SUPPLEMENTAL INFORMATION

for

Deubiquitinating enzyme specificity for ubiquitin chain topology profiled by di-ubiquitin activity probes

[1]Joanna F. McGouran, [1]Selina R. Gaertner, [1,2]Mikael Altun, [3,4]Holger B. Kramer and [1,4]Benedikt M. Kessler

[1]Target Discovery Institute, Henry Wellcome Building for Molecular Physiology, Nuffield Department of Medicine, University of Oxford, Roosevelt Drive, Oxford OX3 7FZ, UK

[2]Present address: Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 17177 Stockholm, Sweden

[3]Department of Physiology, Anatomy and Genetics, University of Oxford, South Parks Road, OX1 3QX, UK

[4]Corresponding authors

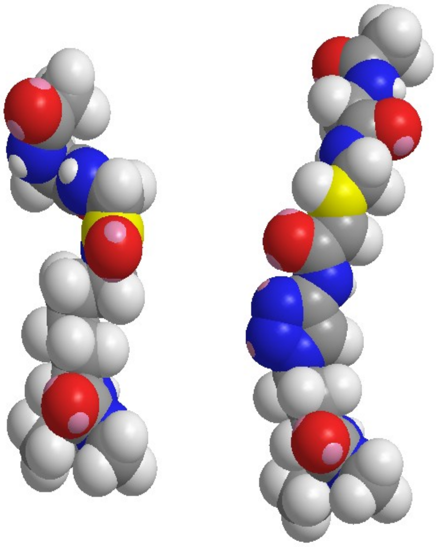


Figure S1 (related to Figure 1). Modeling of native di-Ub linkage as compared to the linkage present in Di-Ub probes generated in this study

Structures were generated and minimized accordingly using the ChemBio 3D package (CambridgeSoft/Perkin Elmer). Structures were then aligned with respect to the distal Ub residues (bottom) to allow for direct comparison. The electrophilic carbon atom of the scissile amide bond (left) or the corresponding electrophilic moiety (carbonyl carbon atom of the vinyl amide, right) are indicated in yellow. (Grey: C, Red:O, Blue:N).

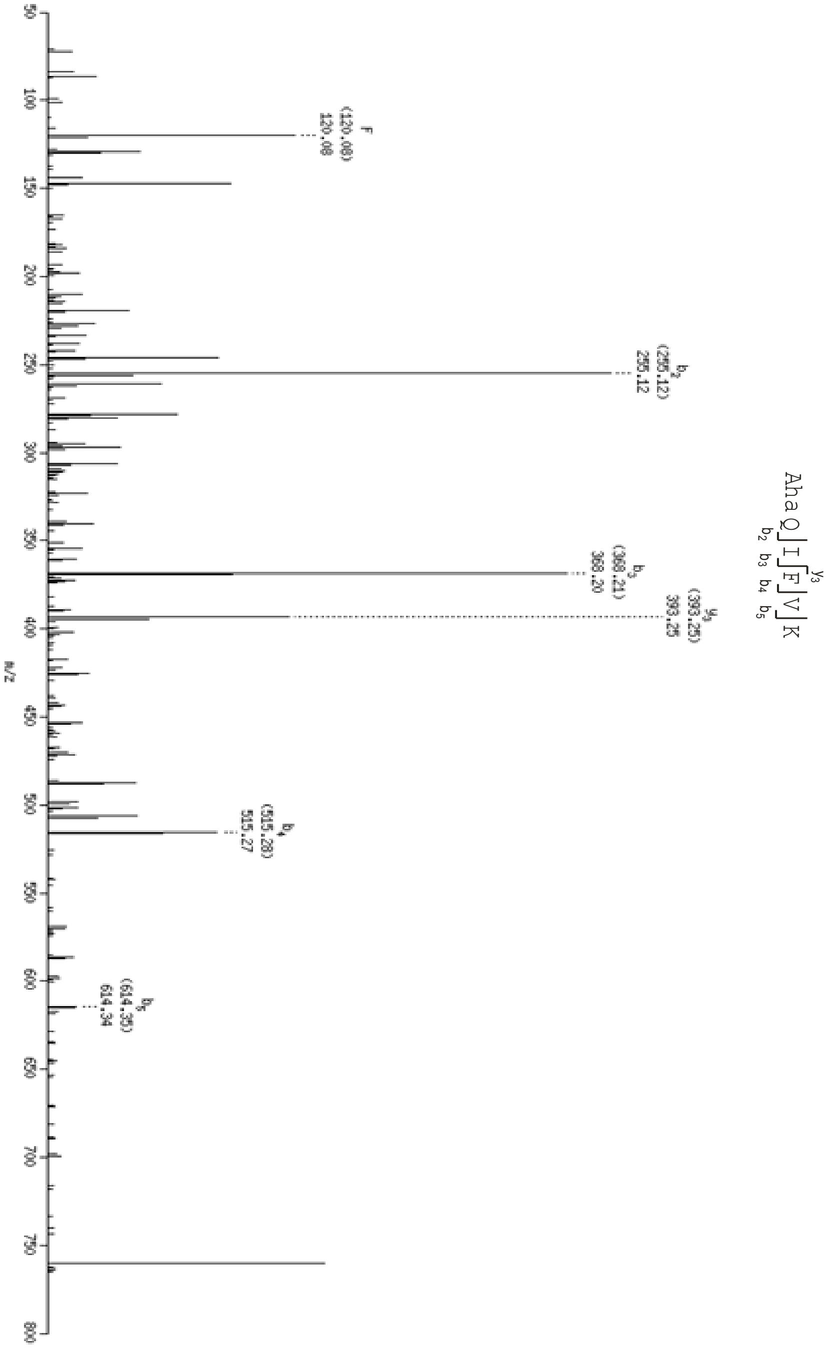
Figure S2 (related to Figure 2). LC-MS/MS results for the ubiquitin mutants

LC-MS/MS spectra of each Ubiquitin mutant proteins with Aha incorporations at the M1 (**A**), K6 (**B**), K11 (**C**), K27 (**D**), K29 (**E**), K33 (**F**), K48 (**G**) and K63 (**H**) are shown in order to confirm the position of Aha incorporation.

A **Aha Ub (M1)**

1 AhaQIFVKTLT GKTITLEVEP SDTIENVKAK IQDKEGIPPD QQRLIFAGKQ

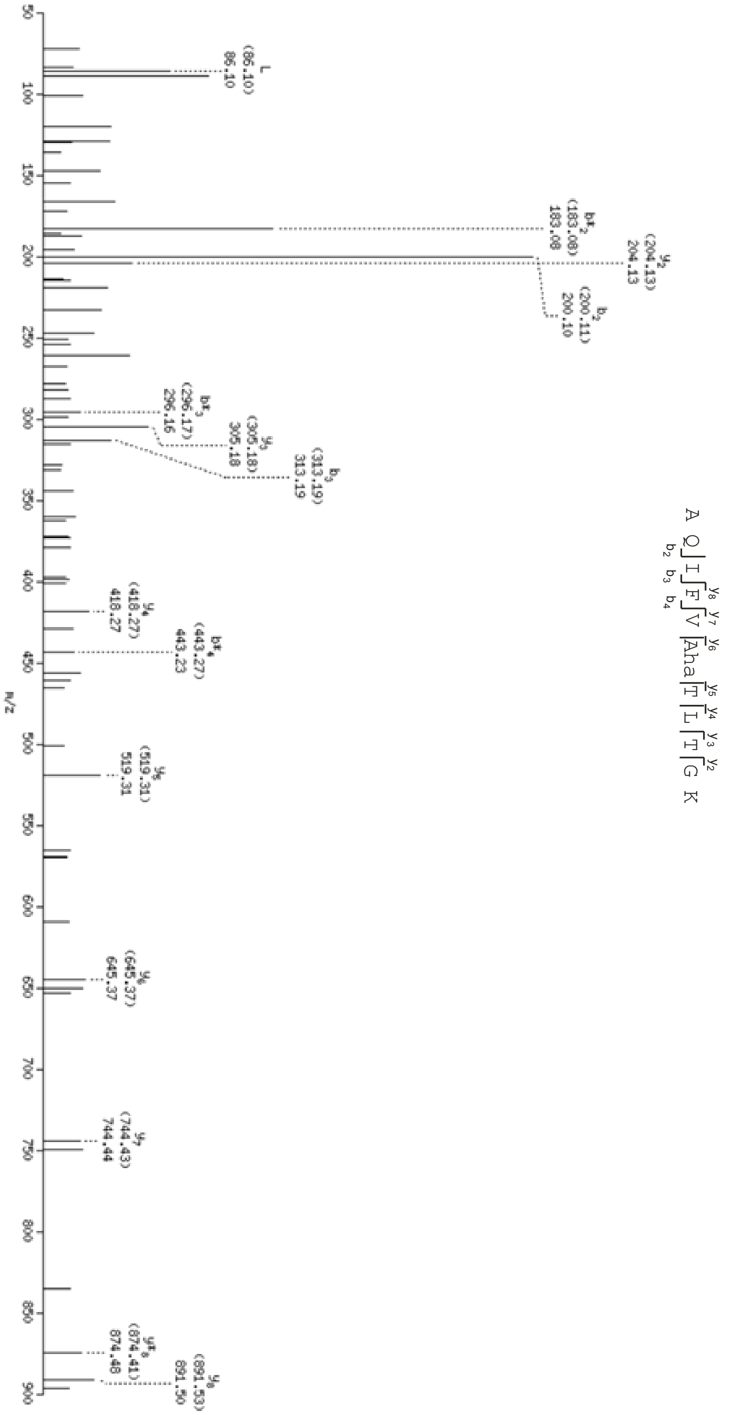
51 LEDGRTLSDY NIQKESTLHL VLRLRGG



B **K6Aha Ub**

1 M**AQIFVAhaTLT GK**TITLEVEP SDTIENVKAK IQDKEGIPPD QQRLIFAGKQ

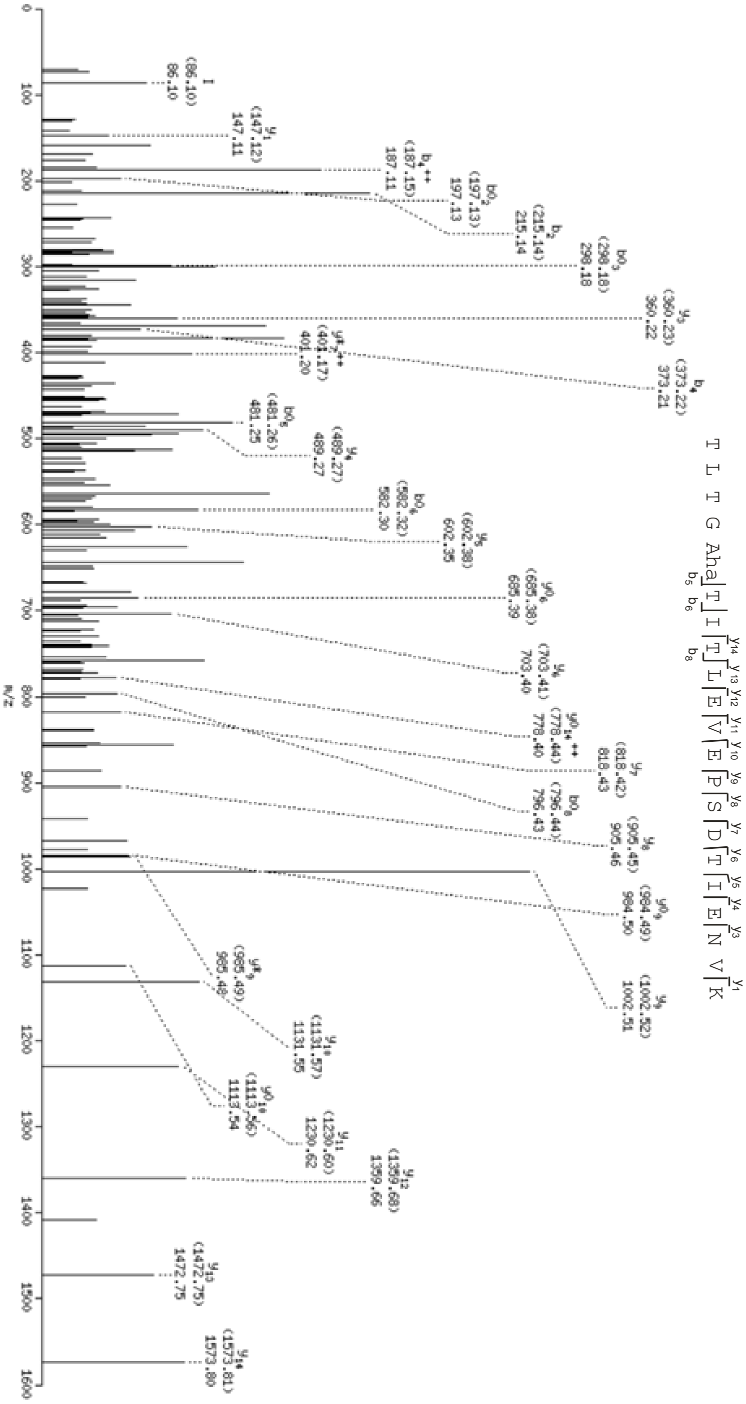
51 LEDGRTLSDY NIQKESTLHL VLRLRGG



C **K11Aha Ub**

1 MAQIFVK**TLT GAhaTITLEVEP SDTIENVK**AK IQDKEGIPPD QQRLIFAGKQ

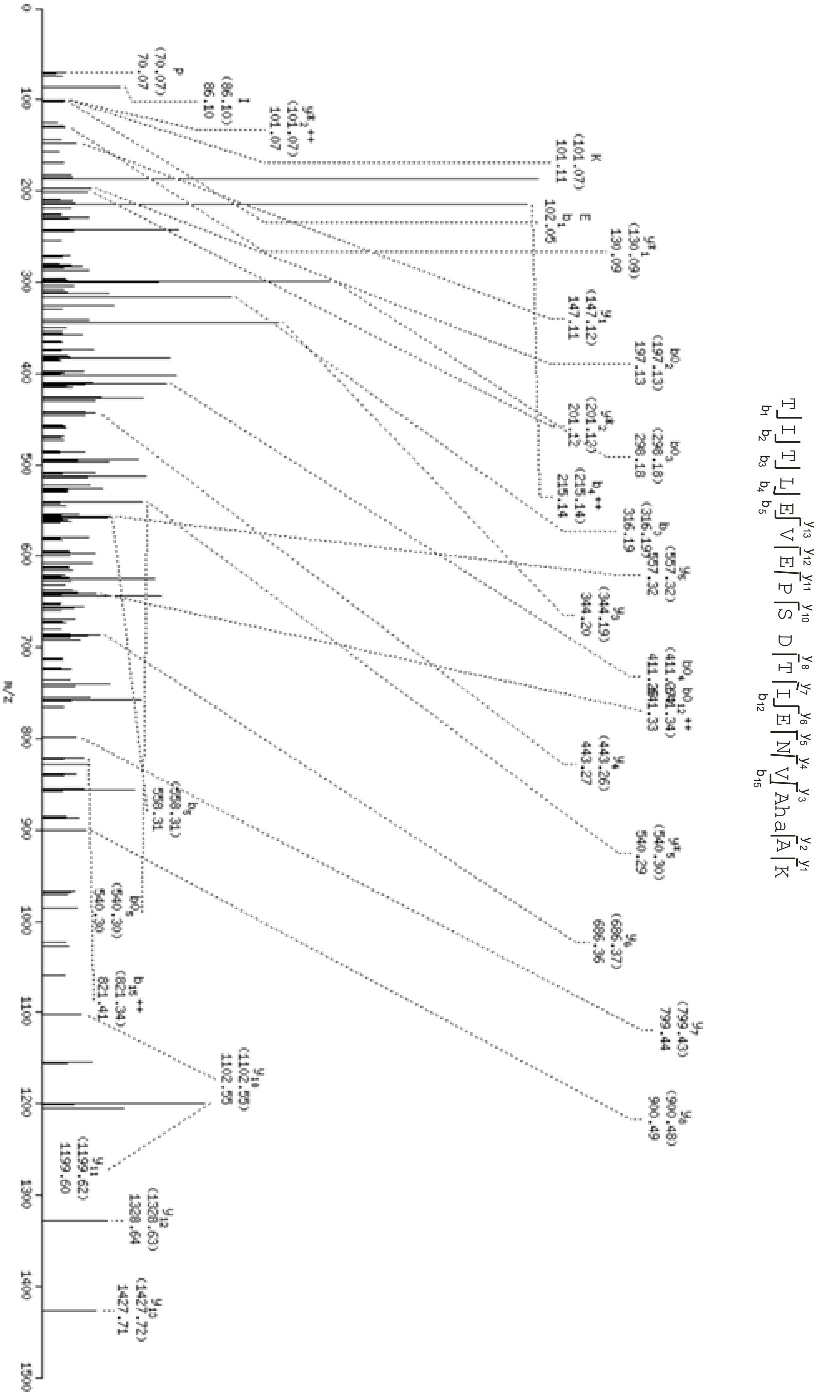
51 LEDGRTLSDY NIQKESTLHL VLRLRGG



D **K27Aha Ub**

1 MAQIFVKTLT GK**TITLEVEP** **SDTIENVAhaAK** IQDKEGIPPD QQRLIFAGKQ

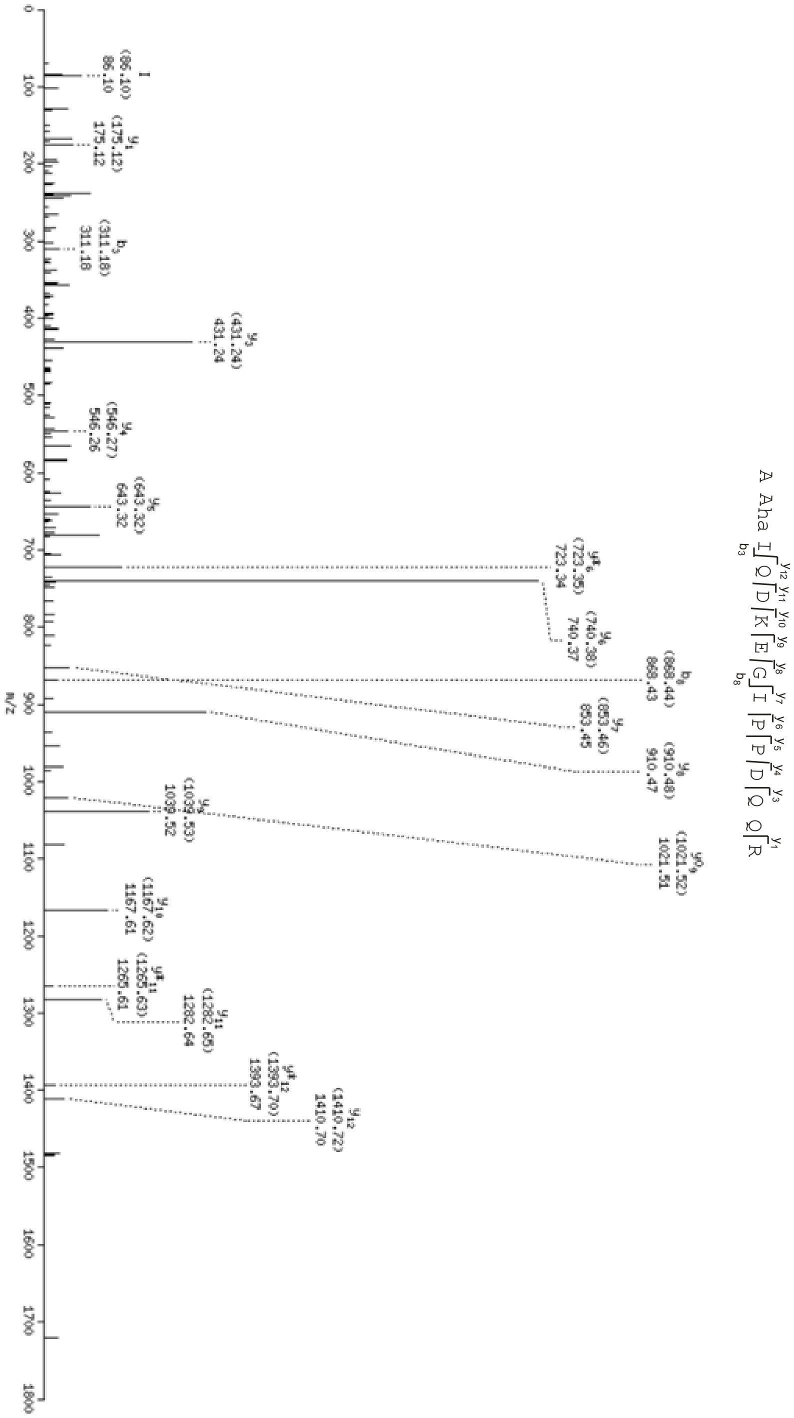
51 LEDGRTLSDY NIQKESTLHL VLRLRGG



E **K29Aha Ub**

1 MAQIFVKTLT GKTITLEVEP SDTIENVK**AAha IQDKEGIPPD QQR**LIFAGKQ

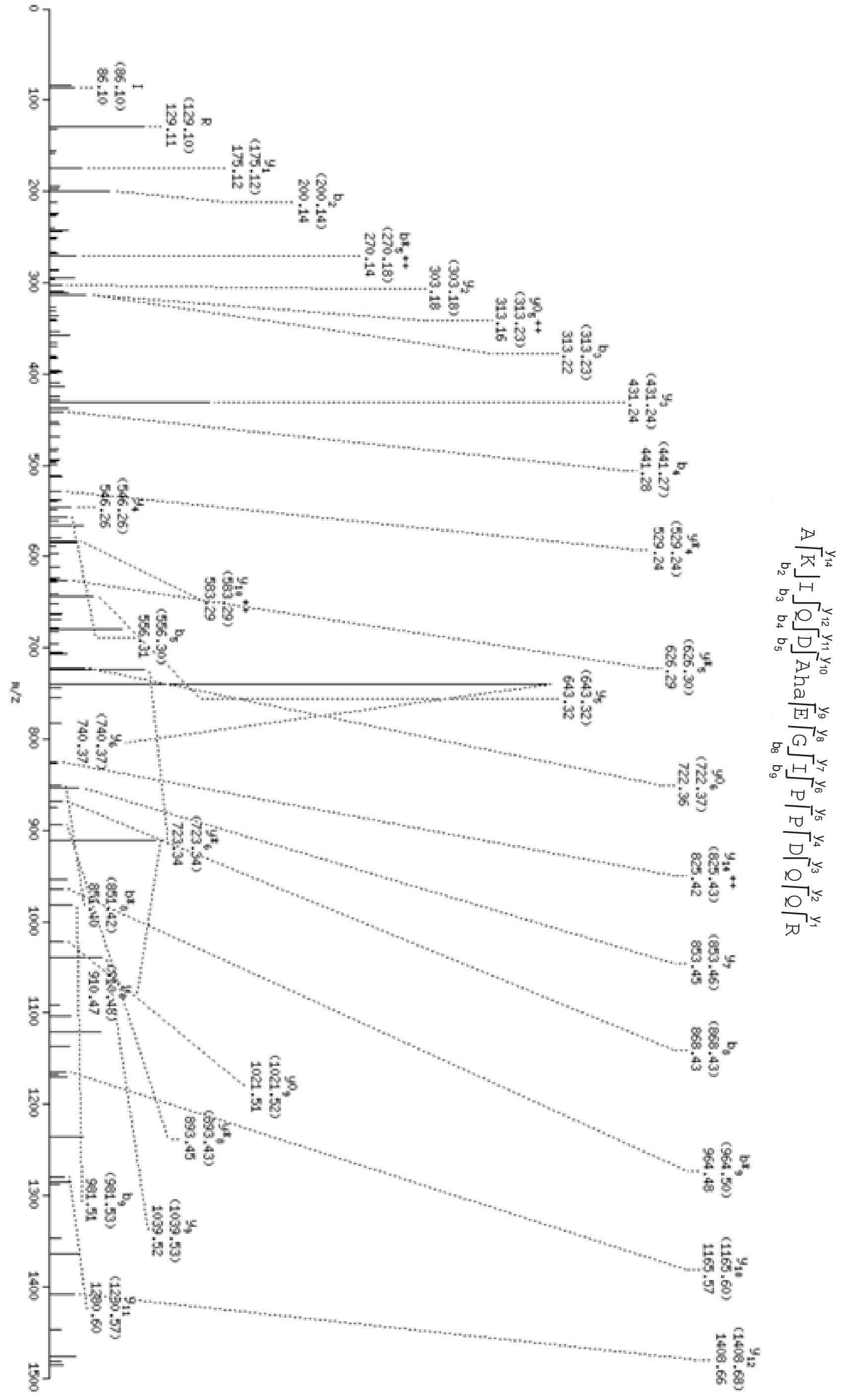
51 LEDGRTLSDY NIQKESTLHL VLRLRGG



F **K33Aha Ub**

1 MAQIFVKTLT GKTITLEVEP SDTIENVK**AK IQDAhaEGIPPD QQR**LIFAGKQ

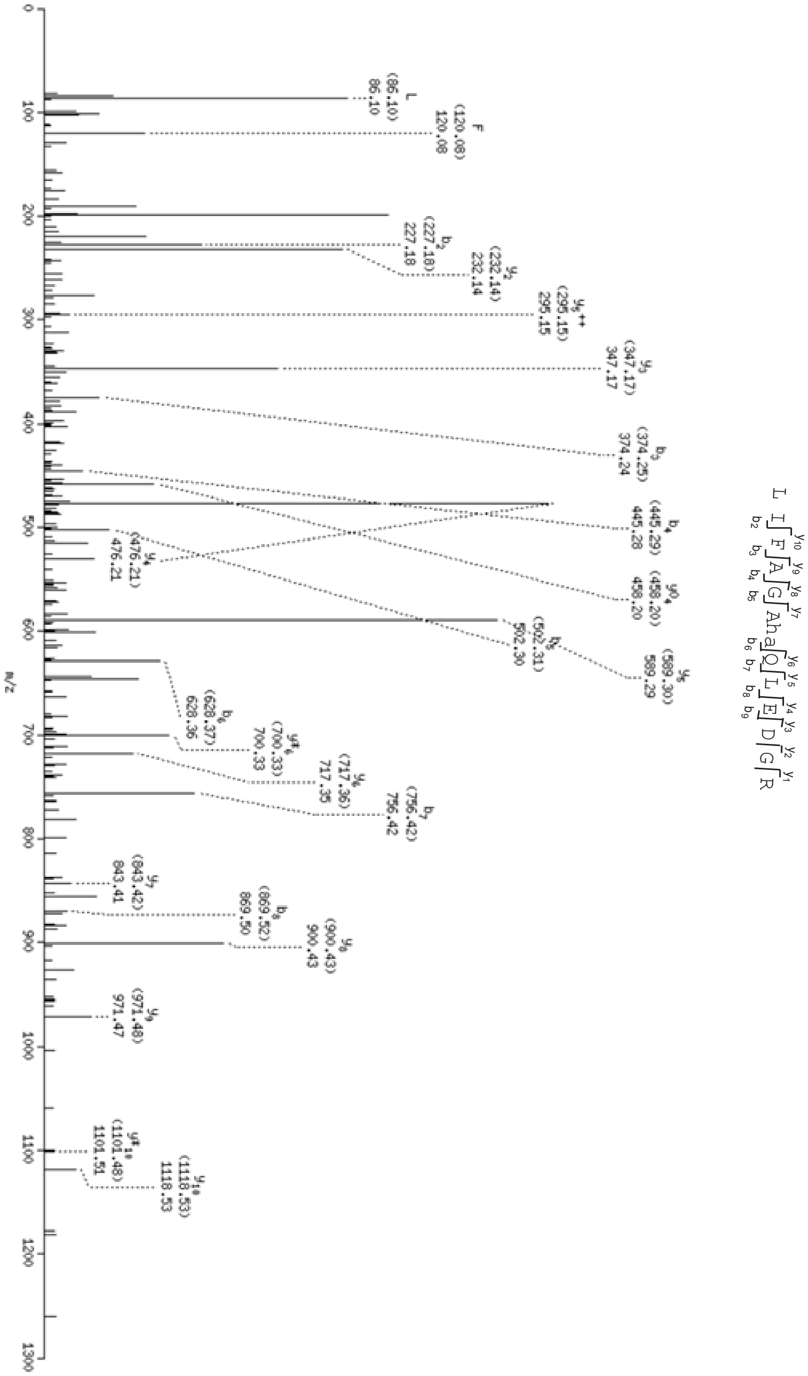
51 LEDGRTLSDY NIQKESTLHL VLRLRGG



G **K48Aha Ub**

1 MAQIFVKTLT GKTITLEVEP SDTIENVKAK IQDKEGIPPD QQR**LIFAGAhaQ**

51 LEDGRTLSDY NIQKESTLHL VLRLRGG



H **K63Aha Ub**

1 MAQIFVKTLT GKTITLEVEP SDTIENVKAK IQDKEGIPPD QQRLIFAGKQ

51 LEDGR**TLSDY NIQAhaESTLHL VLR**LRGG

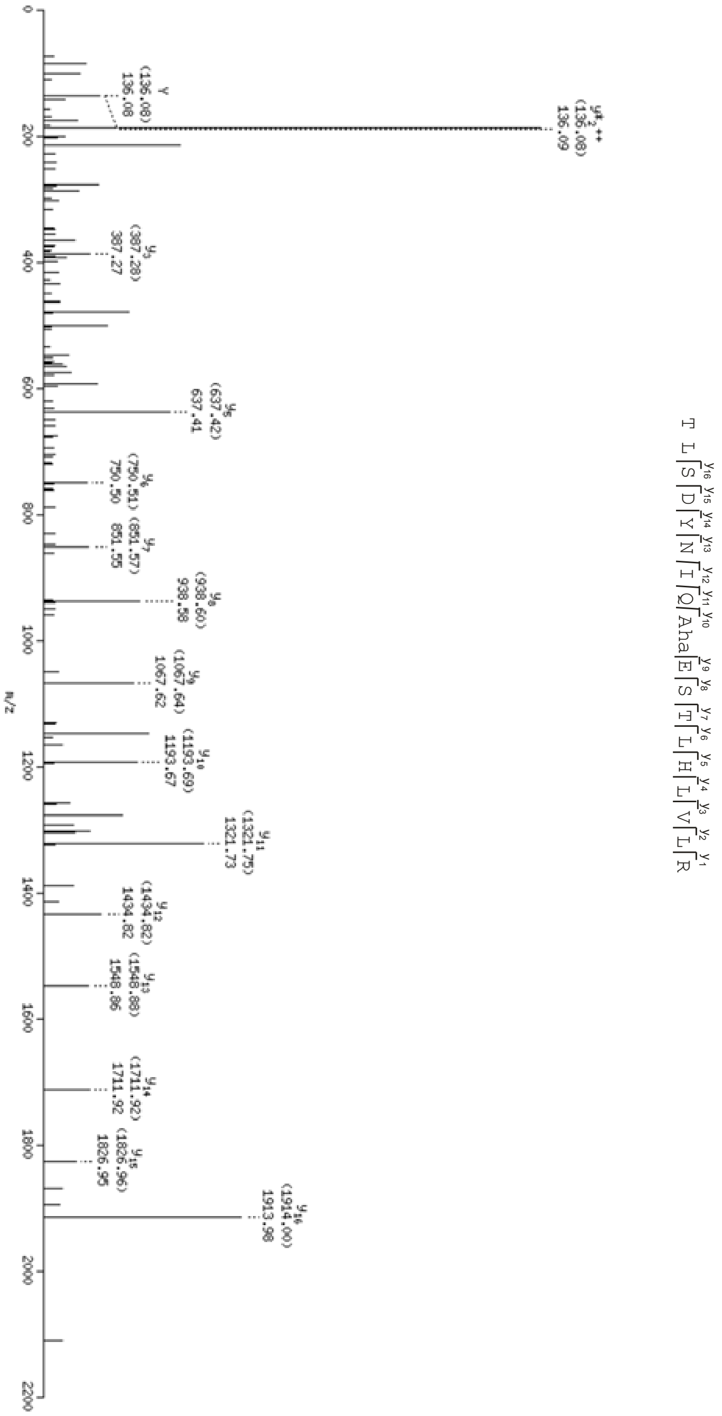
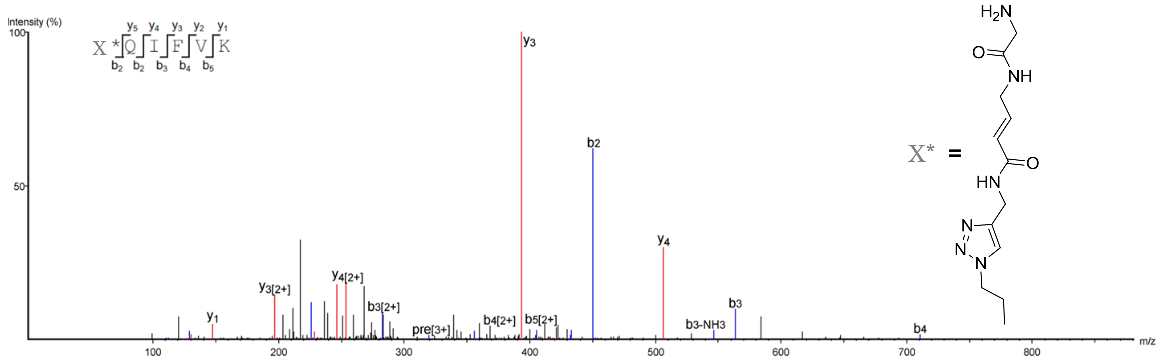


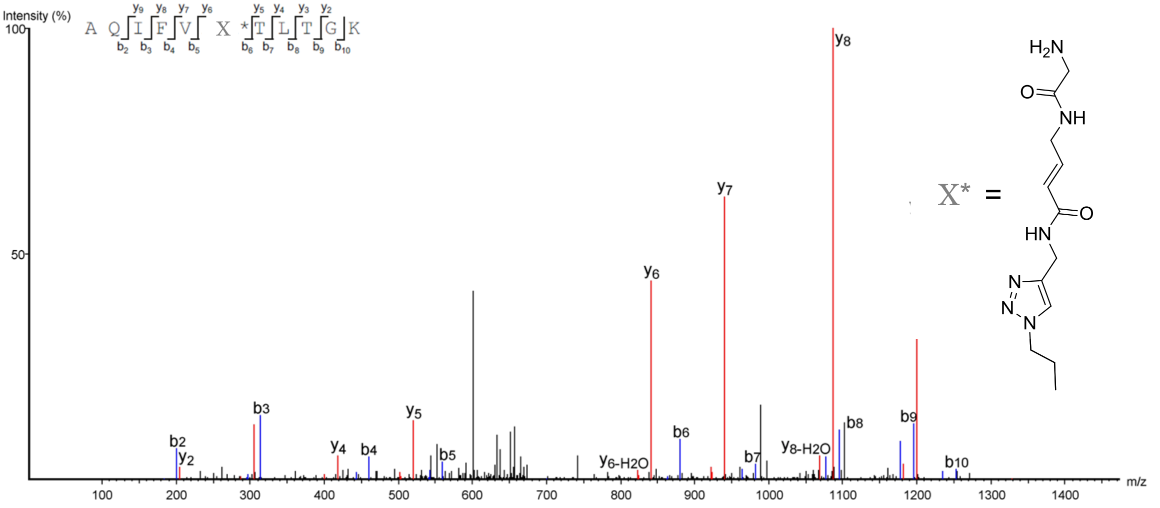
Figure S3 (related to Figure 5A). LC-MS/MS results for Di-Ub probe trap regions

MS/MS spectra of the peptidic fragments of trypsin digested Di-Ub probes bearing the electrophilic trap derived covalent adduct in position M1 (**A**), K6 (**B**), K11 (**C**), K27 (**D**), K33 (**E**), or K63 (**F**), respectively. No MS/MS spectrum containing the probe adduct at the K29 position was detected. The proximal Ubiquitin derived peptides are shown with addition of the distal Ubiquitin C-terminal fragment as a modification. The b and y fragment ions are indicated in blue and red, respectively.

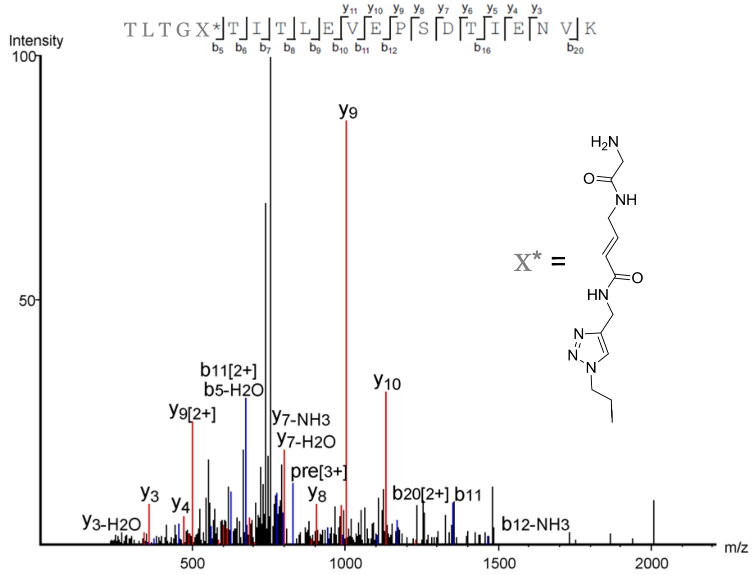
A **M1 Di-Ub probe**



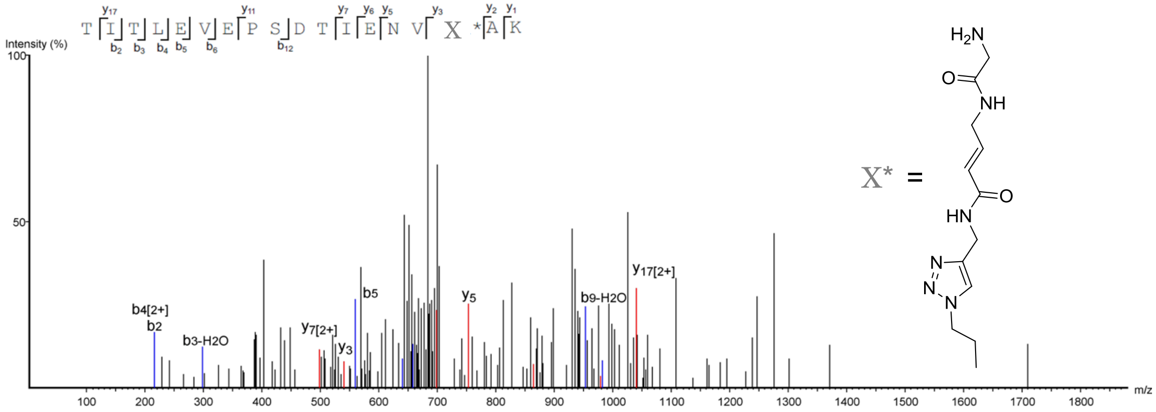
B **K6 Di-Ub probe**



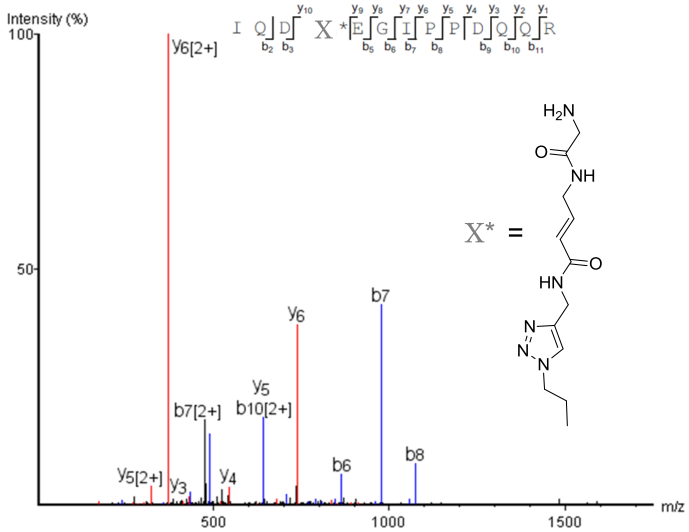
C **K11 Di-Ub probe**



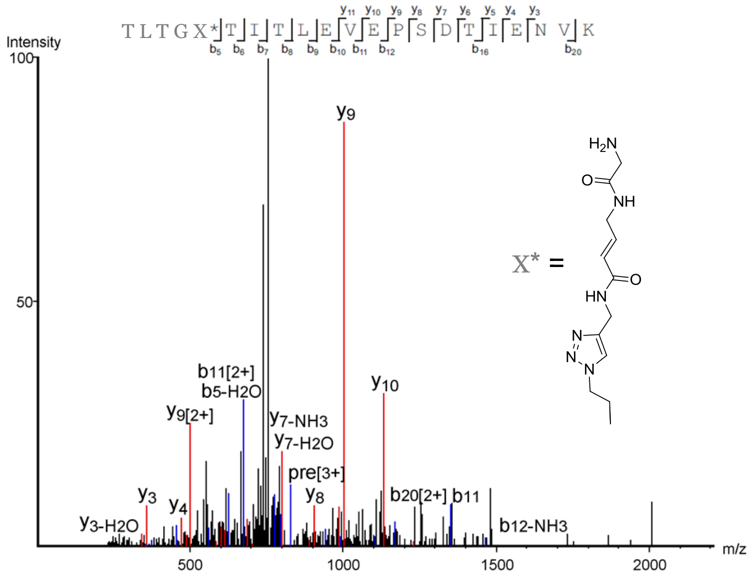
D **K27 Di-Ub probe**



E **K33 Di-Ub probe**



F **K63 Di-Ub probe**



DNA sequences for wildtype and mutant Ubiquitin

Complete DNA sequences of the wildtype and mutant Ubiquitin proteins that were prepared for this study. ATGs are indicated in bold for the introduction of Methionine residues that are then replaced with Aha (see Materials & Methods section).

WT Ub

5´-**ATG**CAGATCTTCGTCAAGACGTTAACCGGTAAAACCATAACTCTGGAAGTTGAACCATCCGATACCAT
CGAAAACGTTAAGGCTAAAATTCAAGACAAGGAAGGAATTCCACCTGATCAACAAAGATTGATCTTTGCC
GGTAAGCAGCTCGAGGACGGTAGAACGCTGTCTGATTACAACATTCAGAAGGAGTCGACCTTACATCTT
GTCTTAAGACTAAGAGGTGGTTGA -3´

K6M Ub

5´-**ATG**GCGCAGATCTTCGTC**ATG**ACGTTAACCGGTAAAACCATAACTCTGGAAGTTGAACCATCCGATAC
CATCGAAAACGTTAAGGCTAAAATTCAAGACAAGGAAGGAATTCCACCTGATCAACAAAGATTGATCTTT
GCCGGTAAGCAGCTCGAGGACGGTAGAACGCTGTCTGATTACAACATTCAGAAGGAGTCGACCTTACAT
CTTGTCTTAAGACTAAGAGGTGGTTGA-3´

K11M Ub

5´-**ATG**GCGCAGATCTTCGTCAAGACGTTAACCGGT**ATG**ACCATAACTCTGGAAGTTGAACCATCCGATAC
CATCGAAAACGTTAAGGCTAAAATTCAAGACAAGGAAGGAATTCCACCTGATCAACAAAGATTGATCTTT
GCCGGTAAGCAGCTCGAGGACGGTAGAACGCTGTCTGATTACAACATTCAGAAGGAGTCGACCTTACAT
CTTGTCTTAAGACTAAGAGGTGGTTGA -3´

K27M Ub

5´-**ATG**GCGCAGATCTTCGTCAAGACGTTAACCGGTAAAACCATAACTCTGGAAGTTGAACCATCCGATAC
CATCGAAAACGTT**ATG**GCTAAAATTCAAGACAAGGAAGGAATTCCACCTGATCAACAAAGATTGATCTTT
GCCGGTAAGCAGCTCGAGGACGGTAGAACGCTGTCTGATTACAACATTCAGAAGGAGTCGACCTTACAT
CTTGTCTTAAGACTAAGAGGTGGTTGA -3´

K29M Ub

5′-**ATG**GCGCAGATCTTCGTCAAGACGTTAACCGGTAAAACCATAACTCTGGAAGTTGAACCATCCGATAC
CATCGAAAACGTTAAGGCT**ATG**ATTCAAGACAAGGAAGGAATTCCACCTGATCAACAAAGATTGATCTTT
GCCGGTAAGCAGCTCGAGGACGGTAGAACGCTGTCTGATTACAACATTCAGAAGGAGTCGACCTTACAT
CTTGTCTTAAGACTAAGAGGTGGTTGA -3′

K33M Ub

5′-**ATG**GCGCAGATCTTCGTCAAGACGTTAACCGGTAAAACCATAACTCTGGAAGTTGAACCATCCGATAC
CATCGAAAACGTTAAGGCTAAAATTCAAGAC**ATG**GAAGGAATTCCACCTGATCAACAAAGATTGATCTTT
GCCGGTAAGCAGCTCGAGGACGGTAGAACGCTGTCTGATTACAACATTCAGAAGGAGTCGACCTTACAT
CTTGTCTTAAGACTAAGAGGTGGTTGA -3′

K48M Ub

5′-**ATG**GCGCAGATCTTCGTCAAGACGTTAACCGGTAAAACCATAACTCTGGAAGTTGAACCATCCGATAC
CATCGAAAACGTTAAGGCTAAAATTCAAGACAAGGAAGGAATTCCACCTGATCAACAAAGATTGATCTTT
GCCGGT**ATG**CAGCTCGAGGACGGTAGAACGCTGTCTGATTACAACATTCAGAAGGAGTCGACCTTACAT
CTTGTCTTAAGACTAAGAGGTGGTTGA-3′

K63M Ub

5′-**ATG**GCGCAGATCTTCGTCAAGACGTTAACCGGTAAAACCATAACTCTGGAAGTTGAACCATCCGATAC
CATCGAAAACGTTAAGGCTAAAATTCAAGACAAGGAAGGAATTCCACCTGATCAACAAAGATTGATCTTT
GCCGGTAAGCAGCTCGAGGACGGTAGAACGCTGTCTGATTACAACATTCAG**ATG**GAGTCGACCTTACAT
CTTGTCTTAAGACTAAGAGGTGGTTGA-3′

Supplemental Table 1 (related to Figure 2). Primer sequences used for generating the Aha Ub mutants

Ub mut*	Forward primer (5'-3')	Reverse primer (5'-3')
Insertion of A pos 2	GGAGAGGATCCATGGCGCAGATCTTCGTCAAG	CTTGACGAAGATCTGCGCCATGGATCCTCTCC
K6M	GGCGCAGATCTTCGTCATGACGTTAACCGGTAAAAC	GTTTTACCGGTTAACGTCATGACGAAGATCTGCGCC
K11M	CAAGACGTTAACCGGTATGACCATAACTCTGGAAG	CTTCCAGAGTTATGGTCATACCGGTTAACGTCTTG
K27M	CCGATACCATCGAAAACGTTATGGCTAAAATTCAAGACAAGG	CCTTGTCTTGAATTTTAGCCATAACGTTTTCGATGGTATCGG
K29M	CGAAAACGTTAAGGCTATGATTCAAGACAAGGAAG	CTTCCTTGTCTTGAATCATAGCCTTAACGTTTTCG
K33M	GCTAAAATTCAAGACATGGAAGGAATTCCACCTG	CAGGTGGAATTCCTTCCATGTCTTGAATTTTAGC
K48M	GATCTTTGCCGGTATGCAGCTCGAGGAC	GTCCTCGAGCTGCATACCGGCAAAGATC
K63M	GATTACAACATTCAGATGGAGTCGACCTTACATC	GATGTAAGGTCGACTCCATCTGAATGTTGTAATC

*Ub mut: Position and amino acid substitution in the Ub protein sequence

Supplemental Table 2 (related to Figure 7). Ubiquitin conjugation machinery detected in probe immunoprecipitation material

Components of the Ub conjugation machinery detected by mass spectrometry analysis of eluted material from immunoprecipitation of cell lysates labeled with Di-Ub probes are displayed: Protein name, UniprotKB accession number, number of peptides detected by MS and mono/Di-Ub probe pulldown where highest amount was detected by LC Progenesis Software analysis (see Experimental Procedures section of the main manuscript).

Protein Name	**UniprotKB**	**Nr. of peptides**	**Di-Ub probe pulldown with highest abundance**
[a]***HECT E3s***			
HECTD1	Q9ULT8	11	M1 Di-Ub
HERC1	Q15751	3	M1 Di-Ub
HUWE1	Q7Z6Z7	117	M1 Di-Ub
E3A	Q05086	6	K27 Di-Ub
E3C	Q15386	3	M1 Di-Ub
HECTD3	Q5T447	1	M1 Di-Ub
HERC4	Q5GLZ8	1	Total HEK293T lysate
[b]***RBR E3s***			
ARIH1	Q9Y4X5	2	M1 Di-Ub
RNF31	Q96EP0	1	M1 Di-Ub
[c]***RING E3s***			
MYCBP2	O75592	10	K11 Di-Ub
RNF25	Q96BH1	4	Total HEK293T lysate
TRIM25	Q14258	2	K27 Di-Ub
TRIM33	Q9UPN9	2	K27 Di-Ub
Listerin	O94822	1	M1 Di-Ub
RING1	Q06587	1	K63 Di-Ub
KCMF1	O94822	1	M1 Di-Ub
RAD18	Q9NS91	1	M1 Di-Ub
BRE	Q9NXR7	2	Total HEK293T lysate
BRE1A	Q5VTR2	3	M1 Di-Ub
BRE1B	O75150	3	Ub-Alkyne
XIAP	P98170	4	M1 Di-Ub
RNF123	Q5XPI4	1	several probes
TRIM37	Q5XPI4	1	several probes
Other E3s			
BRUCE	Q9NR09	3	K27 Di-Ub
CHIP	Q9UNE7	3	K27 Di-Ub
UBR4	Q5T4S7	30	M1 Di-Ub
UBR5	O95071	28	Total HEK293T lysate

E1s			
A1S9	P22314	56	M1 Di-Ub
MOP-4	A0AVT1	11	M1 Di-Ub
E2s			
DDVit 1	Q15819	2	Ub Alkyne
E2 C	O00762	2	M1 Di-Ub
E2 D1	P51668	2	K6 Di-Ub
E2 D3	P61077	5	Total HEK293T lysate
E2 K	P61086	7	M1 Di-Ub
E2 N	P61088	11	M1 Di-Ub
E2 O	Q9C0C9	11	M1 Di-Ub
E2 E2	Q96LR5	1	M1 Di-Ub
E2 L3	P68036	1	Ub VME
E2 R1	P49427	1	M1 Di-Ub
E2 T	Q9NPD8	1	M1 Di-Ub

[a] HECT domain containing E3 ubiquitin ligases

[b] RING-between-RING E3 ubiquitin ligases

[c] Really interesting gene (RING) domain containing E3 ubiquitin ligases