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COMMUNICATION

Fluorescence-based active site probes for profiling deubiquitinating enzymes†

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Novel ubiquitin-based active site probes including a fluorescent tag have been developed and evaluated. A new, functionalizable electrophilic trap is utilized allowing for late stage diversification of the probe. Attachment of fluorescent dyes allowed direct detection of endogenous deubiquitinating enzyme (DUB) activities in cell extracts by in-gel fluorescence imaging.

Ubiquitin (Ub)[1] is a small ~8.5 kDa protein which is covalently attached to other cellular proteins as a post-translational modification constituting either mono-ubiquitination or poly-ubiquitin chains depending upon the modes and multiplicity of attachment.[1] Ubiquitination occurs to a vast array of structurally important and regulatory proteins modulating their lifespan, activity, translocation and interactions with other molecules.

The most well characterized function of ubiquitination is to target proteins for proteasomal degradation within the cell.[3] This process is carried out by ubiquitin conjugating enzymes (ligases), acting in tandem with deubiquitinating enzymes (DUBs), to create a dynamic equilibrium that is critical for many cellular processes. This equilibrium is often perturbed in the context of disease pathologies such as infection, autoimmunity, cancer and neurodegeneration. There is increasing evidence for a pivotal regulatory role of DUBs in these processes, and they are increasingly recognized as attractive targets for drug design.[4,5]

Functional studies of DUBs are challenging due to their frequently overlapping specificities and potential redundancies. Ubiquitin-derived probes based on di-ubiquitin or ubiquitin fusions have proven extremely useful research tools to investigate their biological roles and specificities, but are limited to *in vitro* enzymatic assays using recombinant enzymes.[6–8] To overcome this, site-directed probes were designed using both traditional protein chemistry[9] and intein-based approaches.[10] Activity-based ubiquitin probes are specifically engineered fusion proteins that function by presenting an electrophilic trap in the place of the isopeptide bond between the *N*-lysyl side chain and the C-terminal carboxylate of Ub which is present in most ubiquitinated proteins. Thus features of natural DUB substrates are mimicked aiding covalent trapping of the active site thiol present in the catalytically active site of DUBs that are predominately cysteine proteases. The resulting covalent probe–DUB thioether conjugate is suitable for profiling, isolation and biochemical characterization from cell extracts. This approach is a highly sensitive readout for DUBs and to a certain extent ubiquitin ligases. A further advantage of this activity-based assay is that detection of DUBs using active-site molecular probes is not merely an abundance measurement, it provides information about the level of expression of individual enzymes as well as their activity.[11,12]

Whilst the previously described probes[10] have proven to be highly useful tools, there also remains scope for further expansion and refinement of this concept. Fluorescent tagging offers numerous advantages for profiling active DUBs in cell extracts as well as ultimately facilitating microscopy-based readouts in intact cells when combined with protein transfection or permeabilisation protocols.[13–16] Potential applications include a quicker detection enabled by fluorescence imaging *versus* immuno detection, multiplexing capability inherent to the use of different fluorophores and the scope for intracellular detection within intact cells.

Development of fluorescent probes can be achieved by the incorporation of the fluorescent group within the electrophilic trap section of the molecule. We reasoned that placing a fluorescent moiety at the P′ position of the scissile bond should be feasible as this likely represents the location of a natural DUB substrate protein in many instances. This strategy possesses the advantage of requiring minimal protein modification affording a facile and generally applicable route compared to alternate strategies, leaving the ubiquitin scaffold intact. With this goal in mind we set out to design a probe including a bio-orthogonal handle which could be utilized to increase the scope of the existing probes *via* late stage functionalization. Also the use of a linker functionalizable by "click" chemistry which can be readily used to attach a variety of functional moieties can be further exploited to expand and diversify the panel of existing ubiquitin-based probes.

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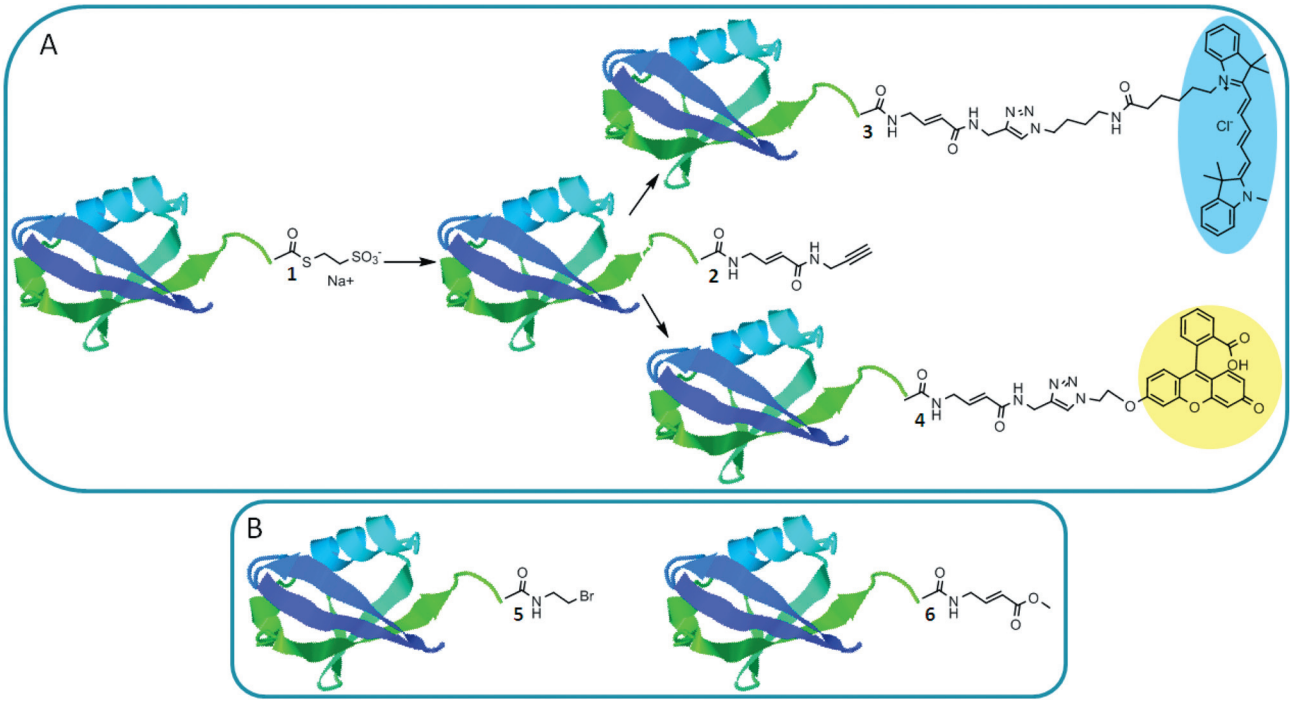


Fig. 1 Novel fluorescence based HA-Ub active site probes. (A) Synthetic route and structures of fluorescent probes (**3**–**4**). Ubiquitin is shown as ribbon structure (green-blue), and the fluorescent Cy 5 and fluorescein moieties are highlighted in blue and yellow respectively. (B) Established probes HA-Ub-Br (**5**) and HA-Ub-VME (**6**) as described previously.[2]

Results and discussion

HA-Ub-thioester **1** was converted to alkyne probe **2** in moderate (30–40%) yield followed by purification *via* strong cation exchange (SCX) high performance liquid chromatography (HPLC, Fig. S1†). With purified probe **2** in hand the Cu(I) catalyzed [3 + 2] cyclo addition[17] was performed with both fluorescein and Cy 5 derived azides (Fig. 1). The reactions and subsequent desalting (PD-10) for removal of unbound fluorophore afforded fluorescent probes **3** and **4** respectively. All probes were characterized by MALDI-TOF (Fig. 2) and SDS-PAGE analysis (data not shown).

Having successfully prepared fluorescent probes **3** and **4**, we then went on to test their activity and properties. One advantage of fluorescent probes **3** and **4** over previously published probe concepts is the much quicker gel-based read out afforded by fluorescence detection in comparison to silver stain or immunoblotting techniques. In an initial experiment, we labelled recombinant DUB UCH-L3 with our fluorescent probe **4** and examined the resulting gel by both fluorescence detection and silver stain. In comparison the HA-Ub-Br probe **5** was also reacted with UCH-L3 as a control (Fig. 3).

Our results demonstrate that UCH-L3 reacted with fluorescein conjugated probe **4** in a stoichiometric fashion and could be detected by UV transillumination with approximately the same sensitivity as the silver stain method. This shows that fluorescent probe **4** is not only reactive towards this DUB but also that the fluorescent DUB–probe conjugate could be readily detected without specialized equipment. Encouraged by this initial result an assay to label active DUBs in whole cell lysates using this probe was conducted, however the extent of the fluorescence based detection was limited (data not shown). A comparison of

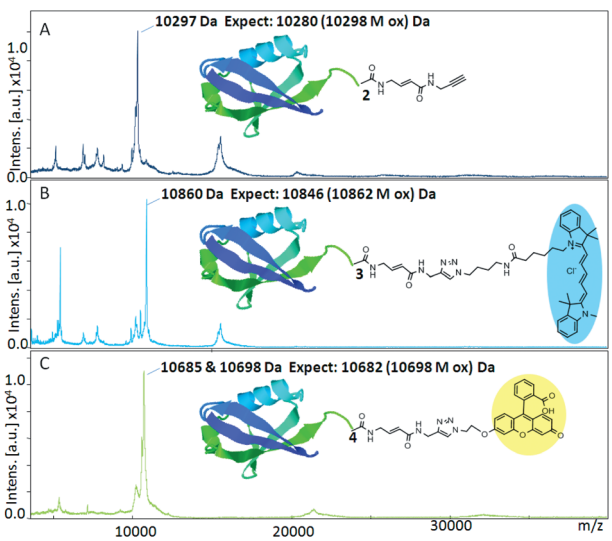


Fig. 2 Characterization of novel HA-Ub active site probes **2**–**4**. (A) Intact mass spectrum of **2**. (B) Intact mass spectrum of **3**. (C) Intact mass spectrum of **4**. Expected $[M + H]^+$ quoted, methionine oxidation product (M ox) expected mass quoted in parentheses.

labelling pattern and efficiency between Cy 5 fluorescent probe **3** and vinyl methyl ester (methyl butenoate) probe HA-Ub-VME **6** not only demonstrated comparable sensitivity of detection to anti-HA immunoblotting but also exhibited a better resolution of high molecular weight bands (Fig. 4). The observed increase in resolution is of particular advantage when inspecting DUBs found in the 100–250 kDa region, where the majority of ubiquitin specific proteases (USPs) are detected. Controls carried out with non-fluorescent HA-Ub-VME probe **6** and the negative

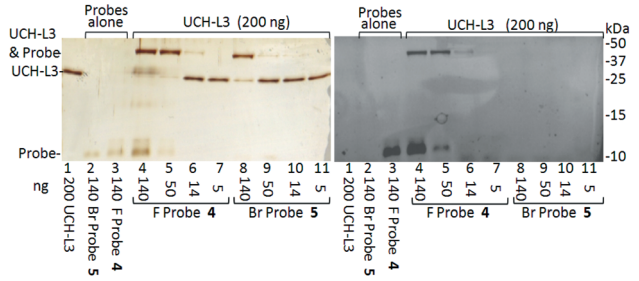


Fig. 3 Comparison of silver stain *versus* fluorescence-based detection of labelling UCH-L3 with HA-Ub-based active site probes. Silver stain (left panel) and fluorescent image (right panel) of UCH-L3 labelling using fluorescein conjugated probe (F) **4** as compared to HA-Ub-Br **5** in a dose-dependent fashion.

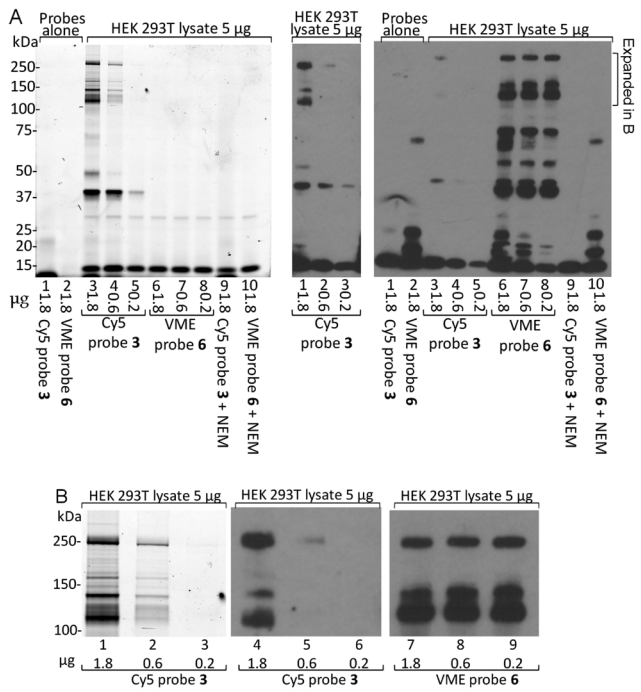


Fig. 4 Fluorescence-based detection of active DUBs in crude cell extracts. (A) SDS-PAGE and fluorescence- (left panel) or immunoblotting- (middle and right panels at long and short exposure times respectively) based detection of DUBs present in whole cell lysates labelled with HA-tagged Cy 5 probe **3** or HA-Ub-VME **6** in a dose-dependent fashion. (B) Expansion of the 100–250 kDa region showing fluorescence- (left panel) or anti HA immunoblotting- (middle and right panel at long and short exposure times respectively) based detection.

control reaction in the presence of 200 µM NEM, a competitive cysteine alkylating agent showed the fluorescence readout to be clearly specific for detection of active DUBs (Fig. 4). The overall labelling profile of DUBs appears to differ between the Cy 5 probe **3** and HA-Ub-VME **6**, indicating that the presence of a fluorophore at the P′ position of the Ub C-terminal cleavage site does have subtle effects on probe specificity. The lower general reactivity observed by immunoblotting between these two probes (Fig. 4) is likely due to the stereoelectronic effects of the Cy 5 fluorophore rather than the change in nature of Michael acceptor from an ester (**6**) to an amide (**3**) containing moiety. This is corroborated by the similar strength of labelling observed in immunoblotting with HA-Ub-VME **6** compared to amide containing alkyne probe **2** (data not shown).

This higher resolution profiling of probe–DUB conjugates coupled with a quicker turn-around time makes DUB probes with a C-terminally incorporated fluorophore a valuable tool in the screening of putative DUB inhibitors. In order to demonstrate the potential in inhibitor screening we treated HEK 293T cell lysate with varying concentrations of the general DUB inhibitor PR619[8,18] (Fig. 5). Qualitative comparisons can be made by inspection of the fluorescence gel readout allowing for fast screening of potential DUB inhibitors. The differential inhibition of two probe reactive species has been highlighted (Fig. 5, panel B) and the loss of fluorescence signal indicating loss of activity toward the probe has been measured (Fig. 5, panel C).

Conclusions

In conclusion, we have presented a brief, facile route to novel fluorescent DUB probes **3**–**4** and demonstrated their activity both with recombinant DUB labelling and in whole cell lysates. DUB enzymatic activity can be visualized using fluorescence detection to give a sensitive, high resolution, rapid gel-based readout. The incorporation of a Cy 5 based fluorophore allows detection in the low ng range and quantification with a dynamic range over two orders of magnitude.[19] In addition to that, traditional protein detection methods such as silver staining or immunoblotting can still be applied. The utility and advantages of novel ubiquitin based probe **3** for screening of DUB inhibitors in cell culture models has also been demonstrated. Furthermore, the scope for imaging of DUB probes within whole cells *via* the use of such fluorescent probes warrants further investigation.

Experimental

Thin layer chromatography (TLC) was carried out on Merk Kieselgel 60F254 precoated aluminium backed plates. Visualization of TLC plates was accomplished with 254 nm UV light and/or ninhydrin (0.2% in ethanol) or potassium permanganate (0.5% in 1 M NaOH). Flash column chromatography was conducted using silica gel (Fluka Kieselgel 60 220–440 mesh). All solvents were used as supplied (analytical or HPLC grade), without further purification. Petrol refers to the fraction of petroleum ether boiling in the range 40–60 °C. Ultrapure water was used for buffers and chemical and biochemical reactions. Reagents were purchased from Aldrich and used as supplied. Precision plus protein standard all blue gel marker (Biorad) was used.

Proton and carbon nuclear magnetic resonance (δH, δC) spectra were recorded on a Bruker DPX 200 (200 MHz), all chemical shifts were quoted in the δ-scale in ppm using residual solvent as the internal standard. Low resolution mass spectra were recorded on a Micromass Platform 1 mass spectrometer using electron spray ionization (ES) with methanol as the carrier solvent. High resolution mass spectra were recorded using a Micromass LCT (resolution = 5000 FWHM) using a lock spray source. The calibration is corrected using a lock-mass. In positive ion this is tetraoctylammonium bromide. *m*/*z* Values are reported in Daltons.

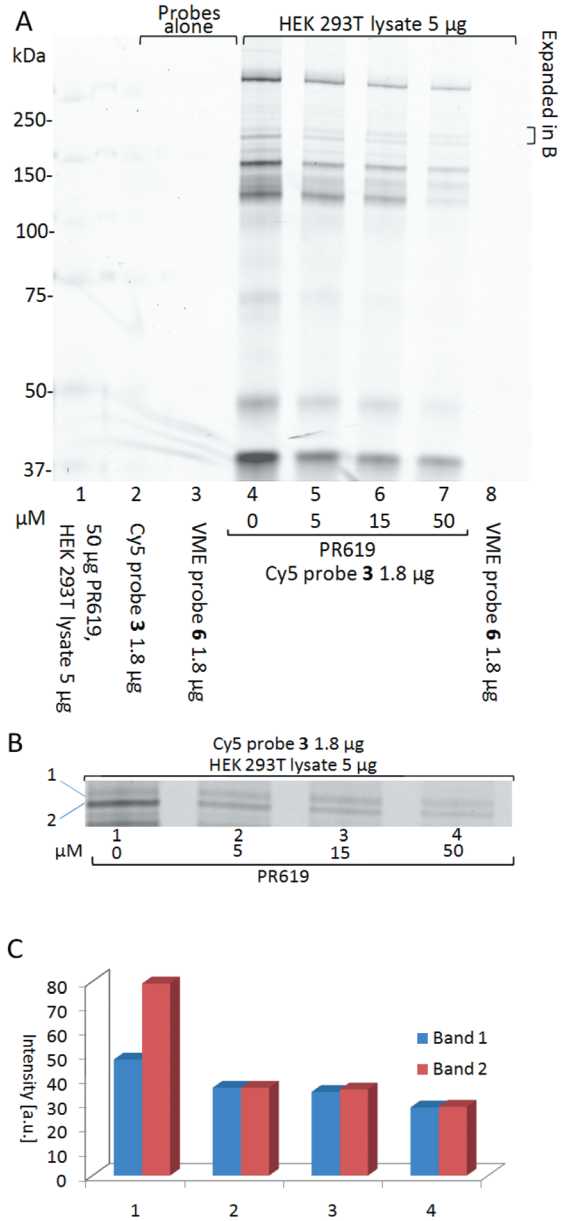


Fig. 5 Fluorescence-based detection of active DUBs in crude cell extracts treated with DUB inhibitor PR619. (A) Fluorescence based detection of DUBs present in whole cell lysates labelled with Cy 5 probe **3** or HA-Ub-VME **6** in a PR619 dose-dependent fashion. (B) Expansion within the 150–250 kDa region showing fluorescence-based detection. (C) Quantitation of fluorescence labelled bands **1** and **2** respectively at different PR619 concentrations.

(*E*)-4-*N*-Boc-but-2-enoic acid 9[20]

Vinyl methyl ester **8**[21] (400 mg, 1.9 mmol) was dissolved in THF (10 mL). H_2O (6 mL) was added along with $LiOH{\cdot}H_2O$ (150 mg, 6.3 mmol) and the reaction stirred for 3 hours. The reaction progression was checked after this time by TLC (4 : 1 petrol–EtOAc) showing complete starting material consumption (R_f 0.3) and product formation (R_f 0.1). THF was removed under reduced pressure after which the aqueous solution was adjusted to pH 3 by the addition of 1 M HCl (aq.). The product was extracted with DCM (3 × 10 mL) and the combined organic layers dried with Na_2SO_4. Concentration under reduced pressure afforded 340 mg (89% yield) of desired product **9** as a white amorphous solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.02 (dt, J = 15.5 and 4.5 Hz, 1H), 5.95 (d, J = 16.3 Hz, 1H), 4.6 (bs, 1H), 3.96–3.93 (m, 2H), 1.46 ppm (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 171.0, 156.8, 147.8, 121.0, 79.5, 42.7 and 28.8 ppm. IR (neat)ν: 2978.5, 1687.1, 1523.3, 1250.6 cm^{-1}. ESI-MS m/z = 224 $(M + Na)^+$.

(*E*)-4-Amino-*N*-Boc-*N*-(prop-2-ynyl)but-2-enamide 10

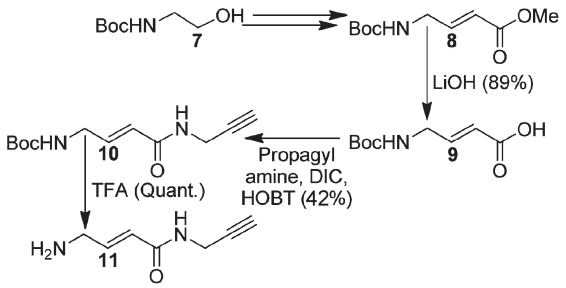
(*E*)-4-*N*-Boc-but-2-enoic acid **9** (120 mg, 0.60 mmol) was dissolved in DCM (5 mL) and cooled to 0 °C. HOBT (72 mg, 0.53 mmol) was added along with DIC (0.10 mL, 6.6 mmol) and the reaction stirred for 1 hour at 0 °C followed by a further hour at room temperature. Propargyl amine (30 mg, 6.6 mmol) was then added as a solution in DCM (2 mL) and the resulting mixture stirred overnight at room temperature. The reaction was diluted with DCM (10 mL) and washed with 5% aq. HCl (10 mL) and sat. $NaHCO_3$ (10 mL) prior to drying with Na_2SO_4. Concentration under reduced pressure followed by column chromatography (2 : 1 petrol–EtOAc to EtOAc) afforded 60 mg (42% yield) of desired product **10** as a white amorphous solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.82 (dt, J = 15.3 and 5.0 Hz, 1H), 6.40 (bs, 1H), 5.96 (dt, J = 15.4 and 1.8 Hz, 1H), 4.13 (bs, 1H), 4.08–4.06 (m, 2H), 3.83–3.80 (m, 2H), 2.04 (s, 1H), 1.13 ppm (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 157.9, 141.7, 125.4, 72.0, 69.8, 42.7, 41.4, 29.6, 28.9 and 23.8 ppm. IR (neat)ν: 3411.4, 1709.8, 1511.7, 1365.0, 1253.3 cm^{-1}. ESI-MS m/z = 261 $(M + Na)^+$. HRMS (ESI) calculated for $C_{12}H_{18}N_2NaO_3$ $(M + Na)^+$ 261.1210, found 261.1208. Anal. Calcd for $C_{12}H_{18}N_2O_3$ C, 60.49; H, 7.61; N, 11.76. Found C, 60.37; H, 7.54; N, 11.8.

(*E*)-4-Amino-*N*-(prop-2-ynyl)but-2-enamide 11

(*E*)-4-Amino-*N*-Boc-*N*-(prop-2-ynyl)but-2-enamide **10** (40 mg, 0.17 mmol) was dissolved in DCM (6 mL). TFA (2 mL) was added and the reaction stirred for 1 hour, after this time TLC (3 : 2 petrol–EtOAc) showed complete starting material consumption (R_f 0.8) and product formation (R_f 0.05). The solution was concentrated under reduced pressure, with re-suspension and co-evaporation from toluene (3 × 5 mL) to afford the desired product **11** in quantitative yield as an off-white amorphous solid (Scheme 1). The product was reacted immediately without further purification. ^{1}H NMR (200 MHz, CD_3OD): δ 7.02 (dt, J = 15.9 and 5.8 Hz, 1H), 6.23 (dt, J = 16.0 and 1.7 Hz, 1H), 4.79 (bs, 1H), 3.46 (d, J = 5.7 Hz, 2H), 3.37 (s, 1H), 2.03–2.00 (m, 2H). ^{13}C NMR (50 MHz, CD_3OD): δ 166.1, 141.2, 125.8, 78.9, 76.9, 53.6 and 41.3 ppm. IR (neat)ν: 2926.3, 1708.5, 1669.8, 1509.0, 1367.0, 1253.3 cm^{-1}. ESI-MS m/z = 139.0 $(M + H)^+$.

Synthesis and purification of HA-Ub-alkyne 2

HA-Ub75-MeSNa **1** was expressed and purified according to literature procedures[2] *via* a HA Ub75-intein-CBD fusion protein to afford a final protein concentration of 4.5 mg mL^{-1} as determined by Bradford assay. 3 mL of thioester **1** was treated with



Scheme 1 Synthesis of **11**.

360 μL of a solution containing 2 M NHS and 1 M NaOH, 75 μL of 1 M Tris base (pH 7.4) was added followed by 360 μL of 1 : 1 MeCN–0.5 M NaOH containing 0.25 M alkyne **11**. The reaction mixture was incubated at 37 °C for three hours, prior to buffer exchange to 50 mM NaOAc (pH 4.5) and concentration. Alkyne probe **2** was purified to >95% purity using a SCX biomonolith column (5.2 × 4.95 mm, Agilent) with a linear gradient from 0% to 100% B, 50 mM NaOAc (pH 4.5, buffer A), 50 mM NaOAc (pH 4.5) and 1 M NaCl (buffer B) at a flow rate of 0.5ml min^{-1} using HPLC (Agilent 1100) (Fig. S1†). Product containing fractions were concentrated and probe **2** was analyzed by MALDI-TOF (Bruker) in linear mode using an α-cyano-4-hydroxycinnamic acid matrix (Fig. 2). A further aliquot was subjected to digestion by trypsin and the resulting peptides analyzed by LC-MS/MS (Fig. S2†).

Synthesis of fluorescent Ub probes 3–4

750 μL of Na_2PO_4/NaH_2PO_4 (50 mM, pH 8) containing a final concentration of 1.1 mg mL^{-1} of alkyne probe **2** was treated with the corresponding fluorescein (synthesis described in ESI†) or Cy 5 azide (Lumiprobe) (1.24 μmol in 25 μL MeCN) followed by tris[(1-ethoxycarbonylmethyl-1*H*-1,2,3-triazol-4-yl)-methyl] amine[22] (0.5 mg in 25 μL MeCN) then Cu(I)Br (0.6 mg, 4.2 μmol in 25 μL MeCN). The reaction mixtures were shaken at room temperature for 30 min prior to desalting (PD-10, GE Healthcare) and concentration. Analysis was carried out by linear mode MALDI-TOF analysis (Bruker).

DUB labelling assays. Recombinant UCH-L3 (Progenra/Lifesensors, 200 ng) was resuspended in 2 μL of Tris buffer (0.5 M, pH 7.4). Varying concentrations of either HA-Ub-Br probe **5** or fluorescent probe **4** were added and the total volume adjusted to 15 μL with 0.5 M Tris buffer, pH 7.4 containing 1 mM DTT. Solutions were incubated at 37 °C for 1 hour, followed by addition of 15 μL of reducing sample buffer and heating to 95 °C for 5 min. Samples were resolved by 18% reducing SDS-PAGE gel and detected using an Autochemi™ UVP bioimaging system. Gels were scanned with UV transillumination using a green (515–570) filter and images captured using a Hamamatsu charged coupled device (CCD) camera. Subsequent silver staining was conducted using standard protocols. HEK293T cell pellets were lysed at 0 °C using glass beads in homogenisation buffer (50 mM Tris, pH 7.5 containing 5 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT and 2 mM ATP) as described.[9] The resulting suspension was centrifuged at 14 000 rpm for 10 minutes at 4 °C. The supernatant was collected and the protein concentration determined by Bradford assay. 5 μg of lysate was incubated with 1.8–0.2 μg of either Cy 5 probe **3** or HA-Ub-VME probe **6** in the presence or absence of NEM (200 nM) in a total volume of 20 μL. Reactions were incubated at 37 °C for 3 hours prior to addition of 20 μL of reducing sample buffer and heating to 95 °C for 5 min. Samples were resolved by 4–12% reducing SDS-PAGE gel and detected by both fluorescence detection using a GE Healthcare Typhoon™ 9400 system with a red laser (633 nm) for excitation and a 670 nm wavelength filter, and western blotting methods using a directly coupled HA-HRP antibody (1 : 10 000 dilution, Sigma Aldrich H6533). PR619 (Progenra/Lifesensors) (0–50 μg) was incubated with cell lysate for 30 min at 0 °C prior to probe labelling and detection as described above.

Acknowledgements

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