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(54) Title: ACTIVE SITE PROBES

(57) **Abstract**: According to the present invention, there is provided a probe for use in the detection of a deubiquitinating enzyme, wherein the probe comprises: a protein comprising an amino acid sequence of a ubiquitin or ubiquitin-like protein; and a group of the formula -L-Z attached at the carboxy terminus of said amino acid sequence, wherein L is a linker group comprising an electrophilic moiety which is capable of covalently binding to an active site of a deubiquitinating enzyme; and Z is a detectable label or a conjugated substance; and wherein the probe comprises at least one detectable label. Also provided are processes for producing said probes, methods of using said probes, and kits comprising said probes.

ACTIVE SITE PROBES

Field of the Invention

The present invention relates to probes which are suitable for use in the detection of deubiquitinating enzymes (DUBs). Processes for producing the present probes, methods of using the probes, and kits comprising the probes are also provided. The probes of the present invention may find application in the fields of molecular biology and proteomics, and may be particularly useful for screening test agents to identify potential DUB inhibitors.

Background to the Invention

Ubiquitin (Ub) is a small 76 amino acid 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. 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. This process is carried out by ubiquitin conjugating enzymes (ligases), acting in tandem with 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 recognized as attractive targets for drug design.

Functional studies of DUBs are challenging due to their frequently overlapping specificities and potential redundancies. Ubiquitin-derived probes based on di-ubiquitin or ubiquitin fusion proteins have proven extremely useful research tools to investigate the biological roles and specificities of DUBs, but such probes can only be used in *in vitro* enzymatic assays using recombinant enzymes.

In order to overcome these limitations, site-directed probes have been designed using both traditional protein chemistry and intein-based approaches. 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 that is present in the catalytically active site of DUBs, which are predominantly cysteine proteases. The resulting covalent probe-DUB thioether conjugate is suitable for profiling, isolation and biochemical characterization from cell extracts. Examples of such probes are described in US 2005/0277762, Borodovsky *et al* (Chemistry & Biology, 9, 1149-1159, 2002) and Altun *et al* (Chemistry & Biology, 18, 1401-1412, 2011).

However, whilst the previously described probes have proven to be highly useful tools, there remains a need to expand and diversify the existing panel of Ub-based probes. In particular, there exists a need for probes which allow for the direct detection of endogenous DUB activities in cell extracts or intact cells, and for probes which can trap and aid in the identification of DUBs that are specific for different Ub-linkages or which are specific for defined substrates in a living cell environment, tasks which are presently difficult to achieve. There also exists a need for analytical techniques which are more rapid than conventional techniques and which have improved multiplexing capability.

Weikart *et al* (ChemBioChem, 11, 774-777, 2010) describe a chemical conjugation approach to site-specifically introduce stable ubiquitin-like (Ubl) modifications into fully recombinant substrate proteins through the use of a bioorthogonal azide-alkyne cycloaddition reaction.

Eger *et al* (J. Am. Chem. Soc., 132, 16337-16339, 2010) describe a method for synthesising site-specifically linked Ub dimers by reaction between an azide-functionalised Ub (AhaUb) and an alkyne-functionalised Ub (PlkUb).

Summary of the Invention

According to the present invention, there is provided a probe for use in the detection of a deubiquitinating enzyme, wherein the probe comprises:

a protein comprising an amino acid sequence of a ubiquitin or ubiquitin-like protein; and

a group of the formula -L-Z attached at the carboxy terminus of said amino acid sequence, wherein L is a linker group comprising an electrophilic moiety which is capable of covalently binding to an active site of a deubiquitinating enzyme; and Z is a detectable label or a conjugated substance;

and wherein the probe comprises at least one detectable label.

Also provided are processes for producing the present probes, methods of using the probes, and kits comprising the probes.

Brief Description of the Drawings

Figure 1 provides a comparison of silver stain (left panel) versus fluorescence-based detection (right panel) of UCH-L3 labelling using fluorescein conjugated probe (F) **4** as compared to HA-Ub-Br probe **5** in a dose-dependent fashion.

Figure 2 shows the results of an experiment in which active DUBs were detected in crude cell extracts by fluorescence-based detection. Shown are: A) results obtained by SDS-PAGE and fluorescence-based detection (left panel) and immunoblotting-based detection (middle and right panels, at long and short exposure times respectively) 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; and B) expansion of the 100-250 kDa region showing fluorescence-based detection (left panel) and anti-HA immunoblotting-based detection (middle and right panel at long and short exposure times respectively).

Figure 3 shows results obtained by the fluorescence-based detection of active DUBs in crude cell extracts treated with a DUB inhibitor (PR619). Shown are: A) results obtained by 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; and C) quantitation of fluorescence labelled bands 1 and 2 respectively at different PR619 concentrations.

Figure 4 shows results obtained from in-cell active DUB imaging experiments. Depicted are results obtained using: (A) HEK293T cells (controls) labelled with Hoechst and (B) cells transfected with HA-tagged ubiquitin vinyl alkyne-Cy5 (HA-Ub-VA-Cy5) using protein chariot reagent.

Figure 5 shows HPLC fractions of di-ubiquitin (Di-Ub) probe formation reactions analysed by SDS-PAGE and anti-HA western blotting. Fractions containing high excess of Di-Ub probe were concentrated (marked with dotted boxes).

Figure 6 shows: (A) OTUB2 labelling with purified M1 HA-Di-Ub and HA-Ub-alkyne probes. Reactions were separated by SDS-PAGE and visualized by silver staining and anti-HA western blotting; and (B) active or inactive M1 Di-Ub probe which was incubated with recombinant DUB or lysate, followed by SDS-PAGE separation and visualization by anti-HA western blotting.

Figure 7 shows lysate labelled with a panel of mono- and di-Ub probes. Reactions were separated by SDS-PAGE and analysed by anti-Ub western blotting. The figure shows two exposure times of the anti-HA western blot: low exposure (left panel) and long exposure (right panel).

Sequence Listing Description

SEQ ID NOs: 1-8 show amino acid sequences of ubiquitin mutants incorporating an azidohomoalanine insertion (Aha; referred to in the sequence listing as Xaa). Shown are: Aha Ub (SEQ ID NO: 1); K6Aha Ub (SEQ ID NO: 2); K11Aha Ub (SEQ ID NO: 3); K27Aha Ub (SEQ ID NO: 4); K29Aha Ub (SEQ ID NO: 5); K33Aha Ub (SEQ ID NO: 6); K48Aha Ub (SEQ ID NO: 7); and K63Aha Ub (SEQ ID NO: 8).

SEQ ID NOs: 9-16 show the nucleic acid sequences of the mutant constructs. Shown are the nucleic acid sequences of constructs encoding: WT Ub (SEQ ID NO: 9); K6M Ub (SEQ ID NO: 10); K11M Ub (SEQ ID NO: 11); K27M Ub (SEQ ID NO: 12); K29M Ub (SEQ ID NO: 13); K33M Ub (SEQ ID NO: 14); K48M Ub (SEQ ID NO: 15); and K63M Ub (SEQ ID NO: 16).

SEQ ID NOs: 17-32 show the nucleic acid sequences of the forward and reverse primers used for generating the Aha Ub mutants. Shown are: A pos 2 forward primer (SEQ ID NO: 17); K6M forward primer (SEQ ID NO: 18); K11M forward primer (SEQ ID NO: 19); K27M forward primer (SEQ ID NO: 20); K29M forward primer (SEQ ID NO: 21); K33M forward primer (SEQ ID NO: 22); K48M forward primer (SEQ ID NO: 23); K63M forward primer (SEQ ID NO: 24); A pos 2 reverse primer (SEQ ID NO: 25); K6M reverse primer (SEQ ID NO: 26); K11M reverse primer (SEQ ID NO: 27); K27M reverse primer (SEQ ID NO: 28); K29M reverse primer (SEQ ID NO: 29); K33M reverse primer (SEQ ID NO: 30); K48M reverse primer (SEQ ID NO: 31); and K63M reverse primer (SEQ ID NO: 32).

Description of Various Embodiments

For the purposes of the present invention, the following terms as used herein shall, unless otherwise indicated, be understood to have the following meanings.

The term "hydrocarbyl" as used herein refers to a group consisting exclusively of hydrogen and carbon atoms, the group having from 1 to 30 carbon atoms. For instance, a hydrocarbyl group may have from 1 to 20 carbon atoms, *e.g.* from 1 to 12 carbon atoms, *e.g.* from 1 to 10 carbon atoms. A hydrocarbyl group may be an acyclic group, a cyclic group, or may comprise both an acyclic portion and a cyclic portion. Examples of hydrocarbyl groups include alkyl, alkenyl, alkynyl, carbocyclyl (*e.g.* cycloalkyl, cycloalkenyl or aryl) and aralkyl. The term “hydrocarbylene” as used herein refers to a divalent hydrocarbyl group.

The term "alkyl" as used herein refers to a straight or branched chain alkyl moiety having from 1 to 30 carbon atoms. For instance, an alkyl group may have from 1 to 20 carbon atoms, *e.g.* from 1 to 12 carbon atoms, *e.g.* from 1 to 10 carbon atoms. In particular, an alkyl group may have 1, 2, 3, 4, 5 or 6 carbon atoms. Examples of alkyl groups include methyl, ethyl, propyl (n-propyl or isopropyl), butyl (n-butyl, sec-butyl or tert-butyl), pentyl, hexyl and the like. The term “alkylene” as used herein refers to a divalent alkyl group.

The term "alkenyl" as used herein refers to a straight or branched chain alkyl group having from 2 to 30 carbon atoms and having, in addition, at least one carbon-carbon double bond, of either E or Z stereochemistry where applicable. For instance, an alkenyl group may have from 2 to 20 carbon atoms, *e.g.* from 2 to 12 carbon atoms, *e.g.* from 2 to 10 carbon atoms. In particular, an alkenyl group may have 2, 3, 4, 5 or 6 carbon atoms. Examples of alkenyl groups include ethenyl, 2-propenyl, 1-butenyl, 2-butenyl, 3-butenyl, 1-pentenyl, 2-pentenyl, 3-pentenyl, 1-hexenyl, 2-hexenyl, 3-hexenyl and the like. The term “alkenylene” as used herein refers to a divalent alkenyl group.

The term "alkynyl" as used herein refers to a straight or branched chain alkyl group having from 2 to 30 carbon atoms and having, in addition, at least one carbon-carbon triple bond. For instance, an alkynyl group may have from 2 to 20 carbon atoms, *e.g.* from 2 to 12 carbon atoms, *e.g.* from 2 to 10 carbon atoms. In particular, an alkynyl group may have 2, 3, 4, 5 or 6 carbon atoms. Examples of alkynyl groups include ethynyl, 1-propynyl, 2- propynyl, 1-butynyl, 2-butynyl, 3-butynyl, 1-pentynyl, 2-pentynyl, 3-pentynyl, 1-hexynyl, 2- hexynyl, 3-hexynyl and the like.

The term "alkoxy" as used herein refers to -O-alkyl, wherein alkyl is as defined above. In some instances, an alkoxy group may have from 1 to 20 carbon atoms, *e.g.* from 1 to 12 carbon atoms, *e.g.* from 1 to 10 carbon atoms. In particular, an alkoxy group may have 1, 2, 3, 4, 5 or 6 carbon atoms. Examples of alkoxy groups include methoxy, ethoxy, propoxy, isopropoxy, butoxy, tert-butoxy, pentoxy, hexoxy and the like.

The term "carbocyclyl" as used herein refers to a saturated (*e.g.* cycloalkyl) or unsaturated (*e.g.* cycloalkenyl or aryl) carbocyclic ring moiety having from 3 to 30 carbon atoms. For instance, a carbocyclyl group may have from 3 to 20 carbon atoms, *e.g.* from 3 to 16 carbon atoms, *e.g.* from 3 to 10 carbon atoms. In particular, a carbocyclyl group may be a 5- or 6-membered ring system, which may be saturated or unsaturated. Examples of carbocyclic groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, phenyl, naphthyl, fluorenyl, azulenyl, indenyl, anthryl and the like. The term "carbocyclylene" as used herein refers to a divalent carbocyclyl group.

The term "cycloalkyl" as used herein refers to an aliphatic carbocyclic moiety having from 3 to 20 ring carbon atoms. For instance, a cycloalkyl group may have from 3 to 16 carbon atoms, *e.g.* from 3 to 10 carbon atoms. In particular, a cycloalkyl group may have 3, 4, 5 or 6 ring carbon atoms. A cycloalkyl group may be a monocyclic, polycyclic (*e.g.* bicyclic) or bridged ring system. Examples of cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, norbornyl and the like. The term "cycloalkylene" as used herein refers to a divalent cycloalkyl group.

The term "cycloalkenyl" as used herein refers to an aliphatic carbocyclic moiety having from 5 to 20 ring carbon atoms and having, in addition, at least one carbon-carbon double bond in the ring. For instance, a cycloalkenyl group may have from 5 to 16 carbon atoms, *e.g.* from 5 to 10 carbon atoms. In particular, a cycloalkenyl group may have 5 or 6 ring carbon atoms. A cycloalkenyl group may be a monocyclic, polycyclic (*e.g.* bicyclic) or bridged ring system. Examples of cycloalkenyl groups include cyclopentenyl, cyclohexenyl and the like. The term "cycloalkenylene" as used herein refers to a divalent cycloalkenyl group.

The term "aryl" as used herein refers to an aromatic carbocyclic ring system having from 6 to 30 ring carbon atoms. For instance, an aryl group may have from 6 to 16 ring carbon atoms, *e.g.* from 6 to 10 ring carbon atoms. An aryl group may be a monocyclic aromatic ring system or a polycyclic ring system having two or more rings, at least one of which is aromatic. Examples of aryl groups include phenyl, naphthyl, fluorenyl, azulenyl, indenyl, anthryl and the like. The term "arylene" as used herein refers to a divalent aryl group.

The term "aralkyl" as used herein refers to an alkyl group substituted with an aryl group, wherein the alkyl and aryl groups are as defined herein. An example of an aralkyl group is benzyl.

The term "heterocyclyl" as used herein refers to a saturated (*e.g.* heterocycloalkyl) or unsaturated (*e.g.* heterocycloalkenyl or heteroaryl) heterocyclic ring moiety having from

3 to 30 ring atoms, wherein said ring atoms include at least one ring carbon atom and at least one ring heteroatom selected from nitrogen, oxygen, phosphorus, silicon and sulphur. For instance, a heterocyclyl group may have from 3 to 20 ring atoms, *e.g.* from 3 to 16 ring atoms, *e.g.* from 3 to 10 ring atoms. In particular, a heterocyclyl group may have 5 or 6 ring atoms, and may be saturated or unsaturated. Examples of heterocyclic groups include imidazolyl, thienyl, furyl, tetrahydrofuryl, pyranyl, thiopyranyl, thianthrenyl, isobenzofuranyl, chromenyl, pyrrolyl, pyrrolinyl, pyrrolidinyl, imidazolidinyl, benzimidazolyl, pyrazolyl, pyrazinyl, pyrazolidinyl, thiazolyl, isothiazolyl, dithiazolyl, oxazolyl, isoxazolyl, pyridyl, pyrazinyl, pyrimidinyl, piperidyl, piperazinyl, pyridazinyl, morpholinyl, thiomorpholinyl, indolizinyl, indolyl, cumaryl, indazolyl, triazolyl, tetrazolyl, purinyl, isoquinolyl, quinolyl, tetrahydroquinolyl, tetrahydroisoquinolyl, decahydroquinolyl, octahydroisoquinolyl, benzofuranyl, dibenzofuranyl, benzothiophenyl, dibenzothiophenyl, phthalazinyl, naphthyridinyl, quinoxalyl, quinazolinyl, quinazolinyl, cinnolinyl, pteridinyl, carbazolyl, phenanthridinyl, acridinyl, perimidinyl, phenanthrolinyl, furazanyl, phenazinyl, phenothiazinyl, phenoxazinyl, chromenyl, isochromanyl, oxiranyl, azirinyl, 1,2-oxathiolanyl, chromanyl and the like. The term “heterocyclylene” as used herein refers to a divalent heterocyclyl group.

The term "heterocycloalkyl" as used herein refers to a saturated heterocyclic moiety having from 3 to 10 ring carbon atoms and 1, 2, 3, 4 or 5 ring heteroatoms selected from nitrogen, oxygen, phosphorus and sulphur. The group may be a monocyclic or polycyclic ring system. Examples of heterocycloalkyl groups include azetidinyl, pyrrolidinyl, tetrahydrofuranyl, piperidinyl, oxiranyl, pyrazolidinyl, imidazolyl, indolizidinyl, piperazinyl, thiazolidinyl, morpholinyl, thiomorpholinyl, quinolizidinyl, tetrahydropyranyl, and the like. The term “heterocycloalkylene” as used herein refers to a divalent heterocycloalkyl group.

The term "heterocycloalkenyl" as used herein refers to a saturated heterocyclic moiety having from 3 to 10 ring carbon atoms and 1, 2, 3, 4 or 5 ring heteroatoms selected from nitrogen, oxygen, phosphorus and sulphur, and having, in addition, at least one carbon-carbon double bond in the ring. The group may be a monocyclic or polycyclic ring system. An example of a heterocycloalkenyl group is pyranyl. The term “heterocycloalkenylene” as used herein refers to a divalent heterocycloalkenyl group.

The term "heteroaryl" as used herein refers to an aromatic heterocyclic ring system having from 5 to 30 ring atoms, wherein said ring atoms include at least one ring carbon atom and at least one ring heteroatom selected from nitrogen, oxygen and sulphur. The group may be a monocyclic ring system or a polycyclic (*e.g.* bicyclic) ring system having two or more rings, at least one of which is aromatic. Examples of heteroaryl groups include pyridazinyl, pyrimidinyl, furanyl, benzo[b]thiophenyl, thiophenyl, pyrrolyl, imidazolyl, pyrrolidinyl, pyridinyl, benzo[b]furanyl, pyrazinyl, purinyl, indolyl, benzimidazolyl, quinolinyl, phenothiazinyl, triazinyl, phthalazinyl, 2H-chromenyl, oxazolyl, isoxazolyl, thiazolyl, isoindolyl, indazolyl, isoquinolinyl, quinazolinyl and the like. The term “heteroarylene” as used herein refers to a divalent heteroaryl group.

The terms "halogen" and “halo” as used herein refer to F, Cl, Br or I.

The term “optionally substituted” as used herein means unsubstituted or substituted.

The term "substituted" as used herein in connection with a chemical group means that one or more (*e.g.* 1, 2, 3, 4 or 5) of the hydrogen atoms in that group are replaced independently of each other by a corresponding number of substituents. It will, of course, be understood that the one or more substituents may only be at positions where they are chemically possible, *i.e.* that any substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound. The term is contemplated to include all permissible substituents of a chemical group or compound. It will be understood by those skilled in the art that one or more hydrogen atoms on a given substituent can themselves be substituted, if appropriate.

Where two or more moieties are described as being "each independently" selected from a list of moieties, this means that the moieties may be the same or different. The identity of each moiety is therefore independent of the identities of the one or more other moieties. Where multiple substituents are indicated as being attached to a structure, it will be understood that the substituents can be the same or different.

The term “electrophilic” or “electrophile” as used herein refers to any chemical moiety (atom or group) which forms a bond to a reaction partner (nucleophile) by accepting a pair of bonding electrons from that partner. Electrophilic moieties are well known in the art and include, without limitation, Michael acceptors and atoms (*e.g.* carbon atoms) that are substituted by one or more electron withdrawing groups.

The term "electron withdrawing group" as used herein refers to any atom or group having an electronegativity which is greater than that of a hydrogen atom, wherein electronegativity is as defined on the Pauling scale. A quantification of the level of electron withdrawing capability is given by the Hammett sigma (σ) constant. This constant is well known in the art (see *e.g.* “Advanced Organic Chemistry”, J. March, McGraw Hill, New York, 2007). The Hammett sigma constant values are generally positive for electron withdrawing groups.

The term "π-bond" as used herein refers to a chemical bond formed by the overlap of *p* orbitals on adjacent atoms, perpendicular to any sigma (σ) bonds between the same atoms. A π-bond is generally a double or triple bond. Examples of π-bonds include C=C, C≡C, C=O, C=N, C≡N, N=O and S=O bonds. In embodiments, the moieties U and V described elsewhere herein may together form one of the aforementioned π-bonds.

The term “alkene bond” as used herein refers to an aliphatic carbon-carbon double (C=C) bond.

The terms "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms include reference to amino acid polymers in which one or more amino acid residues is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to polymers formed of naturally occurring amino acids. The term also refers to variants of the traditional peptide linkage joining the amino acids making up the polypeptide. Examples of peptides and proteins include oligopeptides and polypeptides.

The term "antibody" as used herein refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

The term “specific binding pair” refers to two molecules, usually two different molecules, wherein one of the pair of molecules (the first specific binding member) specifically binds through chemical or physical means to the other molecule (the second specific binding member). The term "specifically binds" refers to a binding reaction which is determinative of the presence of a member of the binding pair in heterogeneous population of molecules, *e.g.* proteins and other biological molecules. Thus, under designated conditions (*e.g.* immunoassay conditions in the case of an antibody), a first member of a binding pair (*e.g.* an antigen) binds to the second member (*e.g.* an antibody), and does not bind in a significant amount to other molecules present in the sample.

The present invention provides active site probes which are suitable for detecting DUBs and other proteases. A probe of the present invention comprises a protein comprising an amino acid sequence of a ubiquitin or ubiquitin-like protein and, attached at the carboxyl terminus of said sequence, a group of the formula -L-Z, wherein L is a linker comprising an electrophilic moiety which is capable of covalently binding to an active site of a deubiquitinating enzyme; and Z is a detectable label or a conjugated substance; and wherein the probe comprises at least one detectable label.

In an embodiment, the probe is a compound of the Formula I:

$$X\text{-}Y\text{-}L\text{-}Z \qquad (I)$$

wherein

X is a detectable label or is absent;

Y is a protein comprising an amino acid sequence of a ubiquitin or ubiquitin-like protein;

L and Z are as defined above; and
at least one of X, Y, L and Z comprises a detectable label.

The probes of the present invention contain a protein which comprises an amino acid sequence of a ubiquitin or ubiquitin-like protein. The protein may contain one or more amino acid residues in addition to said amino acid sequence, or it may consist essentially of said amino acid sequence. In an embodiment, the protein consists of an amino acid sequence of a ubiquitin or ubiquitin-like protein. The protein may comprise ubiquitin or any related proteins or fragments thereof that share the same affinity and enzyme activities of ubiquitin, including any affinities and activities within a 10-fold range of that of ubiquitin. The protein preferably comprises from 50 to 100 amino acid residues, *e.g.* from 50 to 80 amino acid residues, *e.g.* from 70 to 80 amino acid residues, *e.g.* 74, 75 or 76 amino acid residues. In an embodiment, the protein comprises or consists of an amino acid sequence selected from ubiquitin (Ub), ubiquitin-like 5 (UBL5), ubiquitin-fold modifier 1 (UFM1), autophagy-related protein 12 (ATG12/APG12), autophagy-related protein 8 (ATG8/APG8), ubiquitin-related modifier 1 (URM1), interferon stimulated gene 15 (ISG15), ubiquitin-like protein FAT10, small ubiquitin-like modifier 1/2/3 (SUMO 1/2/3) 3, neural precursor cell expressed, developmentally down-regulated 8 (NEDD8), ubiquitin cross-reactive protein (UCRP), homologous to ubiquitin 1 (HUB1), ribosomal protein S30 fused to a ubiquitin-like protein (Fau) and bacterial ubiquitin-like modifier Pup. In an embodiment, an arginine or a glycine residue is present at the carboxy terminus of the protein, and thus, in these instances, the group -L-Z is attached to an arginine or glycine residue. In an embodiment, the carboxyl terminus of said amino acid sequence corresponds to residue Arg_{74} or Gly_{75} of ubiquitin.

The probe comprises at least one detectable label, *e.g.* one, two or more detectable labels. Detectable labels suitable for use in the present invention include any compound or composition that is detectable by spectroscopic, photochemical, electrochemical, biochemical, immunochemical, magnetic, electrical, optical or chemical means. Examples of labels include luminescent labels which emit radiation on exposure to an external source of radiation or other stimulus, *e.g.* fluorescent substances (which emit light when exposed to light), chemiluminescent substances (which emit light during chemical reaction), electroluminescent substances (which emit light on application of an electric current), phosphorescent substances (in which emission of light continues after exposure to light stimulus has ended) and thermoluminescent substances (which emit light once a certain temperature is exceeded).

The one or more detectable labels can be primary labels (where the label comprises an element that is detected directly or that produces a directly detectable element) or, alternatively, they can be secondary labels (where the label binds to a primary label, *e.g.* as is commonly used in immunological labelling). An introduction to labels, labelling procedures and detection of labels is found in Polak and Van Noorden, "Introduction to Immunocytochemistry" (second edition, Springer Verlag, N.Y. (1997)); and in Haugland, "Handbook of Fluorescent Probes and Research Chemicals" (Molecular Probes, Inc., Eugene, Oreg. (1996)). Primary and secondary labels can include undetected elements as well as detected elements. Examples of primary labels include spectral dyes, *e.g.* fluorescent dyes, and radioisotope labels. Examples of second labels include, for example, epitope tags and biotin. The label can be coupled directly or indirectly to a component of the detection assay using methods well known in the art. A wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with, for example, the first nucleic acid, stability requirements, and available instrumentation and disposal provisions.

In an embodiment, the probe comprises at least one detectable label which is a member of a specific binding pair. In a particular embodiment, X in Formula I comprises a member of a specific binding pair. Examples of specific binding pairs include antigen-antibody, hapten-antibody, antibody-antibody pairs, complementary oligonucleotides or polynucleotides, avidin-biotin, streptavidin-biotin, enzyme-substrate, hormone-hormone receptor, lectin-carbohydrate, IgG-protein A, IgG-protein G, nucleic acid-nucleic acid binding proteins, and nucleic acid-anti-nucleic acid antibody pairs.

In an embodiment, the probe comprises an epitope tag. The term "epitope tag" as used herein refers to a molecule or domain of a molecule that is specifically recognized by an antibody or other binding partner. In an embodiment, the epitope tag is a hemagglutinin (HA)-tag, a His-tag, a FLAG-tag, a myc-tag or a nano-tag. Preferably, the epitope tag is attached at the amino terminus of the first protein. In embodiments, X in Formula I comprises an epitope tag, and said epitope tag is optionally attached at the amino terminus of the protein Y.

In an embodiment, the probe comprises at least one detectable label which is biotin or concanavalin A. Biotin binds specifically to avidin and streptavidin, and hence can be detected by addition of an avidin or streptavidin-modified molecule, *e.g.* an avidin- or streptavidin-modified fluorescent dye. In one embodiment, X in Formula I comprises biotin or concanavalin A.

In a preferred embodiment, the probe comprises at least one fluorescent label. Fluorescent labelling offers numerous advantages for profiling active DUBs in cell extracts. For instance, the use of a fluorescent label may facilitate microscopy-based readouts in intact cells, enable more rapid detection as compared with immunodetection techniques, afford multiplexing capability (through the use of different fluorophores) and enable intracellular detection of DUBs within intact cells. In particular, the attachment of fluorescent dyes may allow direct detection of endogenous DUB activities in cell extracts by in-gel fluorescence imaging.

In use, fluorescent labels typically absorb light above about 300 nm, preferably above about 350 nm, and more preferably above about 400 nm, usually emitting at wavelengths greater than about 10 nm higher than the wavelength of the light absorbed. As will be appreciated by those skilled in the art, the absorption and emission characteristics of the bound dye can differ from the unbound dye. Examples of fluorescent labels (fluorophores) include fluoresceins, xanthenes, cyanines, naphthalenes, coumarins, oxadiazoles, pyrenes, oxazines, acridines, arylmethines, tetrapyrroles, BODIPY-based derivatives and quantum dots. Other examples of fluorescent labels include inorganic molecules, multi-molecular mixtures of organic and/or inorganic molecules, crystals, heteropolymers, and the like. Thus, for example, CdSe-CdS core-shell nanocrystals or highly fluorescent quantum dots (*e.g.* zinc sulfide-capped cadmium selenide) may be used. Further examples of fluorescent labels may be found in Haugland, "Handbook of Fluorescent Probes and Research Chemicals", 6th Edition (1996). Particularly preferred for use in the present invention are fluorescein and Cy5. Preferably, Z in Formula I is fluorescein, Cy3 or Cy5.

Detectable signals may also be provided by chemiluminescent and bioluminescent sources. Chemiluminescent sources include a compound which becomes electronically excited by a chemical reaction and can then emit light which serves as the detectable signal or donates energy to a fluorescent acceptor. Alternatively, luciferins can be used in conjunction with luciferase or lucigenins to provide bioluminescence.

Other preferred labels include radioactive labels. Radioactive labels may be introduced into a nanoparticle which then is non-covalently linked to an effector. For example, the isotopes of ^{125}I, ^{131}I, ^{99m}Tc, ^{67}Ga, ^{111}In, ^{14}C, ^{3}H, ^{35}S, and ^{14}P are commonly used as radioactive labels. Methods for detecting or a radioactive label are well known in the art. Magnetic beads are another preferred detectable label. A variety of magnetic beads that are compatible with cells is known in the art, and the beads typically comprise magnetite or superparamagnetic iron oxide and have the size of the order of nm or microns.

Spin labels may also be employed in the present invention. Spin labels are provided comprise an unpaired electron spin which can be detected by electron spin resonance (ESR) spectroscopy. Exemplary spin labels include organic free radicals, transitional metal complexes, particularly vanadium, copper, iron, and manganese, and the like. Exemplary spin labels include nitroxide free radicals.

Attachment of a detectable label to a substrate may be achieved by derivatising the substrate with a chemical group that can react with a corresponding group on the label to produce a chemical bond that chemically links the substrate and label. Thus, before their chemical combination, the substrate and/or the label may comprise one or more linking groups which react together to form a covalent bond between the label and substrate. Examples of such linking groups include hydroxyl, halide (such as chloride, bromide or iodide), amine, amide, azide, hydrazide, carboxyl, imide (such as carbodiimide, maleimide and succinimide), acetyl halide, thiol, nitrile, cyanate, isothiocyanate, organosilane and siloxane groups.

In embodiments: (i) X comprises an epitope tag (*e.g.* an HA-tag, a His-tag, a FLAG-tag, a myc-tag or a nano-tag), and said epitope tag is optionally attached at the amino terminus of the protein Y; (ii) X comprises biotin or concanavalin A; (iii) X is a radioisotope label; or X is a fluorescent label; or (iv) X is absent. Preferably, X is an epitope tag, *e.g.* an HA-tag.

In embodiments, Z is a detectable label, *e.g.* selected from a fluorescent label, a colorimetric label, a luminescent label or a radioactive label. In preferred embodiments, Z is a fluorescent label. In this regard, incorporation of a fluorescent group within the electrophilic trap section of the molecule may be advantageous in that it requires minimal protein modification, leaving the ubiquitin scaffold intact. In particular embodiments, Z is fluorescein, Cy3 or Cy5.

In an embodiment, Z is a conjugated substance. Examples of conjugated substances include peptides, proteins, polymers, nucleic acids, nucleic acid polymers, carbohydrates, lipids, ion-complexing moieties and the like. For instance, Z may be a peptide or a protein, *e.g.* a peptide or protein that is a substrate for a deubiquitinating enzyme. In an embodiment, Z is a protein which comprises an amino acid sequence of a ubiquitin or ubiquitin-like protein, wherein said protein may be the same or different to the protein to which the group -L-Z is attached. Where Z is a peptide or protein, it may be attached to the linker group L via any suitable group known in the art, *e.g.* via a homoazidoalanine residue that is present in said peptide or protein of Z.

In embodiments, X is an epitope tag (*e.g.* an HA-tag, a His-tag, a FLAG-tag, a myc-tag or a nano-tag) and Z is a fluorescent label or a conjugated substance (*e.g.* a conjugated protein or peptide).

The linker group L comprises an electrophilic moiety which is capable of covalently binding to an active site of a deubiquitinating enzyme. In embodiments, the electrophilic moiety is capable of covalently binding to a thiol group at an active site of a deubiquitinating enzyme. The electrophilic group may act as an inhibitory group which is specific for an enzymatic activity of a particular enzyme. The group may reversibly bind to the active site of a DUB or may irreversibly bind to an active site. Examples of suitable electrophilic moieties are described in US 2005/0277762, Borodovsky *et al* (Chemistry & Biology, 9, 1149-1159, 2002) and Altun *et al* (Chemistry & Biology, 18, 1401-1412, 2011), the contents of each of which are incorporated herein by reference.

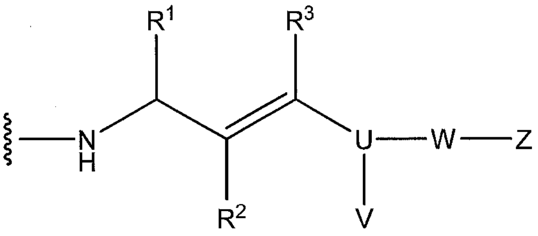
As a result of the presence of the electrophilic moiety, the probe may interact with Ub/Ubl specific enzymes in a reversible fashion or in an irreversible fashion.

Examples of reversibly inhibitory groups include aldehydes and boronates. Examples of irreversibly inhibitory groups include alkylating agents and atoms substituted by electron withdrawing groups, more preferably a Michael acceptor or an alkylating group. Preferably, the electrophilic moiety is a Michael acceptor; an alkylene group containing a carbon atom which is substituted by an electron withdrawing group; or an alkenylene group containing a carbon atom which is substituted by an electron withdrawing group. In an embodiment, the electrophilic moiety comprises a Michael acceptor which is, or is derived from, 3-vinylmethylsulfone; 3-vinylphenylsulfone; 3-vinylnitrile; or 2-carboxyvinylmethane.

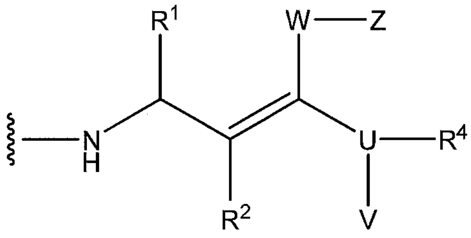
In embodiments, the linker L comprises from 1 to 50 in-chain atoms, *e.g.* from 1 to 40 in-chain atoms, *e.g.* from 1 to 30 in-chain atoms, *e.g.* 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 in-chain atoms. The linker may comprise one or more acyclic portions and/or one or more cyclic portions. In embodiments, each of the in-chain atoms of the linker L is independently selected from carbon, nitrogen, oxygen and sulphur. The linker may be linear or branched, and may be substituted with one or more substituents in addition to the group Z.

In certain compounds, L comprises a hydrocarbylene chain which is optionally interrupted by one or more (*e.g.* 1, 2, 3 or 4) in-chain heteroatoms, *e.g.* selected from oxygen, nitrogen and sulphur, and may be substituted with one or more (*e.g.* 1, 2, 3, 4 or 5) substituents in addition to Z. Where the hydrocarbylene chain is substituted, the one or more substituents may, for example, be selected from R^a, amino, hydroxy, C_{1-6} alkyl, C_{1-6} alkoxy, carbonyl, carbocyclyl (e.g. aryl or C_{3-6} cycloalkyl) and heterocyclyl (e.g. heteroaryl) groups.

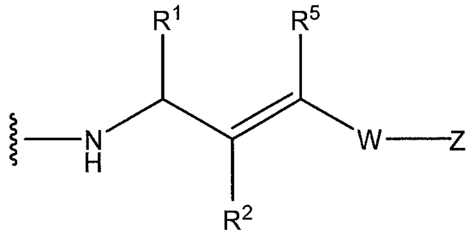
In an embodiment, the linker -L-Z is a group of the formula (II), (III), (IV) or (V):



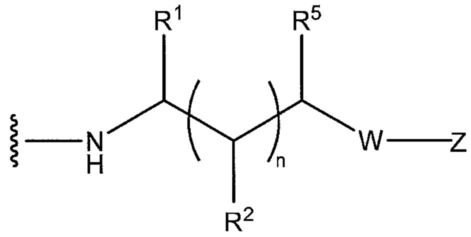
(II)



(III)



(IV)



(V)

wherein

R^1 is selected from hydrogen; R^a; hydrocarbyl optionally substituted with 1, 2, 3, 4 or 5 R^a; and -$(CH_2)_j$-heterocyclyl optionally substituted with 1, 2, 3, 4 or 5 R^a;

R^2 is selected from hydrogen; R^a; hydrocarbyl optionally substituted with 1, 2, 3, 4 or 5 R^a; and -$(CH_2)_j$-heterocyclyl optionally substituted with 1, 2, 3, 4 or 5 R^a;

R^3 is selected from hydrogen; R^a; hydrocarbyl optionally substituted with 1, 2, 3, 4 or 5 R^a; and -$(CH_2)_j$-heterocyclyl optionally substituted with 1, 2, 3, 4 or 5 R^a;

R^4 is selected from hydrogen; R^a; hydrocarbyl optionally substituted with 1, 2, 3, 4 or 5 R^a; and -$(CH_2)_j$-heterocyclyl optionally substituted with 1, 2, 3, 4 or 5 R^a; or R^4 is absent;

R^5 is an electron withdrawing group;

U and V taken together form an electron withdrawing group in which the bond between U and V is a π-bond;

W is a linker;

n is 0 or 1;

Z is as defined above;

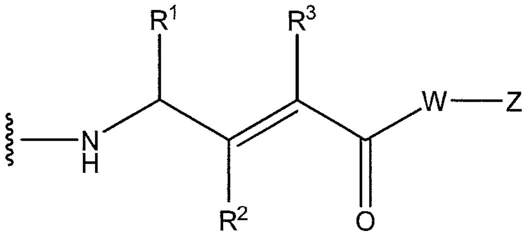
each R^a is independently selected from halogen, trifluoromethyl, cyano, nitro, oxo, $=NR^b$, $-OR^b$, $-C(O)R^b$, $-C(O)N(R^b)R^c$, $-C(O)OR^b$, $-C(O)SR^b$, $-OC(O)R^b$, $-S(O)_kR^b$, $-S(O)_kN(R^b)R^c$, $-N(R^b)R^c$, $-N(R^b)N(R^b)R^c$, $-N(R^b)C(O)R^c$ and $-N(R^b)S(O)_kR^b$;

R^b and R^c are each independently hydrogen or selected from hydrocarbyl and -$(CH_2)_j$-heterocyclyl, either of which is optionally substituted with 1, 2, 3, 4 or 5 substituents independently selected from halogen, oxo, cyano, amino, hydroxy, alkyl and alkoxy;

j is 0, 1, 2, 3, 4, 5 or 6; and

k is 0, 1 or 2.

In a particularly preferred embodiment, the group -L-Z is of the formula (IIa):



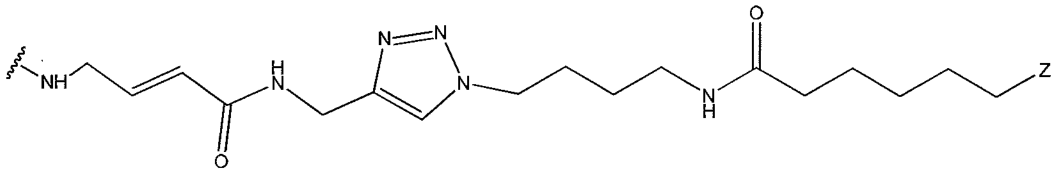
(IIa).

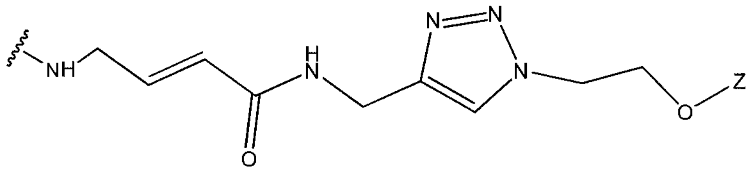
In embodiments of the above formulae, R^1 and/or R^2 is hydrogen. In embodiments, R^3 is hydrogen. In embodiments, R^1, R^2 and R^3 are each hydrogen.

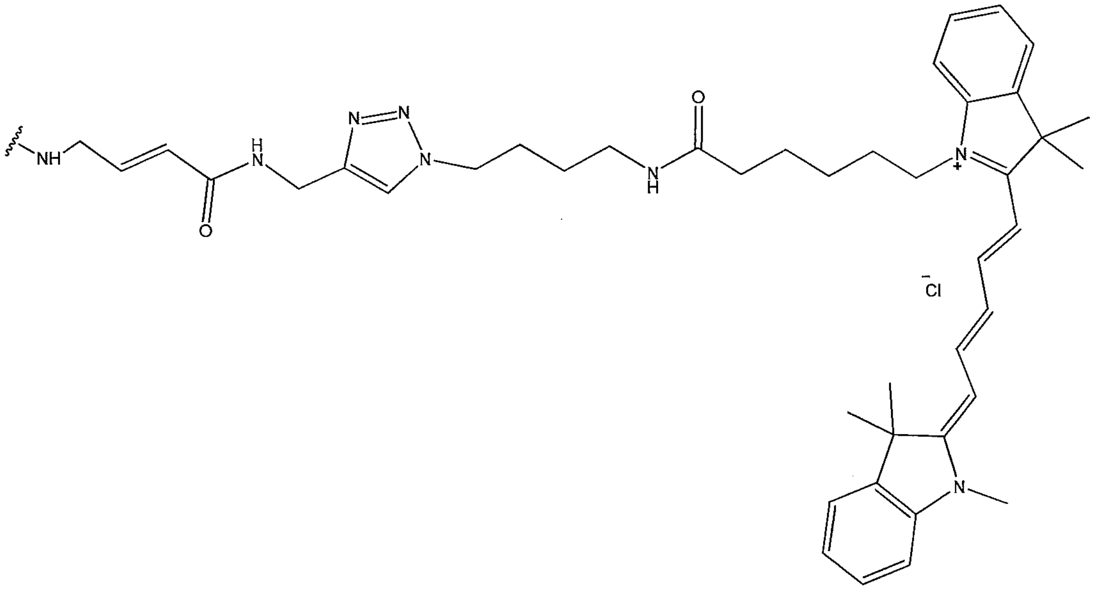
In embodiments, U and V taken together form a group containing a π-bond selected from C=C, C≡C, C=O, C=N, C≡N, N=O and S=O bonds. In embodiments, U and V taken together form -C(O)-.

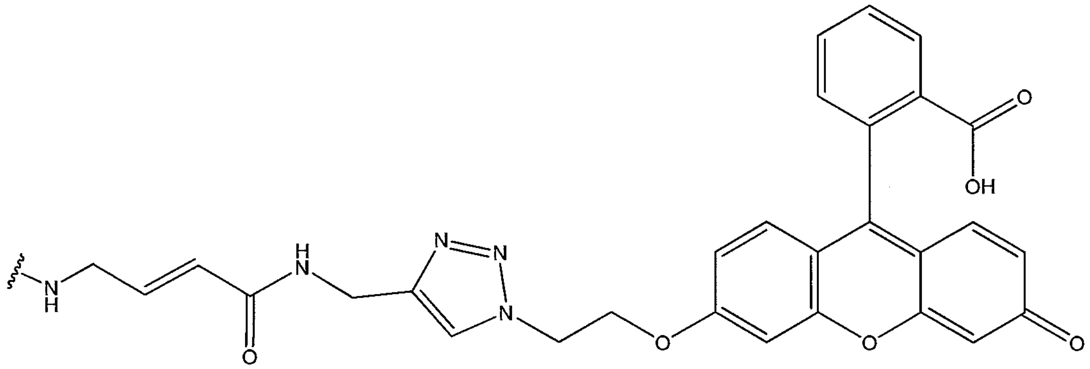
In embodiments of the above formulae, W is a linker containing from 1 to 30, *e.g.* from 1 to 20, *e.g.* from 1 to 15, *e.g.* from 1 to 10, *e.g.* from 5 to 10 in-chain atoms. In embodiments, W comprises a triazole group in the chain. In embodiments, W is formed by the reaction of an alkyne and an azide.

In a preferred embodiment, the group -L-Z is selected from one of the following groups:









The probes of the present invention may be obtained using suitable techniques known in the art. Preferably, the probes are synthesised by a bioorthogonal procedure involving the use of a functionalisable electrophilic trap which is allows for late stage functionalisation of the compound. In this regard, by attaching the moiety Z to a bio-orthogonal handle, the range of existing ubiquitin-based probes may be expanded by late stage functionalisation. Thus, the use of a linker functionalisable by "click" chemistry may permit the attachment of a variety of functional moieties, such that it can be further exploited to expand and diversify the panel ubiquitin-based probes. Thus, in embodiments, the present probes may be synthesised by conjugating the group Z to the remainder of the probe.

In particular, the present invention provides a process for producing a probe of the Formula I which comprises contacting a compound of the Formula VI:

$$X\text{-}Y\text{-}R^x \qquad (VI)$$

with a compound of the Formula VII:

$$R^y\text{-}Z \qquad (VII)$$

wherein X, Y and Z are as defined above; R^x and R^y are reactive groups and R^x contains said electrophilic moiety; and the contacting is performed under conditions such that R^x and R^y react together to form said linker group L, thereby forming a compound of the Formula I.

The reactive groups R^x and R^y may be any suitable groups known in the art. In an embodiment, one of R^x and R^y comprises an azide group, and the other comprises an alkyne group (*e.g.* a strained alkyne), a phosphine (*e.g.* an activated phosphine), or a diene or dieneophile. Reaction between R^x and R^y, may be accomplished using techniques known in the art, *e.g.* via a Huisgen cycloaddition reaction, a Staudinger reaction or a Diels-Alder reaction. Preferably, a reaction falling within the class of reactions collectively referred to as “click chemistry” is utilised.

In an embodiment, one of R^x and R^y, comprises an azide and the other is an alkyne. In preferred embodiments, R^x comprises an alkyne group (*e.g.* an ethynyl group) and R^y comprises an azide group. In these embodiments, formation of the compound of Formula I may be accomplished by performing an azide-alkyne Huisgen cycloaddition reaction, which is typically performed using a copper catalyst (see Chan *et al*, Org. Lett., 2004, 6, 2853). Preferably, said alkyne and azide groups are terminal groups.

The compound of Formula VI may be synthesised using techniques described in the Examples herein and/or following procedures known in the art, *e.g.* those described in US 2005/0277762, Borodovsky *et al* (Chemistry & Biology, 9, 1149-1159, 2002) and Altun *et al* (Chemistry & Biology, 18, 1401-1412, 2011). For instance, the compound of

Formula VI may be synthesised by a method comprising: (a) providing a fusion protein encoded by a nucleic acid vector, the fusion protein having: an epitope tag, a domain having an amino acid sequence from a member protein of the subset, an intein, and an affinity creating binding peptide; (b) breaking a peptide bond located between the domain and the intein, to yield a sulfoxide thioester at the carboxy terminus of the representative peptide; (c) further reacting the sulfoxide thioester to form the reactive group R^a, said group including the electrophilic moiety. In particular, an electrophilic moiety may be introduced by direct nucleophilic substitution of intein-derived sequences (for example Ub/Ubl thioesters), or by performing the following steps: adding 1-amino-2,2-dimethoxyalkane; performing acid catalysed hydrolysis of the resulting acetal; and isolating peptide aldehydes (reversible inhibitors of a variety of ubiquitin-related enzymes). Further, the use of the Wittig reaction with stabilized ylides of the thus obtained aldehydes in aqueous solution may directly lead to a compound of the Formula VI containing a Michael acceptor as the electrophilic moiety. Preferably, the fusion protein is purified prior to breaking the peptide bond between the intein and the domain. Purification may be accomplished by contacting a preparation comprising the fusion protein with an immobilized binding partner of the affinity binding peptide. In some embodiments, the affinity binding domain is a chitin binding domain, and the immobilized binding partner is immobilized chitin.

In a particular embodiment, a probe of the present invention is obtained from the alkyne derivative **2** referred to in the Examples herein. Thus, in one embodiment, the compound of Formula VI is said alkyne derivative **2**.

Probes of the present invention may be used to identify or inhibit genomic proteins, and may find particular application in diagnosis and/or treatment of medical diseases or conditions. In particular, the present probes may efficiently label enzymes involved in Ub-conjugation and deconjugation, and may be useful as chemoselective reaction tools for the elucidation of enzymatic specificities of DUBs and to identify new targets for drug design. In this regard, the Ub pathway has been shown to play an important regulatory role in processes such as cell cycle control, signal transduction and the immune response, and has been implicated in the development of cancers and neurodegenerative diseases. Ub and Ubl proteins have been implicated in a variety of cellular processes such as autophagy, interferon response, nuclear translocation, cell cycle progression and apoptosis. Inhibitors of DUBs may be useful in the treatment of a variety of diseases and conditions, including cancer, HPV infection, HSV infection, Fanconi anaemia, breast cancer, Von Hippel Lindau syndrome, Liddle's syndrome, immune dysregulation, Parkinson's disease, Opitz syndrome, infertility, familial cylindromatosis and adenoviral infection. The present probes may be active both with recombinant DUB labelling and in whole cell lysates.

The present probes may be used to detect a variety of DUBs. DUBs can be subdivided into Ub C-terminal hydrolases (UCHs) and Ub-specific processing proteases (UBPs). Examples of DUBs which may be detected using the present probes include UCHL1, Unp, Usp6, Usp7, Usp8, Usp9Y, Usp14, CYLD, BAP1, VDU1, VDU2 and adenovirus protease.

Methods of detecting DUBs using the present probes are also provided. In particular, there is provided a method of detecting a DUB in a sample, which method comprises: contacting the sample containing a DUB with a probe of the invention such that one or more components of the sample bind to the probe; and detecting the presence of the probe in the sample. In an embodiment, the sample is a cell lysate or comprises intact cells. Cell lysates treated with the Ub or Ubl-derived probes can be generated from a wide variety of types of cells and cell tissues. The probes and methods herein can be used to determine the activities of various proteins in crude extracts, for diagnostic purposes. Further, probes can be used to localize proteins, for example, during cell fractionation to separate cell membrane, cytoplasmic, nuclei, nuclear membrane, and mitochondrial fractions, for further localisation of specific enzymes. Virtually any cell can be used with the methods of this invention. Such cells include both eukaryotic and prokaryotic cells. Bacterial cells, fungal cells, algal cells, plant cells, animal cells are all well suited to the methods of this invention. In particularly preferred embodiments, the cells are vertebrate cells, more preferably mammalian cells, and most preferably human cells. The cells can be cultured *ex vivo*, obtained from fresh preparations, present in a tissue culture, or in a tissue *in vivo*. In high-throughput screening applications, cultured cells are most preferred.

Detection may be accomplished using any suitable technique known in the art. For instance, where the probe comprises a fluorescent label, detection may be made by methods such as *e.g.* fluorimetry or fluorescent microscopy. Where the probe comprises a magnetic bead, detection may be made by magnetometry. Where the probe comprises a radioisotope, detection may be performed using methods such as scintillography or autoradiography. Where the detectable label is a colorimetric tag, detection may be performed using optical means.

Preferably, the probe comprises a fluorescent label (*e.g.* Z is a fluorescent label) and fluorescent detection is performed. DUB enzymatic activity may be visualized using fluorescence detection to give a sensitive, high resolution, rapid gel-based readout, and is much quicker in comparison to silver stain or immunoblotting techniques. The incorporation of fluorophore allows detection in the low ng range and quantification with a dynamic range over various orders of magnitude. In addition, traditional protein detection methods such as silver staining or immunoblotting can still be applied. The utility and advantages of the present proves for screening of DUB inhibitors in cell culture models has also been demonstrated. Furthermore the probes may be used to image within whole cells.

In particular, there is provided a method of detection comprising: contacting a sample (*e.g.* a cell lysate or a sample containing intact cells) with a probe of the present invention comprising an epitope tag (*e.g.* X is an epitope tag); and analysing the sample by reducing SDS gel electrophoresis and immunoblotting with an antibody specific for the tag, such that one or more components bind to the probe and react with the antibody to visualize bands on the gel. Thus, for instance, enzymes and proteins modified by interaction with the present probes can be immunoprecipiated by an antibody specific for an epitope tag, and then visualized by SDS-PAGE followed by staining (*e.g.* silver staining). The analytes may optionally be further characterised by *e.g.* mass spectrometry, for example tandem mass spectrometry.

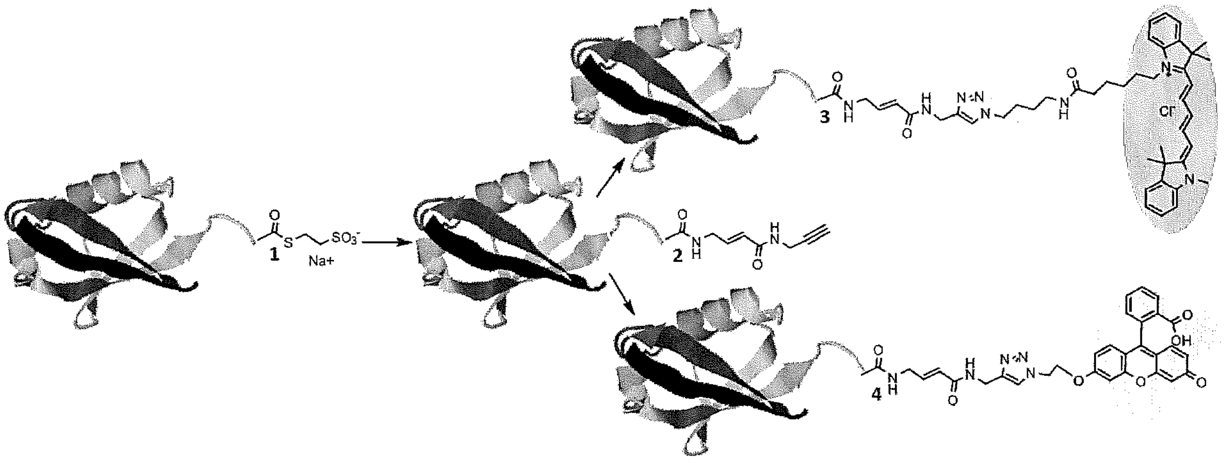
The present probes may be particularly useful for screening test agents for a desired activity, *e.g.* as potential DUB inhibitors. The term "test agent" refers to any agent or composition that is to be screened for a desired activity and may comprise a molecule or mixture of molecules, optionally in a suitable carrier. Examples of test agents include small molecule compounds, for example organic compounds having a molecular weight of 5000 Da or less, 2000 Da or less, 1000 Da or less, 750 Da or less or 500 Da or less. Other examples of test agents include biological macromolecules, *e.g.* proteins, nucleic acids and polymer conjugates. The test agent may be a member of a library of compounds. Such libraries may comprise at least 1000, at least 10,000, or at least 100,000 different members.

Also provided are kits comprising one or more probes of the present invention. The kits may be used for identifying ubiquitination and deubiquitination proteins in a cell. The kit may comprise two or more containers, one of which contains a probe of the present invention. The kit may also include other instruments and/or reagents for practice of the methods of this invention. Such reagents and instruments include, but are not limited to microtiter plates, cells, buffers, filters for detection of fluorescent labels, software for running assays on high throughput robotic systems, and the like. In addition, the kits can include instructional materials providing general directions and/or specific protocols for the methods of this invention.

The following non-limiting Examples illustrate the present invention.

EXAMPLE 1

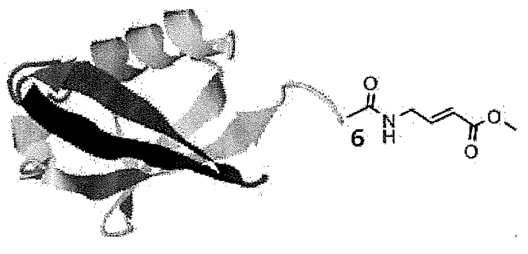
Fluorescent active site probes **3** and **4** were synthesised according to the following reaction scheme, in which ubiquitin is shown as a ribbon structure and the fluorescent Cy5 and fluorescein moieties are highlighted.



Scheme 1

Following the above reaction scheme, HA-Ub-thioester **1** was reacted with (E)-4-amino-N-(prop-2-ynyl)but-2-enamide **11** to afford alkyne derivative **2** in moderate (30-40%) yield, and the latter was then purified by *via* strong cation exchange (SCX) high performance liquid chromatography (HPLC). Purified alkyne derivative **2** was then reacted with Cy 5-derived azide or fluorescein-derived azide **6** via a Cu catalysed [3+2] cycloaddition reaction (as described in Chan *et al*, Org. Lett., 2004, 6, 2853). 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 and SDS-PAGE analysis.

The activity and properties of the probes **3** and **4** were then tested, and compared with those of two known probes, namely HA-Ub-Br probe **5** and HA-Ub-VME (vinyl methyl ester) probe **6**:



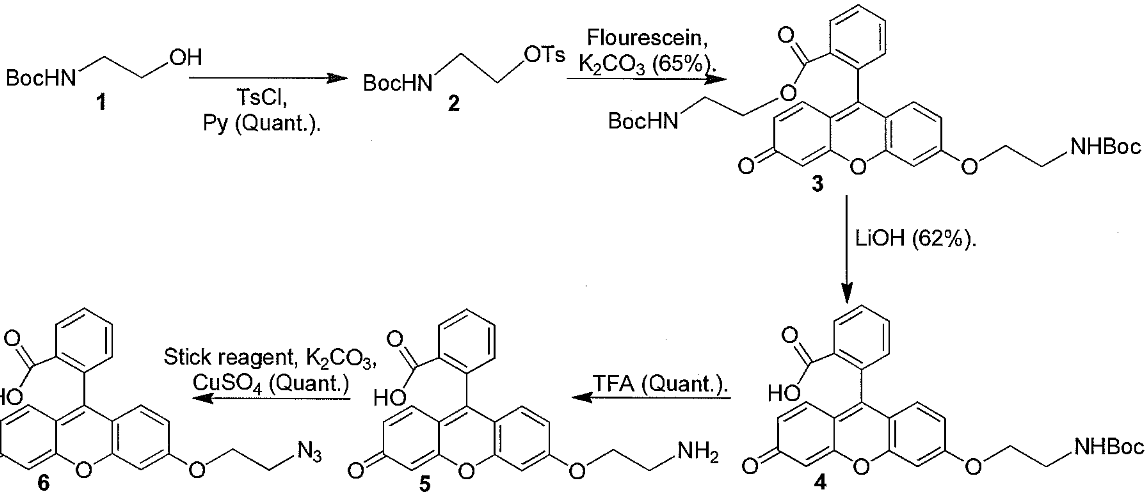
Experimental

Thin layer chromatography (TLC) was carried out on Merck 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 1M NaOH). Flash column chromatography was conducted using silica gel (Fluka Kiegselgel 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). 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 was corrected using a lock-mass. In positive ion mode, this was tetraoctylammonium bromide. M/z values are reported in Daltons.

Fluorescein-derived azide (**6**)

Fluorescein azide **6** was synthesised according to the following reaction scheme



Scheme 2

*O-Tosyl-N-Boc-ethanolamine (**2**)*

N-Boc-ethanolamine **1** (1.0 g, 6.2 mmol) was dissolved in DCM (5 mL). Pyridine (1.3 mL) was added and the solution cooled to 0 °C. p-Toluene sulfonyl chloride (2 g, 10.5 mmol) was added and the reaction stirred for 18 hours. The resultant solution was washed with 6:1 H_2O:pyridine (2 × 30 mL) and 4:1 H_2O:37% aq. HCl (30 mL) prior to drying with Na_2SO_4 and concentration under reduced pressure. This afforded 1.8g (92% yield) of **2** as a white amorphous solid. ESI-MS *m/z* = 653 $(2M+H)^+$. IR (neat) ν: 3413.5, 1711.2, 1589.5, 1511.3 cm^{-1}.

*Fluorescein-di-O,O'-N-Boc Ethylamine (**3**)*

Fluorescein disodium salt, (300 mg, 1.0 mmol), K_2CO_3 (400 mg, 2.9 mol) and tosylate **2** (800 mg, 2.5 mmol) were dissolved in DMF (5 mL) and heated to 60 °C for 18 hours. The mixture was then diluted with EtOAc (10 mL) and extracted with brine (10 mL). The aqueous layer was washed with EtOAc (10 mL) and the combined organic extracts further washed with brine (2 × 10 mL). The organic layer was dried with Na_2SO_4 and concentrated prior to loading onto a silica plug. The product was washed with DCM followed by elution with warm acetone which after concentration afforded desired product **3** in 89% yield as an orange foam. IR (neat) ν: 3409.5, 3000.3, 2985.6, 1706.2, 1688.1, 1514.5 cm^{-1}. ESI-MS *m/z* = 619 $(M+H)^+$. HRMS (ESI) calculated for $C_{34}H_{38}N_2NaO_9$ $(M+Na)^+$ 641.2470 found 641.2489. Anal. Calcd for $C_{34}H_{38}N_2O_9$ C,66.01; H,6.19; N, 4.53, found C,65.92; H,6.12; N, 4.48.

*Fluorescein-O'-N-Boc Ethylamine (**4**)*

Fluorescein derivative **3** (500 mg, 0.80 mmol) was dissolved in THF (10 mL). H_2O (10 mL) was added along with $LiOH{\cdot}H_2O$ (600 mg, 25 mmol) and the reaction stirred for 3 hours. The reaction progression was checked after this time by t.l.c. (3:2 petrol:EtOAc) showing complete starting material consumption (R_f 0.1) and product formation (R_f 0.3). 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 x 10 mL) and the combined organic layers dried with Na_2SO_4. Concentration under reduced pressure followed by flash column chromatography on silica (3:2 petrol:EtOAc to EtOAc) afforded 260 mg (68% yield) of desired product **4** as a viscous orange oil. IR (neat) ν: 3367.9, 2998.7, 2980.2, 1688.4, 1611.3, 1504.0 cm^{-1}. ESI-MS *m/z* = 474 $(M-H)^-$. HRMS (ESI) calculated for $C_{27}H_{25}NaO_7$ $(M+Na)^+$ 498.1523 found 498.1519.

*Fluorescein-O'-Ethylamine (**5**)*

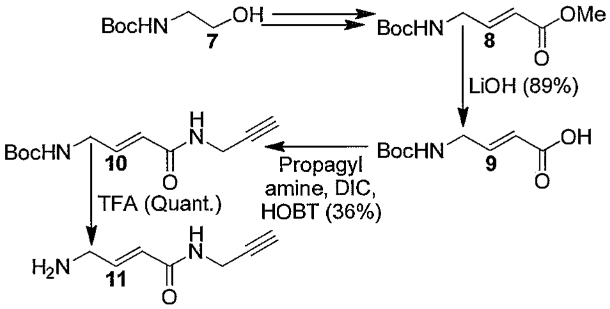
N-Boc protected **4** (160 mg, 0.34 mmol) was dissolved in DCM (6 mL). TFA (2 mL) was added and the reaction stirred for 1 hour, and after this time t.l.c. (3:2 petrol:EtOAc) showed complete starting material consumption (R_f 0.3) and product formation (R_f 0.01). The solution was concentrated under reduced pressure, with re-suspension and co-evaporation with toluene (3 ×5 mL) to afford the desired product **5** in quantitative yield which was reacted immediately without further purification.

*Fluorescein-O'-N-azido Ethylamine (**6**)*

Amine **5** (140 mg, 0.34 mmol) was dissolved in MeOH (15 mL), K_2CO_3 (225 mg, 1.7 mmol) and $CuSO_4{\cdot}5H_20$ (6 mg, 0.024 mmol). Imidazole-1-sulfonyl azide hydrochloride (150 mg, 0.41 mmol) was added and the reaction mixture stirred at room temperature. After 18 hours, t.l.c (9:1 EtOAc:MeOH) showed complete starting material consumption (R_f 0.2) and product formation (R_f 0.8). The solvent was removed under reduced pressure and the resulting mixture taken up in H_2O (5 mL), the aqueous solution was adjusted to pH 3 by the addition of 1 M HCl (aq.). The product was extracted with EtOAc (3 × 10 mL) and the combined organic layers dried with Na_2SO_4. Concentration under reduced pressure afforded 120 mg (88% yield) of desired product **6** as an orange oil. IR (neat) ν: 3234.6, 2998.4, 2981.7, 2106.22, 1752.9, 1610.2, 1503.9 cm^{-1}. ESI-MS *m/z* = 402 $(M+H)^+$. HRMS (ESI) calculated for $C_{22}H_{16}N_3O_5$ $(M+H)^+$ 402.1084, found 402.1076

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

(E)-4-amino-N-(prop-2-ynyl)but-2-enamide **11** was synthesised according to the following reaction scheme:



Scheme 3

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

The procedure described by Grison *et al* (Tetrahedron, 2001, 57, 4903) was followed. Vinyl methyl ester **8** (400 mg, 1.9 mmol; see Hanzlik *et al*, J. Med. Chem, 1984, 27, 711) 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. 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. Propagyl 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 saturated $NaHCO_3$ (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. 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. The product was reacted immediately without further purification. IR (neat) ν: 2926.3, 1708.5, 1669.8, 1509.0, 1367.0, 1253.3 cm^{-1}. ESI-MS *m/z* = 139.0 $(M+H)^+$.

HA-Ub-alkyne (**2**)

HA-Ub75-MeSNa **1** was expressed and purified according to literature procedures *via* a

HA Ub75-intein-CBD fusion protein to afford a final protein concentration of 4.5 mg/mL as determined by Bradford assay (see Borodovsky *et al*, Chemistry & Biology, 9, 1149-1159, 2002). 3 mL of thioester **1** was treated with 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 using HPLC (Agilent 1100) (Figure S1). Product containing fractions were concentrated and probe **2** was analysed by linear mode MALDI-TOF (Bruker) analysis. A further aliquot was subjected to digestion by trypsin and the resulting peptides analysed by LC-MS/MS.

Fluorescent probes (**3** & **4**)

Purified alkyne derivative **2** was reacted with Cy5-derived azide or fluorescein-derived azide **6** via a Cu catalysed [3+2] cycloaddition reaction (following the procedure described by Chan *et al*, Org. Lett., 2004, 6, 2853) to yield fluorescent active site probes **3** and **4**. In more detail, 750 µL of Na_2PO_4/NaH_2PO_4 (50 mM, pH 8) containing a final concentration of 1.1 mg/mL of alkyne derivative **2** was treated with the fluorescein-derived azide **6** or Cy 5-derived azide (Lumiprobe Corporation) (1.24 µmol in 25 µL MeCN) followed by tris[(1-ethoxycarbonylmethyl-1H-1,2,3-triazol-4-yl)methyl] amine (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 minutes prior to desalting (PD-10, GE Healthcare) and concentration. The resulting compounds were then analysed by linear mode MALDI-TOF analysis (Bruker).

Detection of DUBs

DUB UCH-L3 was labelled with fluorescent probe **4** and the resulting gel examined by both fluorescence detection and silver stain. HA-Ub-Br probe **5** was also reacted with UCH-L3 as a control. In more detail, 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 mins. Samples were resolved by 18% reducing SDS-PAGE gel and detected using an Autochemi™ UVP bioimaging system. Gels were scanned with UV transilumination 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.

The results of this initial experiment are presented in Figure 1. It was observed 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.

The labelling pattern and efficiency of Cy 5 fluorescent probe **3** and HA-Ub-VME probe **6** were then compared. 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 by Borodovsky *et al* (cited *supra*). 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 minutes. 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 (1:10000 dilution, Sigma Aldrich H6533).

A comparison of labelling pattern and efficiency between Cy5 fluorescent probe **3** and

HA-Ub-VME probe **6** not only demonstrated comparable sensitivity to anti HA immunoblot, but also exhibited a better resolution of high molecular weight bands (see Figure 2). 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 control reaction in the presence of 200 nM NEM, a competitive cysteine alkylating agent showed the fluorescence readout to be clearly specific for detection of active DUBs (see Figure 2). The overall labelling profile of DUBs appears to be similar between the Cy 5 conjugated 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. This higher resolution profiling of probe-DUB conjugates coupled with 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 for use in inhibitor screening, HEK 293T cell lysate was then treated with varying concentrations of the general DUB inhibitor PR619 (see Tian *et al,* Assay Drug Dev. Technol., 2011, 9, 165). 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.

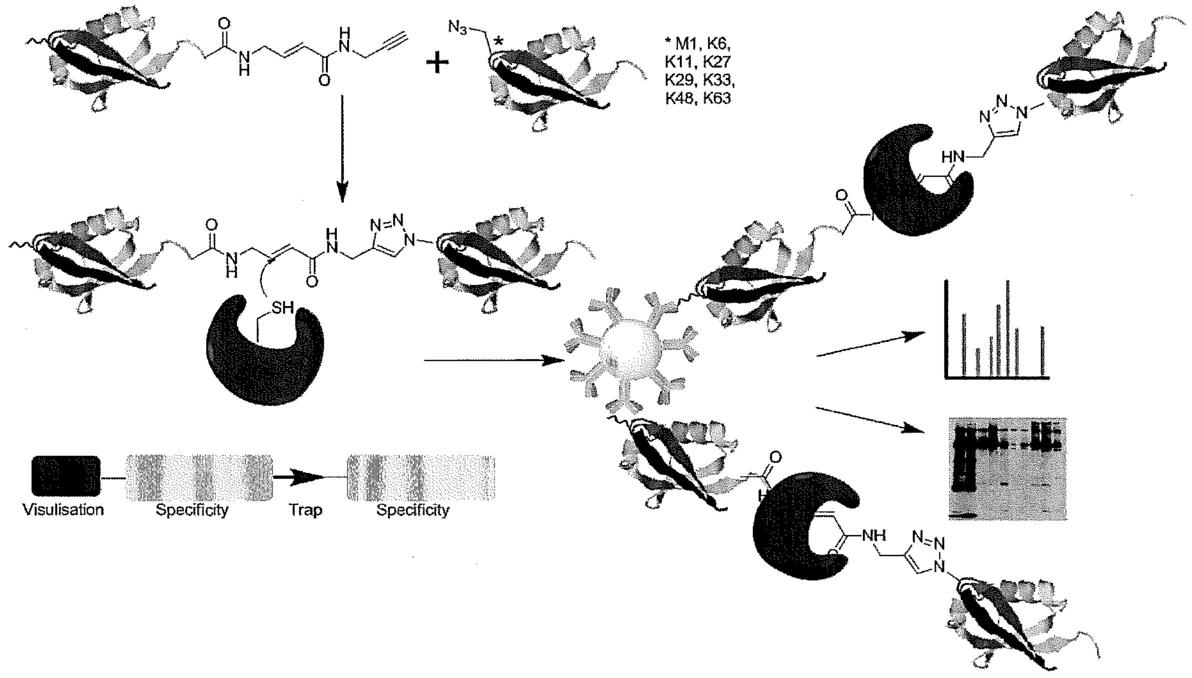
The results of this experiment are presented in Figure 3. Qualitative comparisons can be made by inspection of the fluorescence gel readout allowing for fast screening of potential DUB inhibitors. The differential inhibition of two probe reactive species was highlighted (see Figure 3, panel B), and the loss of fluorescence signal indicating loss of activity toward the probe was measured (see Figure 3, panel C).

The above-mentioned probes were also used to track active DUBs in cells using a fluorescence-based microscopy approach. Figure 4 shows results obtained from such experiments. Cell-based imaging of active DUBs (development of a "DUB-tracker") will complement antibody-based fluorescent staining methodologies to further elucidate the function role of individual DUBs. Also, it is envisaged that these approaches can be further developed in the context of high throughput cell imaging.

In a further experiment, a ubiquitin derivative carrying a homoazidoalanine in position of lysine 48 was attached to a HA-Ub-VA-alkyne probe, yielding a di-ubiquitin active site probe. The probe was found to target recombinant UCH-L3, demonstrating that the creation of di-Ub/Ubl active site probes is feasible.

EXAMPLE 2

Active site probes were engineered on a di-ubiquitin (Di-Ub) scaffold according to the following reaction scheme.



The Di-Ub probes comprised two linked Ub moieties representing all Ub-linkages determining the specificity for DUBs that bind to the probe. Linkage specificity was achieved by incorporating Aha at the N-terminal methionine or in the positions of lysine residues in the proximal Ub. An electrophilic moiety in the “warhead’ linking the two Ub molecules allowed covalent trapping of DUBs/Ub processing enzymes with a cysteine in the active site. DUBs/Ub processing enzymes bound to the probe were then identified after an immunoprecipitation (IP) by tandem mass spectrometry (LC-MS/MS) analysis.

Materials

Reagents were purchased from Aldrich and used as supplied unless otherwise stated. Deionised water was used for buffers and chemical and biochemical reactions. Precision plus protein standard all blue gel marker (Biorad) was used. HA-Ub-alkyne probe was prepared according to the procedures set forth in Example 1 above. HA-Ub-VME probe was prepared according to Borodovsky *et al* (*supra*). Azidohomoalanine was prepared according to Link *et al* (Nature Protocols, 2007, 2, 8, 1879-1883). HEK293T cells were cultured in DMEM (DUBELCO) medium supplemented with 10% FCS (Sigma) and 1% penicillin/streptomycin at 37 °C with 5% CO_2.

Preparation of Ubiquitin mutants

Following overnight culture, bacteria were harvested and the wild type plasmid DNA containing the Ubiquitin sequence cloned into the pET15B vector (Addgene; see also Borodovsky *et al*, cited *supra*) was isolated, using Wizard® Plus SV Minipreps DNA Purification System (Promega, Fitchburg USA) according to the manufacturer's protocol. DNA concentration was analysed by measuring absorption at 260 nm wavelength using a NanoDrop spectrophotometer. Point mutants were created using the QuickChange II site directed mutagenesis kit by strategene (La Jolla, CA. USA) to create an alanine insertion at position 2 of Ub followed by K6M, K11M, K27M, K29M, K33M, K48M & K63M substitution. The primers used for the design of the Ubiquitin mutants were designed and obtained from Metabion (Germany). The primer sequences are given in the sequence listing (see SEQ ID NOs: 17-32). Mutant constructs were verified by DNA sequencing. Sequencing results are also given in the sequence listing (see SEQ ID NOs: 9-16).

Ubiquitin mutant transfection, azidohomoalanine (Aha) incorporation and purification

For the expression of WT and mutant Ub methionine auxotroph B834(DE3) competent cells were used (Novagen Merck, Darmstadt Germany) according to the manufacturer's protocol. Glycerol stocks were prepared by picking one colony from an agar plate and incubating it in LB medium with constant shaking at 37 °C for 18 h. 100 µL cell suspension was mixed with 900 µL of 60% glycerol in water and stored at -80 °C. 10 mL overnight culture was prepared from a glycerol stock. Bacteria were incubated in SelenoMet medium (Molecular Dimensions Newmarket, UK) containing 40 µg/mL methionine at 37 °C for 18 h. After transferring the bacteria to up to 500 mL SelenoMet medium containing and methionine, bacteria were grown with vigorous shaking at 37 °C until they reached an OD_{600} of 0.9. After harvesting the cells they were washed two times with methionine free SelenoMet medium before resuspension in SelenoMet medium containing 40 µg/mL azidohomoalanine and 0.4 mM IPTG. Protein expression was accomplished at 37 °C for 4 hours under vigorous shaking. Subsequently the harvested bacteria were resuspended in lysis buffer (100 mM Tris pH 7.5, protease inhibitor cocktail tablet (Roche Mannheim Germany), 0.02% NP40, 0.4 mg/mL lysozyme) before 1.2 mg/mL $MgSO_4$ and 12 µg/mL DNAseI were added. Vigorously vortexed cell lysate was cleared and treated with 0.5% perchloric acid on ice. Remaining Ub in the supernatant was dialysed in 50 mM NaOAc pH 4.5 for 18 h at 4 °C.

To check the purity and Aha incorporation the Ub mutants were resolved on an 18% Bis-Tris gel and visualized by silver staining. Linear mode MALDI-TOF MS analysis (Bruker) was also performed. A further aliquot of each mutant was subjected to digestion by trypsin and the resulting peptides analysed by LC-MS/MS. In brief, proteins were resuspended in 6 M Urea, reduced with dithiotreitol and alkylated with iodacetamide before proteolysis with Trypsin. Desalted samples were separated on a 75 µm x 250 mm reversed phase Nano-Acquity-UPLC (Waters, En Yvelines Cedex France) and analyzed by a coupled Q-TOF Premier mass spectrometer (Waters, En Yvelines Cedex France). MS/MS spectra were searched against a human database containing the Ub mutant sequences (86,755 sequences) in Mascot v2.4 allowing one missed cleavage and 50 ppm/0.1 Da mass deviation in MS and MS/MS respectively. Oxidation of methionine, methionine to azidohomoalanine exchange and deamidation of asparagine and glutamine were used as variable modifications. The sequences of the ubiquitin mutants incorporating an azidohomoalanine insertion are shown in the sequence listing (see SEQ ID NOs: 1-8, wherein Xaa represents azidohomoalanine [Aha]).

Di-Ub probe synthesis

HPLC purified HA-Ub-alkyne (77 µL, 2 mg/mL) was mixed with the appropriate azidohomoalanine incorporated Ub mutant (805 µL, 0.56 mg/mL) in 50 mM Na_2HPO_4/NaH_2PO_4 pH 8 buffer. Tris[(1-ethoxycarbonylmethyl-1*H*-1,2,3-triazol-4-yl)-methyl]amine (90 µg in 15 µL CH_3CN) and Cu(I)Br (120 µg in 15 µL CH_3CN) were added before shaking at room temperature. After 30 min a second portion of Cu(I)Br (120 µg in 15 µL CH_3CN) was added, prior to a further 30 min shaking the Di-Ub probe was buffer exchanged into 50 mM NaOAc pH 4.5 (PD-10, GE Healthcare). The resulting probes were HPLC (Agilent 1100) purified 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. Product containing fractions were identified based on SDS-PAGE separation and anti-HA immunoblotting and concentrated.

In vitro DUB labelling

Recombinant DUBs (OTUB2, UCH-L3, UCH-L5, USP-15, Progenra USA) or HEK293T cell lysate were incubated with the relevant Ub probe with varying concentrations in presence or absence of 10 mM NEM. Volume was adjusted to 15 µl with 50 mM NaH_2PO_4/Na_2HPO_4 pH 8.0 buffer. Solutions were incubated at 37 °C for 3 hours followed by addition of 5 µL of 3 x reducing sample buffer and heating to 95 °C for 5 mins. Samples were resolved on a NuPAGE 4-12% Bis-Tris gradient gel and visualized by silver staining or western blotting methods using a directly coupled HA-HRP antibody (1:10000 dilution, Sigma Aldrich H6533).

As a control for C-terminal activity, an inactivated linear Di-Ub probe was also generated. 2 µg of linear Di-Ub probe was incubated in 10 µL inactivation buffer (50 mM MeSNa, 50 mM NaH_2PO_4/Na_2HPO_4 pH 8.0) at 37 °C for 24 h. 1.5 µl of 50 mM MeSNa was added along with 10 µL 50 mM NaH_2PO_4/Na_2HPO_4 pH 8.0 and Di-Ub probe incubated for further 40 h at RT. The buffer was exchanged to 50 mM NaH_2PO_4/Na_2HPO_4 pH 8.0 by centrifugal filter devices.

Immunoprecipitation (IP) and analysis by mass spectrometry

The immunoprecipitated solution was desalted using chloroform-methanol extraction, followed by overnight in-solution tryptic digestion and subsequent desalting as described previously (Fischer et al., 2012). In brief, proteins were chloroform-methanol precipitated prior to resuspension in 6 M Urea, reduced with dithiotreitol and alkylated with iodacetamide before proteolysis with Trypsin. Tryptic digests were desalted and subjected to LC-MS/MS (75 µm x 250 mm reversed phase Nano-Acquity-UPLC, Waters, En Yvelines Cedex France) analysis using a Thermo LTQ Orbitrap Velos (30,000 Resolution, Top 20, collision-induced dissociation) workflow and a gradient of 1–40% acetonitrile in 48 min at a flow rate of 250 nL/min. Samples were analyzed in duplicate. Peptides were detected and quantified with Progenesis LC-MS software (version 3.1.4003.30577) using default settings (no deconvolution/deisotoping, 200 most intense MS/MS peaks). A merged peaklist generated by Progenesis LC-MS was searched against the uniprot human database (version.3.80, 86719 entries) using Mascot v2.4, allowing one missed cleavage and 20 ppm/ 0.5 Da mass deviations in MS and MS/MS. Carbamidomethylation of cysteine was a fixed modification. Oxidation of methionine and deamidation of asparagine and glutamine were used as variable modifications.

Using a significance threshold of $p < 0.05$, a total of 158,054 out of 251,351 spectra could be matched to the Uniprot human database. For label-free protein quantitation Mascot results were imported into Progenesis LC-MS. Raw protein abundance values from Progenesis LC-MS were first normalized using quantile normalization, and technical replicates aggregated by taking the median normalized value for each protein in each sample. Quantitation of enzyme abundance based on the different probe pulldowns was based on at least two unique peptides in order to generate heatmaps. MS/MS spectra matching to enzymes identified by one peptide were inspected manually.

Results

Di-Ub probes were engineered comprising two recombinant Ub proteins joined by a linkage. The linkage contained an electrophilic trap in the position of the scissile isopeptide bond between the C-terminal glycine residue of a distal Ub and the lysine residue of a proximal Ub molecule, thus allowing covalent capture of DUBs with a cysteine in the active site. The linkage position was predefined by site directed incorporation of the unnatural amino acid Aha into the proximal Ub, linked to an alkyne moiety at the C-terminus of the distal Ub. Introduction of an alanine residue at position two of Ub to facilitate N-terminal Met/Aha cleavage was followed by a K to M point mutation at each of the lysine residues to afford the seven Ub mutant plasmids. The Ub mutants and wild type (WT) Ub were expressed with Aha incorporation (M1Aha, K6Aha, K11Aha, K27Aha, K29Aha, K33Aha, K48Aha and K63Aha; see SEQ. ID NOs: 1 to 8) and purified. The purified proteins were characterized by silver staining, MALDI-TOF MS of the intact proteins and LC-MS/MS analysis after digestion with trypsin to verify site specific Aha incorporation. HA-Ub-alkyne and VME probes were created and purified as described above. The HA tag present on the N terminal of the distal Ub (derived from HA-Ub-alkyne) allowed for retrieval and visualization. The HA-Ub-alkyne was reacted with the relevant azide bearing Ub by Cu(I) catalysed triazole formation to afford the corresponding HA-tagged Di-Ub probes.

HPLC purification of all Di-Ub probes was performed to increase the purity, reducing the residual amount of HA-Ub-alkyne. HPLC fractions were analyzed by western blotting (see Figure 5). The fractions marked with a dotted box were concentrated, and used for subsequent Ub processing enzyme profiling experiments.

The reactivity of the purified M1 Di-Ub probe was tested by incubation with the deubiquitinating enzyme OTUB2 alongside the monomeric alkyne probe and visualized by both silver stain and western blotting (see Figure 6a). Covalent trapping of OTUB2 with the probes was observed as a characteristic mass shift and shown to be inhibited by NEM. As a test for the competing use as conjugation substrates due to the presence of a free C-terminus in the proximal Ub of the M1 Di-Ub probe, a portion was reacted with MeSNa to deactivate the electrophilic trap whilst maintaining the carboxy terminus which is required for conjugation. Inactivated and active M1 Di-Ub probes were incubated with four different recombinant DUBs (OTUB2, UCH-L3, UCH-L5 and USP15) and also extract prepared from HEK293T cells (see Figure 6b). All DUBs were labelled with the active M1 Di-Ub probe, but did not react with the inactivated counterpart. Incubating the active probe with lysate resulted in strong labelling of proteins whereas lysate incubation with the inactive probe did not lead to any labelling. This demonstrated that the electrophilic trap is required for tagging.

To compare the cellular labelling pattern of the different di-Ub probes and the HA-Ub-VME and HA-Ub-alkyne probes, HEK 293T cell lysate and the relevant probe were incubated and analysed by SDS-PAGE and anti-HA immunoblotting (Fig. 7). Interestingly, Di-Ub probe labelling profiles appeared distinct from one another and notably so from the mono-Ub probes, suggesting a higher DUB selectivity. To investigate this in more detail, cell lysate probe labelling followed by immunoprecipitation (IP) with anti-HA antibody agarose beads was carried out for all ten Ub probes. The elution fractions of all IPs were analyzed by tandem mass spectrometry to identify ubiquitin processing machinery trapped by the probes.

In total, 1067 proteins were identified by LC-MS/MS in all the ten probe pulldowns combined including 36 DUBs; 23 USPs, 4 UCHs, 5 OTUs 1 MJD, 1 JAMM, 1 subunit of the BRCA1 complex and 1 sumo deconjugase. The intensities of peptide signals for detected DUBs were subjected to relative quantitation in order to compare DUB selectivity for the various Ub linkages. To compare and further explore DUBs preferences for Ub linkages, poly-Ub chains of defined linkage were incubated with various recombinant members of the UCH, USP and OTU families in an *in vitro* assay. Samples were analyzed by SDS-PAGE and anti-Ub immunoblotting.

The monomeric HA-Ub-VME and HA-Ub-alkyne probes showed generally the highest relative reactivity across the panel of cellular DUBs detected and the M1 Di-Ub probe was the most broadly reactive among the Di-Ub probes. Whilst the lysine linkage mimicking probes will have the electrophilic trap in the appropriate position to mimic the native isopeptide bond the M1 probe is linked *via* an azide side chain rather than an N-terminal azide. The different conformation of the distal ubiquitin and the greater degree of flexibility afforded by this linker in comparison to the native peptide bond appear to have resulted in a greater than anticipated reactivity of this probe. Several DUBs appeared to be selective for specific probes tested.

5 OTU containing enzymes were detected in the IP material. For OTU5 a preference for K11 was observed amongst the di-Ub probes. With OTU6B and VCP135 little reactivity towards the Di-Ub probes in comparison to the monomeric probes was observed. OTU7B reflects specificity for K11 in the assay showing the highest reactivity amongst the Di-Ub probes. OTUB1 displayed K48 linkage specificity amongst the Di-Ub probes, in line with both literature observations and the results of the recombinant assay.

For USPs, the recombinant assays and probe labelling experiments showed that these enzymes are generally specific to ubiquitination substrates rather than the linkage of the ubiquitin chain, with a low degree of selectivity observed between the various ubiquitin linkages. However, some examples of apparent specificity in the reactivity toward different Di-Ub probes were seen.

For instance, USP13, 25 and 40 showed high relative affinity for the M1 probe. USP 15 in the IP results showed a degree of selectivity for the K27 linked probe. The corresponding recombinant assay showed that USP 15 was active in cleaving K27 chains, but also other linkages. USP 16 showed a high relative reactivity towards the K27 probe and to a lesser extent the K29 probe in the assay. Di-Ub probe labelling data also suggested K11 selectivity by USP 5 whilst the recombinant data suggested a very low degree of reactivity generally. USP 47 was found to have a high relative reactivity towards K27 linkages based on reactivity to the Di-Ub probes and the recombinant DUB assays, and USP 24 was highlighted to have high relative reactivity towards the K27 and K11 probes. Ataxin 3 was also detected, principally in the M1 and K29 probe IPs. UB fusion protein 1 was also detected, the highest relative abundance observed with the M1 Di-Ub probe. BRCA1-A showed little selectivity amongst the probes. Taken together, the probes representing the various Di-Ub linkages provide a topology of cellular DUB specificities for different poly-Ub chains, some of which were validated *in vitro*.

Quantitative mass spectrometry analysis of Di-Ub probe IP material revealed that components of the Ub conjugation machinery were enriched by the different Di-Ub probes. Higher levels of E1, E2 and E3 enzymes were recovered with the M1 di-Ub probe as compared to mono-Ub and other di-Ub probes. Other HECT domain E3 ligases were detected including E3A (E6-AP), E3C (HECTH2), HECTD1, HECTD3, HERC1 and HERC4. Enzymes belonging to the RBR family of Ubiquitin E3 ligases were also detected for example AROH1 and RNF31 which bear both RING and HECT domains, suggesting that these enzymes were directly labelled by the di-Ub probes. Di-Ub probe pulldowns revealed enzymes of the RING family such as MYCBP2, RNF25, TRIM25, TRIM33, IRF2-BP1, KCMF1, Listerin, MID2, RAD18, RING1 and TTC3 (DCRR1). As RING E3 ligases are believed to mediate the transfer of Ub from the cognate E2 to the substrate directly, it is likely that these E3 enzymes were pulled down by the di-Ub probes as multi-protein complexes. Consistent with this, a panel of E1 and E2 enzymes was also detected in the Di-Ub probe IP experiments. The unexpected reactivity of the M1 Di-Ub probe towards Ub conjugation machinery may provide a highly useful tool in activity based profiling of these classes on enzyme.

In conclusion, it can be seen that the above-mentioned activity probes based on Di-Ub scaffolds may be used to analyse DUB specificity in a cellular context. In particular, the probes may be used to assess the selectivity of DUBs within the cognate environment of substrates as part of multi-protein complexes, which can be problematic to reconstitute in *in vitro* experiments. The present probes may therefore be useful in understanding the role of DUBs and Ub conjugating enzymes in cellular functions under normal physiology, as well as disease.

It will be understood that the present invention has been described above purely by way of example, and modification of detail can be made within the scope of the invention. Each feature disclosed in the description, and where appropriate the claims and drawings may be provided independently or in any appropriate combination.

CLAIMS

1. A probe for use in the detection of a deubiquitinating enzyme, wherein the probe comprises:

a protein comprising an amino acid sequence of a ubiquitin or ubiquitin-like protein; and

a group of the formula -L-Z attached at the carboxy terminus of said amino acid sequence, wherein L is a linker group comprising an electrophilic moiety which is capable of covalently binding to an active site of a deubiquitinating enzyme; and Z is a detectable label or a conjugated substance;

and wherein the probe comprises at least one detectable label.

2. A probe according to claim 1, wherein the probe comprises at least one detectable label which is member of a specific binding pair.

3. A probe according to claim 2, wherein the probe comprises an epitope tag, *e.g.* a hemagglutinin (HA) tag.

4. A probe according to claim 3, wherein said epitope tag is attached at the amino terminus of said first protein.

5. A probe according to any one of the preceding claims, wherein the probe comprises at least one detectable label which is a fluorescent label or a radioisotope label.

6. A probe according to any one of the preceding claims, wherein the probe comprises an epitope tag and a fluorescent label.

7. A probe according to any one of the preceding claims, wherein the probe is of the Formula I:

$$X\text{-}Y\text{-}L\text{-}Z \qquad (I)$$

wherein

X is a detectable label or is absent;

Y is a protein comprising an amino acid sequence of a ubiquitin or ubiquitin-like protein;

L and Z are as defined in claim 1; and

at least one of X, Y, L and Z comprises a detectable label.

8. A probe according to claim 7, wherein X and/or Z comprises a detectable label; and optionally Y and L do not comprise a detectable label.

9. A probe according to claim 7 or claim 8, wherein X is a detectable label selected from an epitope tag, a radioisotope label and a fluorescent label.

10. A probe according to claim 9, wherein X is an epitope tag, *e.g.* a hemagglutinin (HA) tag.

11. A probe according to any one of the preceding claims, wherein the probe is synthesised from a fusion protein.

12. A probe according to any one of the preceding claims, wherein the ubiquitin or ubiquitin-like protein is selected from ubiquitin (Ub), ubiquitin-like 5 (UBL5), ubiquitin-fold modifier 1 (UFM1), autophagy-related protein 12 (ATG12/APG12), autophagy-related protein 8 (ATG8/APG8), ubiquitin-related modifier 1 (URM1), interferon stimulated gene 15 (ISG15), ubiquitin-like protein FAT10, small ubiquitin-like modifier 1/2/3 (SUMO 1/2/3) 3, neural precursor cell expressed, developmentally down-regulated 8 (NEDD8), ubiquitin cross-reactive protein (UCRP), homologous to ubiquitin 1 (HUB1), ribosomal protein S30 fused to a ubiquitin-like protein (Fau) and bacterial ubiquitin-like modifier Pup.

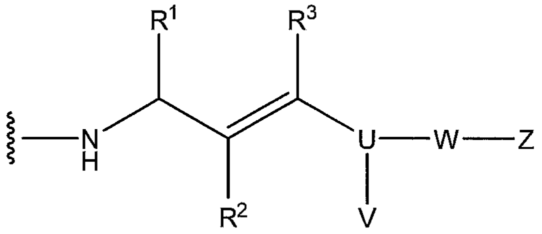
13. A probe according to any one of the preceding claims, wherein an arginine or a glycine residue is present at the carboxy terminus of said protein.

14. A probe according to any one of the preceding claims, wherein the electrophilic

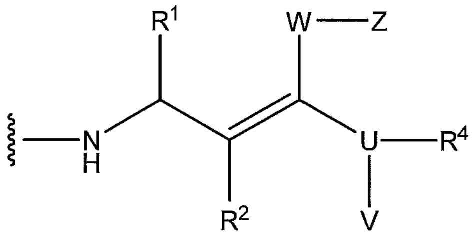
moiety is capable of covalently binding to a thiol group that is present at an active site of the deubiquitinating enzyme.

15. A probe according to any one of the preceding claims, wherein the electrophilic moiety comprises a Michael acceptor; an alkylene group which contains a carbon atom that is substituted by an electron withdrawing group; or an alkenylene group which contains a carbon atom that is substituted by an electron withdrawing group.

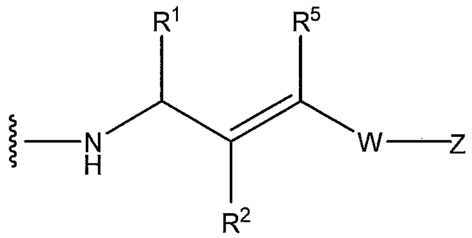
16. A probe according to any one of the preceding claims, wherein the group -L-Z is of the formula (II), (III), (IV) or (V):



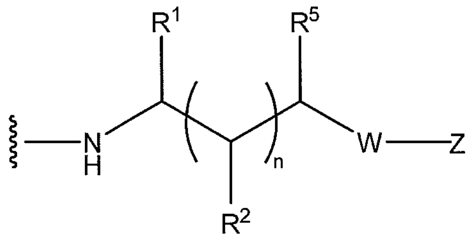
(II)



(III)



(IV)



(V)

wherein

R^1 is selected from hydrogen; R^a; hydrocarbyl optionally substituted with 1, 2, 3, 4 or 5 R^a; and $-(CH_2)_j$-heterocyclyl optionally substituted with 1, 2, 3, 4 or 5 R^a;

R^2 is selected from hydrogen; R^a; hydrocarbyl optionally substituted with 1, 2, 3, 4 or 5 R^a; and $-(CH_2)_j$-heterocyclyl optionally substituted with 1, 2, 3, 4 or 5 R^a;

R^3 is selected from hydrogen; R^a; hydrocarbyl optionally substituted with 1, 2, 3, 4 or 5 R^a; and $-(CH_2)_j$-heterocyclyl optionally substituted with 1, 2, 3, 4 or 5 R^a;

R^4 is selected from hydrogen; R^a; hydrocarbyl optionally substituted with 1, 2, 3, 4 or 5 R^a; and $-(CH_2)_j$-heterocyclyl optionally substituted with 1, 2, 3, 4 or 5 R^a; or R^4 is absent;

R^5 is an electron withdrawing group;

U and V taken together form an electron withdrawing group in which the bond between U and V is a π-bond;

W is a linker;

n is 0 or 1;

Z is as defined in claim 1;

each R^a is independently selected from halogen, trifluoromethyl, cyano, nitro, oxo, $=NR^b$, $-OR^b$, $-C(O)R^b$, $-C(O)N(R^b)R^c$, $-C(O)OR^b$, $-C(O)SR^b$, $-OC(O)R^b$, $-S(O)_kR^b$, $-S(O)_kN(R^b)R^c$, $-N(R^b)R^c$, $-N(R^b)N(R^b)R^c$, $-N(R^b)C(O)R^c$ and $-N(R^b)S(O)_kR^b$;

R^b and R^c are each independently hydrogen or selected from hydrocarbyl and -$(CH_2)_j$-heterocyclyl, either of which is optionally substituted with 1, 2, 3, 4 or 5 substituents independently selected from halogen, oxo, cyano, amino, hydroxy, alkyl and alkoxy;

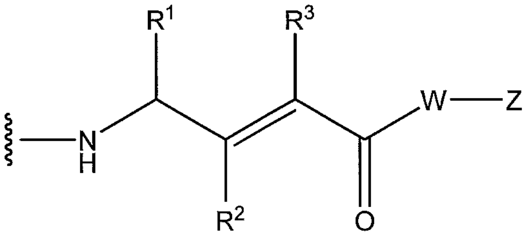
j is 0, 1, 2, 3, 4, 5 or 6; and

k is 0, 1 or 2.

17. A probe according to claim 16, wherein U and V taken together form -C(O)-.

18. A probe according to claim 16 or claim 17, wherein the group -L-Z is of the formula (II).

19. A probe according to claim 18, wherein the group -L-Z is of the formula (IIa):



(IIa).

20. A probe according to any one of claims 16 to 19, wherein R^1 and/or R^2 is hydrogen.

21. A probe according to any one of claims 16 to 20, wherein R^3 is hydrogen.

22. A probe according to claim 16, wherein R^5 is an electron withdrawing group selected from R^a.

23. A probe according to any one of claims 16 to 22, wherein W is a linker containing from 1 to 30, *e.g.* from 1 to 20, *e.g.* from 1 to 15, *e.g.* from 1 to 10, *e.g.* from 5 to 10 in-chain atoms.

24. A probe according to any one of claims 16 to 23, wherein W is a linker comprising a triazole group in the chain.

25. A probe according to claim 24, wherein W is a linker obtainable by the reaction of an alkyne group and an azide group.

26. A probe according to any one of the preceding claims, wherein Z is a detectable label.

27. A probe according to claim 26, wherein Z is a fluorescent label, *e.g.* selected from fluorescein, Cy3, Cy5 and BODIPY-based derivatives.

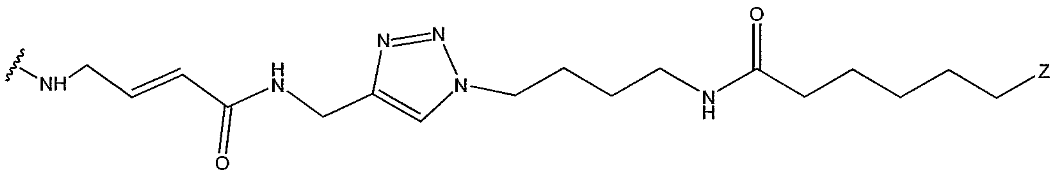
28. A probe according to any one of claims 1 to 25, wherein Z is a conjugated substance.

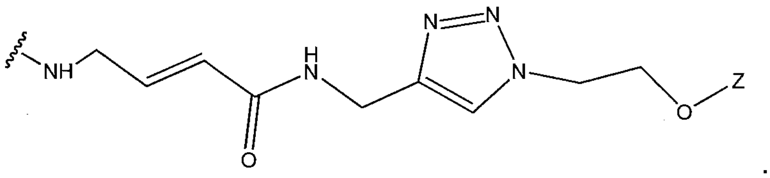
29. A probe according to claim 28, wherein Z is a peptide or a protein.

30. A probe according to claim 29, wherein Z comprises an amino acid sequence of a ubiquitin or ubiquitin-like protein.

31. A probe according to claim 29 or claim 30, wherein Z is attached to the linker group L *via* a homoazidoalanine residue that is present in said peptide or protein of Z.

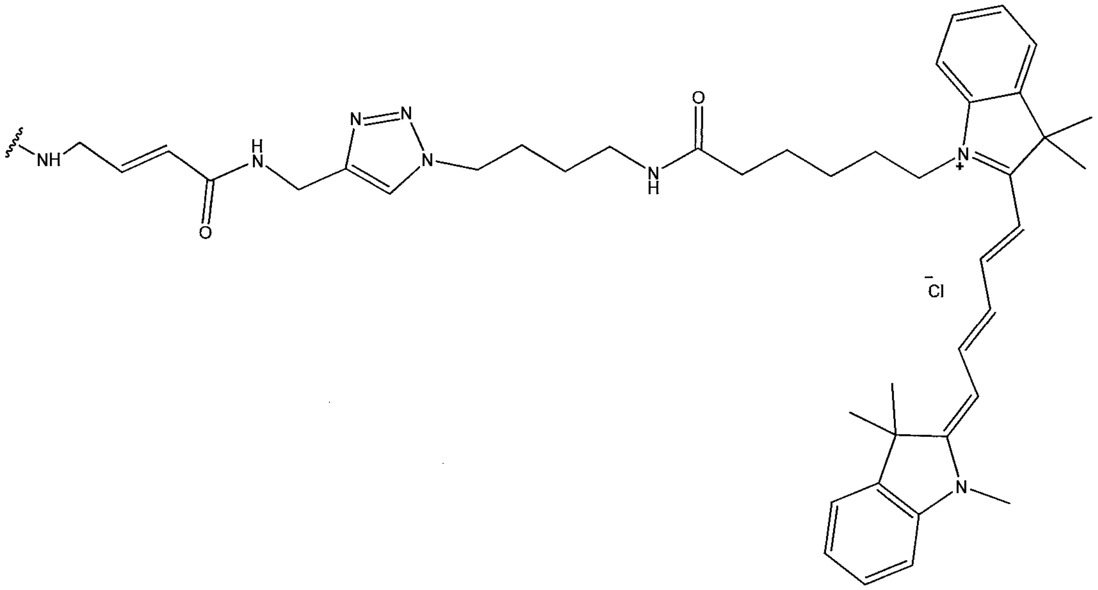
32. A probe according to any one of the preceding claims, wherein -L-Z is a group selected from:

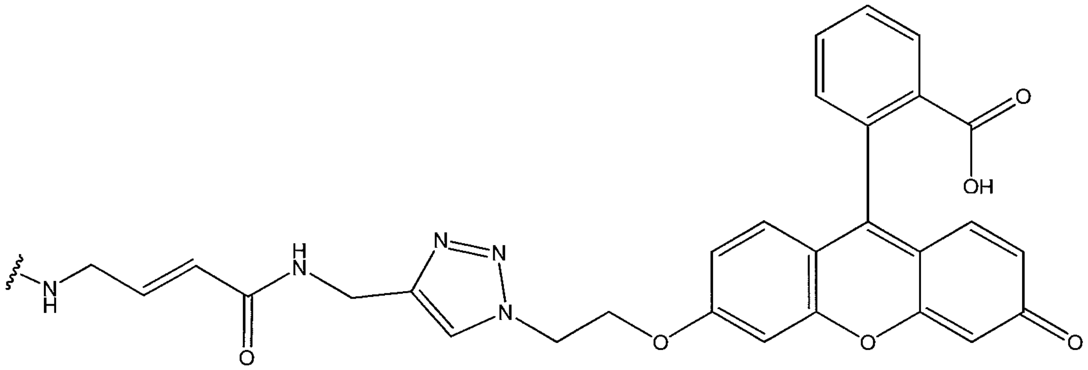




33. A probe according to claim 32, wherein Z is a fluorescent label.

34. A probe according to any one of claims 1 to 27, wherein -L-Z is a group selected from:





35. A process for producing a probe of the Formula I:

$$X\text{-}Y\text{-}L\text{-}Z \qquad (I)$$

wherein

X is a detectable label or is absent;

Y is a protein comprising an amino acid sequence of a ubiquitin or ubiquitin-like protein;

L and Z are as defined in claim 1; and

at least one of X, Y, L and Z comprises a detectable label;

wherein the process comprising contacting a compound of the Formula VI:

$$X\text{-}Y\text{-}R^x \qquad (VI)$$

with a compound of the Formula VII:

$$R^y\text{-}Z \qquad (VII)$$

wherein R^x and R^y are reactive groups and R^x contains said electrophilic moiety; and wherein the contacting is performed under conditions such that R^x and R^y react together to form said linker group L, thereby forming a compound of the Formula I.

36. A process according to claim 35, wherein one of R^x and R^y comprises an azide group and the other comprises an alkyne group; and said groups react to form a linker group L which comprises a triazole group in the chain.

37. A process according to claim 36, wherein R^x comprises an alkyne group (*e.g.* a terminal alkyne group) and R^y comprises an azide group (*e.g.* a terminal azide group).

38. A process according to claim 36 or claim 37, wherein the process comprises performing an azide-alkyne Huisgen cycloaddition reaction using a copper catalyst.

39. A probe obtainable by the process of any one of claims 35 to 38.

40. A method of detecting a deubiquitinating enzyme, which method comprises:

contacting a probe of any one of claims 1 to 34 with a sample comprising at least one deubiquitinating enzyme; and

detecting for the presence of said probe in said sample.

41. A method according to claim 40, wherein the probe comprises a fluorescent label and the method comprises detecting the fluorescence of the probe.

42. A method according to claim 40 or claim 41, wherein the probe comprises an epitope tag and the method comprises contacting the sample with an antibody which specifically binds to said epitope tag.

43. A method according to any one of claims 40 to 42, wherein the probe is detected by immunoblotting and/or by tandem mass spectrometry.

44. A method according to any one of claims 40 to 43, wherein the sample further comprises an inhibitor of a deubiquitinating enzyme.

45. A method according to any one of claims 40 to 44, wherein the sample comprises a cell lysate or intact cells.

46. A kit comprising a probe of any one of claims 1 to 34.

47. A kit according to claim 46, wherein the probe comprises an epitope tag and the kit further comprises an antibody specific for the epitope tag.

48. A kit according to claim 46 or claim 47, further comprising one or more reagents for lysing a cell and/or one or more reagents for fractionating cell components.

49. Use of a probe of any one of claims 1 to 34 for detecting a deubiquitinating enzyme.

50. The use according to claim 49, wherein the probe comprises a fluorescent label and detection is performed by *e.g.* fluorescent imaging or fluorescent microscopy.

51. Use of a probe of any one of claims 1 to 34 for diagnosing a medical disease or condition.

52. Use of a probe of any one of claims 1 to 34, for screening one or more test agents to identify potential enzyme inhibitors, *e.g.* potential deubiquitinating enzyme inhibitors.

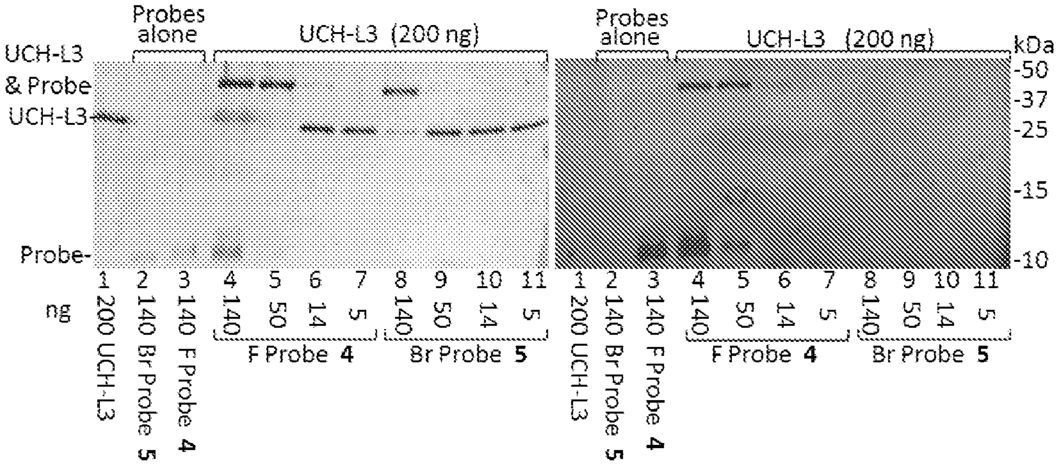

Probes alone
UCH-L3 (200 ng)
UCH-L3 & Probe
UCH-L3
Probe
kDa
-50
-37
-25
-15
-10
1 2 3 4 5 6 7 8 9 10 11
ng
200 UCH-L3
140 Br Probe 5
140 F Probe 4
140 50 14 5
F Probe 4
140 50 14 5
Br Probe 5

Fig. 1

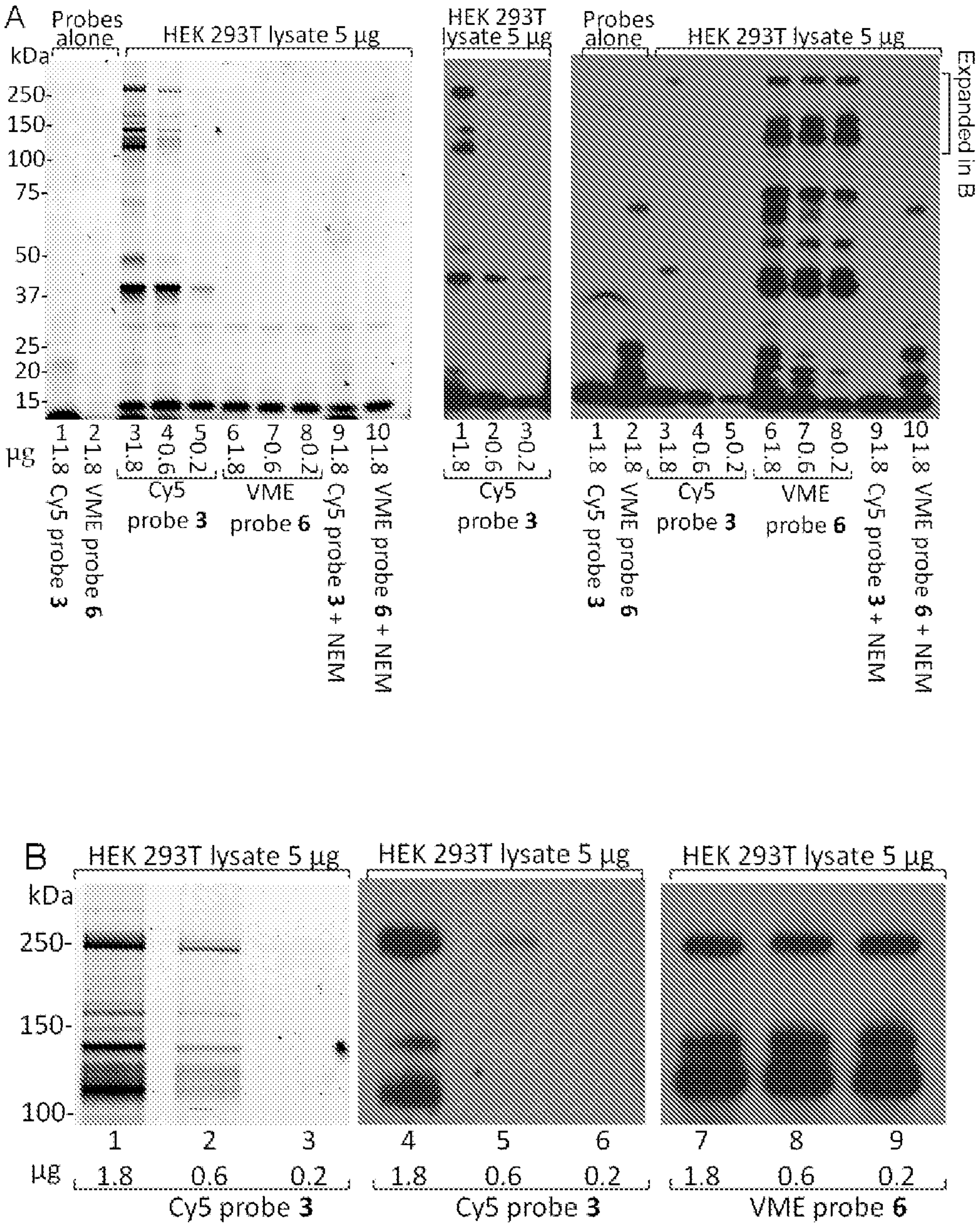

A
Probes alone
HEK 293T lysate 5 µg
kDa
250-
150-
100-
75-
50-
37-
25-
20-
15-
1 1.8 Cy5 probe 3
2 1.8 VME probe 6
3 1.8
4 0.6
5 0.2
Cy5 probe 3
6 1.8
7 0.6
8 0.2
VME probe 6
9 1.8 Cy5 probe 3 + NEM
10 1.8 VME probe 6 + NEM
µg
HEK 293T lysate 5 µg
1 1.8
2 0.6
3 0.2
Cy5 probe 3
Probes alone
HEK 293T lysate 5 µg
Expanded in B
B
HEK 293T lysate 5 µg
HEK 293T lysate 5 µg
HEK 293T lysate 5 µg
kDa
250-
150-
100-
1 2 3 4 5 6 7 8 9
µg 1.8 0.6 0.2 1.8 0.6 0.2 1.8 0.6 0.2
Cy5 probe 3
Cy5 probe 3
VME probe 6

Fig. 2

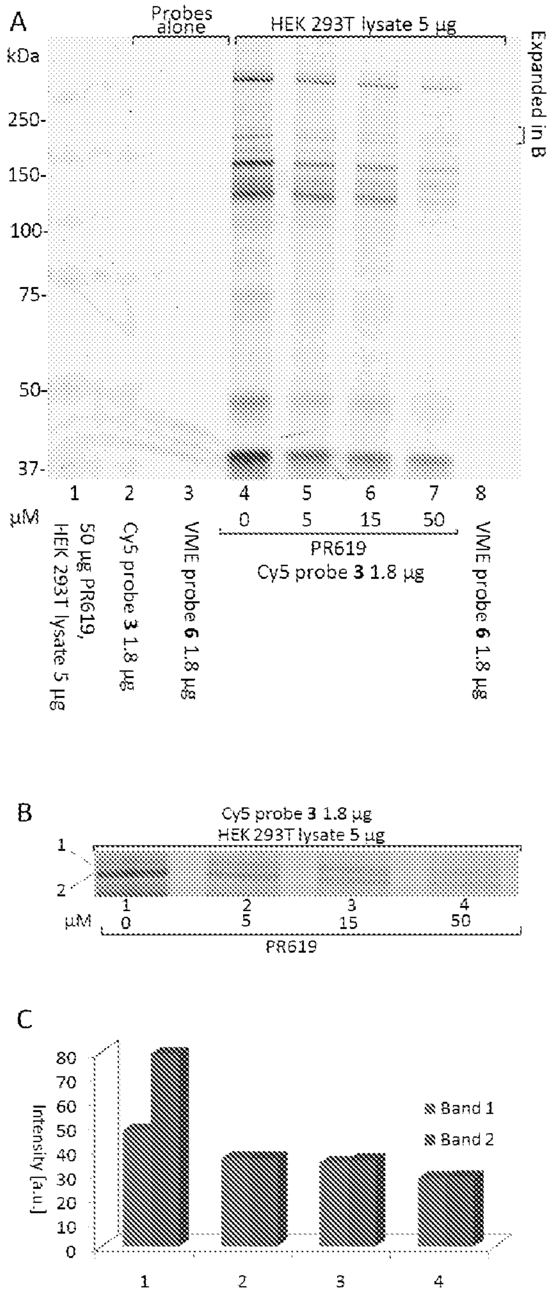

A
Probes alone
HEK 293T lysate 5 µg
kDa
250-
150-
100-
75-
50-
37-
Expanded in B
1 2 3 4 5 6 7 8
µM
0 5 15 50
PR619
Cy5 probe 3 1.8 µg
50 µg PR619, HEK 293T lysate 5 µg
Cy5 probe 3 1.8 µg
VME probe 6 1.8 µg
VME probe 6 1.8 µg
B
Cy5 probe 3 1.8 µg
HEK 293T lysate 5 µg
1
2
1 2 3 4
µM 0 5 15 50
PR619
C
Intensity [a.u.]
0 10 20 30 40 50 60 70 80
Band 1
Band 2
1 2 3 4

Fig. 3

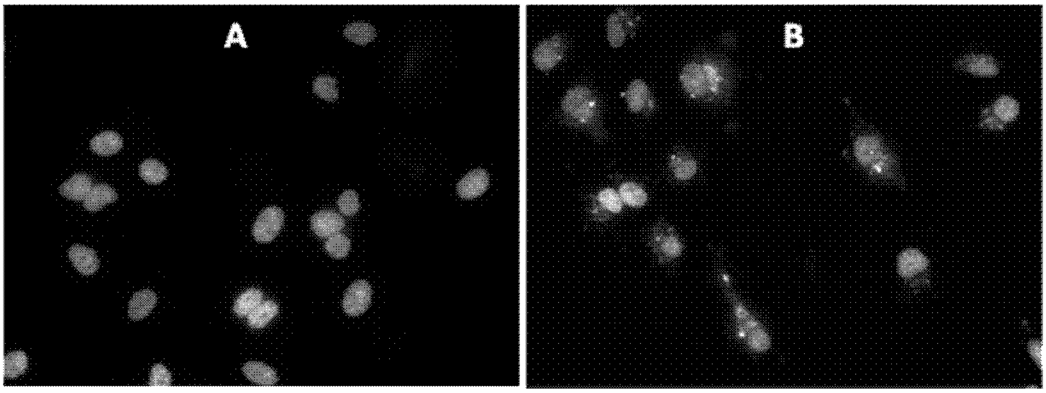


Fig. 4

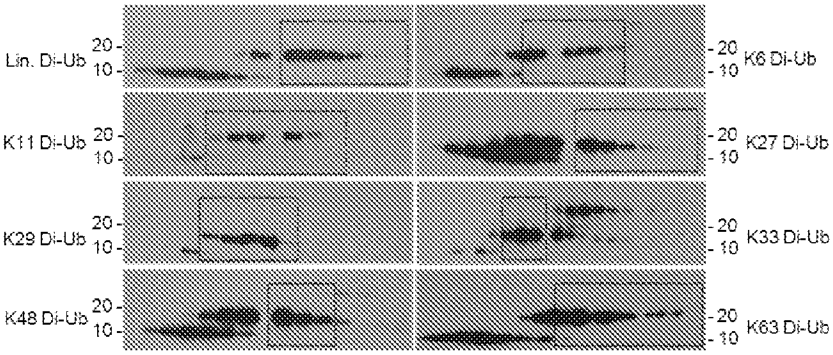

Lin. Di-Ub
20 -
10 -
K11 Di-Ub
20 -
10 -
K29 Di-Ub
20 -
10 -
K48 Di-Ub
20 -
10 -
- 20
- 10
K6 Di-Ub
- 20
- 10
K27 Di-Ub
- 20
- 10
K33 Di-Ub
- 20
- 10
K63 Di-Ub

Fig. 5

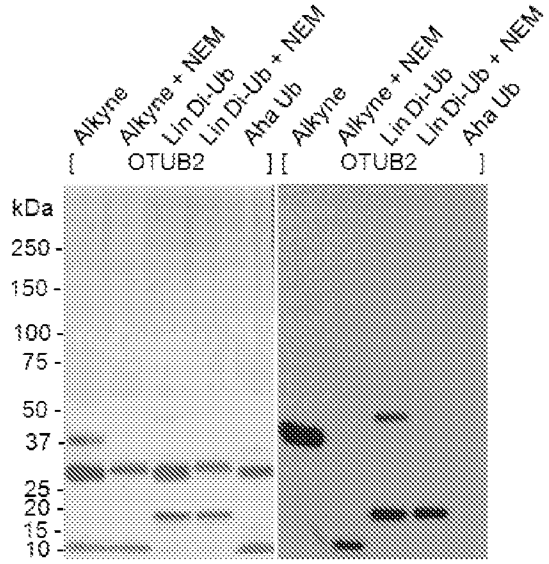

Alkyne
Alkyne + NEM
Lin Di-Ub
Lin Di-Ub + NEM
Aha Ub
Alkyne
Alkyne + NEM
Lin Di-Ub
Lin Di-Ub + NEM
Aha Ub
[OTUB2]
[OTUB2]
kDa
250 -
150 -
100 -
75 -
50 -
37 -
25 -
20 -
15 -
10 -

Fig. 6a

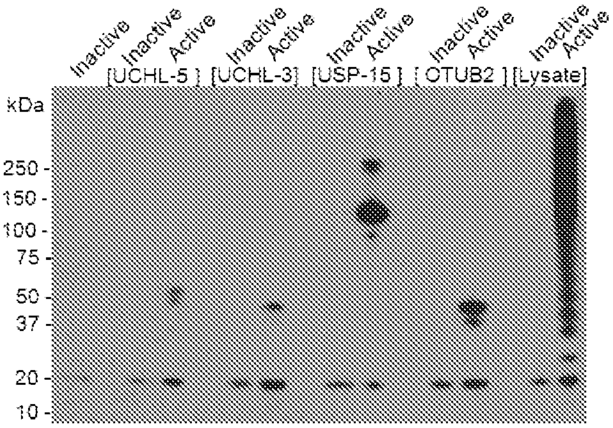

Inactive
Inactive
Active
Inactive
Active
Inactive
Active
Inactive
Active
Inactive
Active
[UCHL-5]
[UCHL-3]
[USP-15]
[OTUB2]
[Lysate]
kDa
250 -
150 -
100 -
75 -
50 -
37 -
20 -
10 -

Fig. 6b

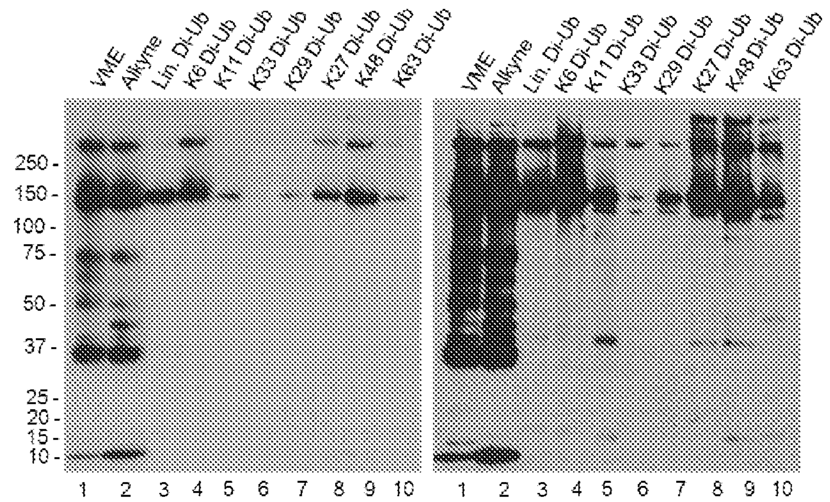

VME
Alkyne
Lin. Di-Ub
K6 Di-Ub
K11 Di-Ub
K33 Di-Ub
K29 Di-Ub
K27 Di-Ub
K48 Di-Ub
K63 Di-Ub
250 -
150 -
100 -
75 -
50 -
37 -
25 -
20 -
15 -
10 -
1
2
3
4
5
6
7
8
9
10

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/050848

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/37 G01N33/532
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, CHEM ABS Data, WPI Data, BEILSTEIN Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LOVE KERRY ROUTENBERG ET AL: "Ubiquitin C-Terminal Electrophiles Are Activity-Based Probes for Identification and Mechanistic Study of Ubiquitin Conjugating Machinery", ACS CHEMICAL BIOLOGY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC, US, vol. 4, no. 4, 1 April 2009 (2009-04-01), pages 275-287, XP008133068, ISSN: 1554-8929 Abstract; Fig. 1-5; Methods. -----	1-31, 35-52
X	US 2012/058499 A1 (ORCUTT STEVEN J [US] ET AL) 8 March 2012 (2012-03-08) Abstract; Fig. 1; par. 17, 36, 88-128; claims 1, 14, 15. ----- -/--	1-30, 35-52

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 12 July 2013	Date of mailing of the international search report 23/07/2013
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer López García, F

2

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/050848

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HASSIEPEN ET AL: "A sensitive fluorescence intensity assay for deubiquitinating proteases using ubiquitin-rhodamine110-glycine as substrate", ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol. 371, no. 2, 31 October 2007 (2007-10-31), pages 201-207, XP022323546, ISSN: 0003-2697, DOI: 10.1016/J.AB.2007.07.034 Abstract. -----	1-30, 35-52
X	SILVIA EGER ET AL: "Synthesis of Defined Ubiquitin Dimers", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 132, no. 46, 24 November 2010 (2010-11-24), pages 16337-16339, XP55066880, ISSN: 0002-7863, DOI: 10.1021/ja1072838 Abstract; Figs. 1, 1S. -----	1-31, 35-52
X	WO 2011/157982 A1 (MEDICAL RES COUNCIL [GB]; KOMANDER DAVID [GB]; YE YU [GB]) 22 December 2011 (2011-12-22) Abstract; examples -----	1-31, 35-52
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