16 h with vigorous shaking. The cells were centrifuged at 8,000 rpm for 15 min. The resulting pellet was re-suspended in column buffer and lysed using a sonication tip. Sonication was carried out for 5 mins with the tip set to pulse for 3 sec on and 3 sec off. The lysate was centrifuged at 14,000 rpm for 45 min. A column containing chitin resin (2.5 mL) (New England Biolabs) was equilibrated with column buffer (25 mL). The clarified supernatant was run over this column. The column was washed with column buffer (25 mL). After this wash, column buffer containing sodium 2-sulfanylethanesulfonate (MeSNa) (7.5 mL; 50 mM) was run through the column before incubation in this buffer for 18 h at 37 °C with gentle shaking. HA-Ub_{75}-MeSNa **3**

was eluted in column buffer (5 mL) before concentration by spinning at 14,000 rpm in 5 kDa MW cut-off Vivaspin 500 centrifugal concentrators (Sartorious, Göttingen Germany). HA-Ub_{75}-MeSNa was desalted using a NAP-5 column (GE Healthcare, Illinois USA) and eluted in column buffer according to manufacturer's instructions. The sample was concentrated again at 14,000 rpm using 5 kDa MW cut-off Vivaspin centrifugal concentrators and the protein concentration was measured on a nanodrop (4.8 mg/mL, 100 µL).

2.5.2 Synthesis of probe 1 (HA-Ub_{75}-Allylamine)



1

N-Hydroxysuccinimide solution (0.2 M, 45 µL) and Tris-Cl (100 mM, 10 µL, pH 7.5) were added to HA-Ub_{75}-MESNa **3** in column buffer (1.2 mg/mL, 500 µL) and incubated for 10 min at rt. Allylamine (23 µL, 0.3 mmol) was added to a solution of MeCN-H_2O (1:1, 56 µL). This solution was added to the reaction mixture and the pH was adjusted to 9.0. The reaction was incubated for 18 h at 37 °C with gentle shaking. After this time, the reaction mixture was desalted using a NAP-5 column according to manufacturer's instructions and concentrated in a 5 kDa MW cut off Vivaspin centrifugal concentrator at 14,000 rpm. The protein concentration was measured on a nanodrop (3.4 mg/mL, 100 µL).

2.6 *In vitro* DUB labelling

2.6.1 HEK 293T cell lysate preparation

A HEK 293T cell pellet was lysed using glass beads. To a 100 µL cell pellet, 100 µL of glass beads (0.5 mm, Sigma-Aldrich, Missouri, USA) were added. Homogenisation buffer (200 µL) was added. The mixture was vortexed for 20 s before being placed on ice for 90 s. This sequence was repeated 20 times. Cell debris and glass beads were pelleted by centrifuging at 14,000 rpm for 5 min. The resulting supernatant was aspirated off. The protein concentration of the clarified extract was measured by nanodrop (19.9 mg/mL, 200 µL)

2.6.2 *In vitro* thiol-ene labelling using DPAP and MAP

All thiol-ene reactions were carried out in 2 mL transparent glass vials with a PTFE/silicone septum (Sigma-Aldrich, Missouri, USA). UV light (365 nm) was applied in a Luzchem LZC photoreactor oven containing three top and six top 8 W UV lamps. The relevant alkene probe (1 – 4 µg) was incubated with HEK 293T cell lysate (2.5 µL, 19.9 mg/mL in homogenisation buffer) or OTUB1 (2 µg). The final volume of the labelling was adjusted to 30 µL with homogenisation buffer containing TCEP (1 mM) for the lysate labelling, or phosphate buffer (pH 8.0) containing TCEP (1 mM) for the OTUB1 labelling. The probes were pre-incubated with the DUBs for 90 min at 37 °C with gentle shaking before the addition of radical initiator DPAP (0.25 µM) and radical stabiliser MAP (0.25 µM). The reaction mixture was degassed for 2 min with N_2 and exposed to UV light (365 nm) for 2 min. Upon completion, 2X reducing sample buffer (30 µL) was added and the proteins were heated to 95 °C for 5 min. Proteins where visualised using silver staining and anti-HA western blotting following separation on a 12% SDS-PAGE

2.6.3 Optimised Eosin Y lysate labelling

Probe **1** (1 µg) was incubated with HEK 293T cell lysate (2.5 µL, 19.9 mg/mL in homogenisation buffer). The final volume of the labelling was adjusted to 30 µL with homogenisation buffer containing TCEP (1 mM). A stock solution of Eosin Y (2.9 mM in DMSO - homogenisation buffer, 4:1) was prepared fresh before use and protected from light. The reaction was preincubated for 90 min at 37 °C with gentle shaking before the addition of Eosin Y (0.5 µL of stock, final conc. = 50 µM). Samples were exposed to white light (10 W) from 10 cm for 5 min at 37 °C or ambient light for 30 min at 37 °C. Upon completion, 2X reducing sample buffer (30 µL) was added and the proteins were heated to 95 °C for 5 min. Proteins where visualised using silver staining and anti-HA western blotting after being separated on a 12% SDS-PAGE.

2.6.4 Optimised Eosin Y recombinant enzyme labelling

Probe **1** (2 µg) was incubated with OTUB1 (0.2 µL, 13.78 µg/µL in storage buffer) or UCHL1 (3.3 µL, 0.9 µg/µL in storage buffer). The final volume of the labelling was adjusted to 30 µL with homogenisation buffer containing TCEP (1 mM). A stock solution of Eosin Y (0.29 mM in DMSO - homogenisation buffer, 4:1) was prepared fresh before use and protected from light. The reaction was preincubated for 90 min

at 37 °C with gentle shaking before the addition of Eosin Y (0.5 µL of stock for recombinant enzyme labelling, final conc. = 5 µM). Samples were exposed to white light (10 W) from 50 cm for 5 min or ambient light for 30 min at 37 °C. Upon completion, 2X reducing sample buffer (30 µL) was added and the proteins were heated to 95 °C for 5 min. Proteins where visualised using silver staining and anti-HA western blotting after being separated on a 12% SDS-PAGE.

2.6.5 Amino acid spike OTUB1 labelling using Eosin Y

A stock solution (0.15 M in H_2O) of the amino acids Gly, Lys, Cys, Trp and Ser and the tripeptide GSH were prepared and adjusted to pH 7.5. Each amino acid or tripeptide (1 µL, for 5 mM final conc.) was added to OTUB1 (0.2 µL, 13.78 µg/µL in storage buffer) and incubated for 5 min at 37 °C. Probe **1** (1.2 µL, 3.4 mg/mL in column buffer) was added to the reaction and the final volume of the labelling was adjusted to 30 µL with homogenisation buffer containing TCEP (1 mM). The solution was preincubated for 90 min at 37 °C with gentle shaking before the addition of Eosin Y (0.5 µL of 0.29 mM stock, final conc. = 5 µM). Samples were exposed to white light (10 W) from 50 cm for 5 min. Upon completion, 2X reducing sample buffer (30 µL) was added and the proteins were heated to 95 °C for 5 min. Proteins where visualised using silver staining and anti-HA western blotting after being separated on a 12% SDS-PAGE.

3. Full images of gels in the main body

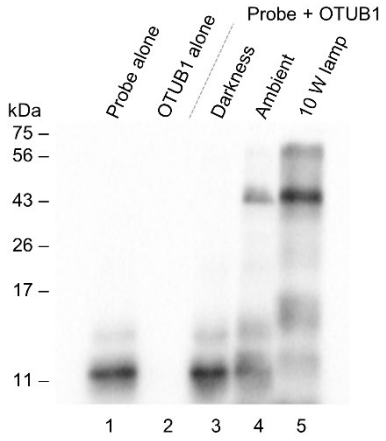


Figure S10. (Full image of main body Figure 1). Labelling of OTUB1 (2 µg) with probe **1** (3 µg) visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C before the addition of Eosin Y. Samples were incubated for a further 10 min at 37 °C in the dark (lane 3) in ambient light (lane 4) or exposed to a 10 W white light lamp from 10 cm (lane 5).

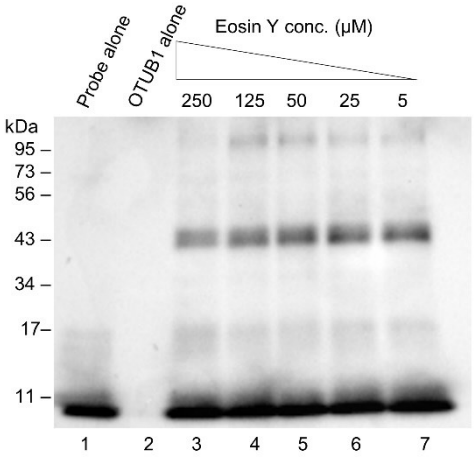


Figure S11. (Full image of main body Figure 2). Concentration dependence of Eosin Y for an OTUB1 (2 μg) labelling with probe **1** (3 μg) visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C before the addition of Eosin Y at the indicated concentration. Samples were incubated for a further 30 min at 37 °C in ambient light.

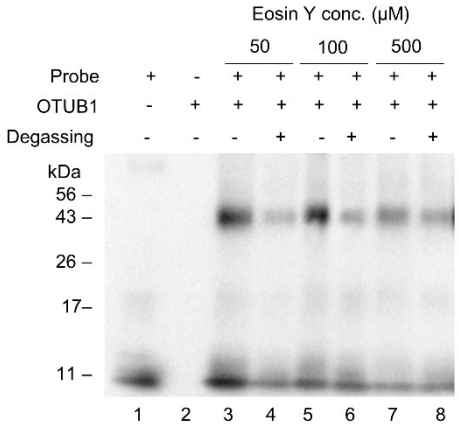


Figure S12. (Full image of main body Figure 3A). The effect of degassing on OTUB1 (2 μg) labelling with probe **1** (3 μg) visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C. Lane 4, 6 and 8 were degassed for 2 min using N_2 before Eosin Y was added to all samples. A further incubation was carried out for 10 min at 37 °C while exposed to a 10 W white light lamp from 10 cm.

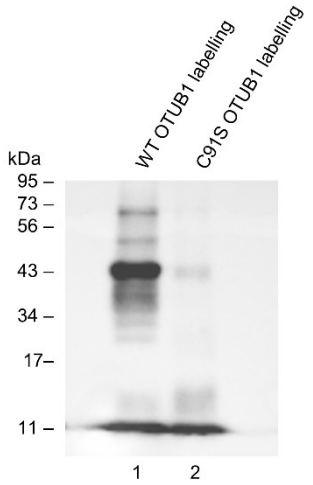


Figure S13. (Full image of main body Figure 3B). Eosin Y mediated labelling of (A) WT OTUB1 (2 µg) or (B) C91S OTUB1 (2 µg) with probe **1** (3 µg) visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C before the addition of Eosin Y (5 µM). Samples were incubated for a further 30 min at 37 °C in ambient light.

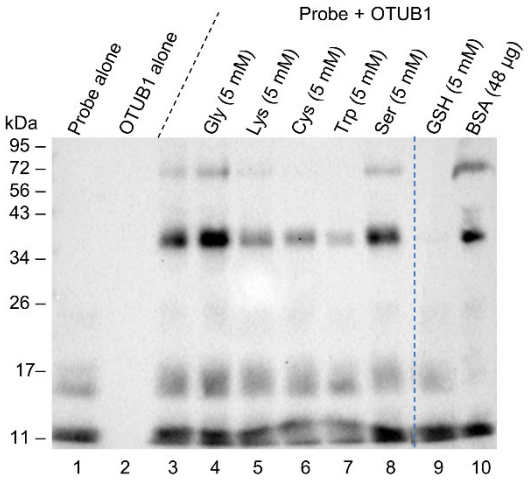


Figure S14. (Full image of main body Figure 5). The effect of individual amino acids, GSH and BSA on OTUB1 (2 µg) labelling with probe **1** (3 µg) using Eosin Y, visualised by anti-HA western blot. Samples were preincubated for 90 min at 37 °C with the probe and the indicated additive. Eosin Y was added to all samples and a further incubation was carried out for 10 min at 37 °C while exposed to a 10 W white light lamp from 10 cm.

References

1. Chong, S.; Mersha, F. B.; Comb, D. G.; Scott, M. E.; Landry, D.; Vence, L. M.; Perler, F. B.; Benner, J.; Kucera, R. B.; Hirvonen, C. A.; Pelletier, J. J.; Paulus, H.; Xu, M. Q., Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene* **1997,** *192* (2), 271-81.

2. Borodovsky, A.; Ovaa, H.; Kolli, N.; Gan-Erdene, T.; Wilkinson, K. D.; Ploegh, H. L.; Kessler, B. M., Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. *Chem Biol* **2002,** *9* (10), 1149-59.