A diubiquitin-based photoaffinity probe for profiling

K27-linkage targeting deubiquitinases

Xiao-Dan Tan,^{a,b} Man Pan,^a Shuai Gao,^a Yong Zheng,^{b,c} Jing Shi,^{c*} and Yi-Ming Li^{b*}

^a Tsinghua-Peking Center for Life Sciences, Ministry of Education Key Laboratory of Bioorganic Phosphorus Chemistry and Chemical Biology, Department of Chemistry, Tsinghua University, Beijing 100084, China;

^b School of Biological and Medical Engineering, Hefei University of Technology, Hefei, Anhui 230009, China, E-mail: <u>ymli@hfut.edu.cn;</u>

^c Department of Chemistry, University of Science and Technology of China; and High Magnetic Field Laboratory, Chinese Academy of Sciences, Hefei 230026, China, E-mail: <u>shijing@ustc.edu.cn</u>

Table of Contents

1. General methods	S2
2. Characterizations of peptide 1-3 and K29-diub photoaffinity probe	S5
3. LC-MS/MS identification of captured proteins	S7
4. References	S13

1. General methods

Materials. 2-Chlorotrityl resin and rink amide resin were purchased from Hecheng Technology (Tianjing, China). Fmoc-amino acids and 1-Hydroxy-7-azabenzotriazole (HOAt) were purchased from GL Biochem (Shanghai, China). Ethyl cyanoglyoxylate-2-oxime (oxyma), 1-(4-azidophenyl)-2-bromoethan-1-one and α,α "-di-bromo-adipyl(bis)amide reagent was purchased from Adamasbeta (Shanghai, China). N,N-Diisopropyl-carbodiimide (DIC), 1,2-ethanedithiol, N,N-diisopropylethylamine (DIPEA), triisopropylsilane (TIPS) and 4-mercaptophenylacetic acid (MPAA) were purchased from Ouhe Technology (Beijing, China). Dithiothreitol (DTT) was purchased from Aladdin (Shanghai, China). Acetonitrile (HPLC grade) was purchased from J. T. Baker (Phillipsburg, NJ, USA). Streptavidin agarose resin were purchased from Thermo Pierce and General Electric Company (GE). Na₂HPO₄·12H₂O, CH₃OH, guanidine hydrochloride (Gn·HCl), Et₂O, Fmoc-hydrazine and N,N-Dimethylformamide (DMF) were purchased from Sinopharm Chemical Reagent. Thioanisole and trifluoroacetic acid (TFA) (HPLC grade) were purchased from J&K Scientific (Beijing, China). CH₂Cl₂ (DCM) and NaNO₂ were purchased from Beijing Chemical Works (Beijing, China). All primers were ordered from Biomed Biotech (Beijing). All restriction enzymes were ordered from New England Biolabs (NEB).

Peptide synthesis. All synthetic peptides used in this work were obtained from the 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide synthesis (SPPS). With the assistance of CEM microwave peptide synthesizer, single coupling time was less than 15 min at 75 °C and 50 °C (His and Cys). To remove the Fmoc group, 20% piperidine in DMF with 0.1 M oxyma was added to the resins for 1 min at 90 °C. While the peptides synthesis completed, the resins were transferred into customized Sand core funnel, and incubating with cleavage cocktail (thioanisole: water: 1,2-Ethanedithiol: trifluoroacetic acid=5:5:3:87) for 3 h at room temperature. The eluent was concentrated by blowing with N₂. The crude peptides were obtained by precipitating with cold ether and centrifuge at 4500 rpm. The crude peptides were purified by RP-HPLC (20-60% H₂O/CH₃CN over 30min) with welch xb-C4 or xb-C18 semi-preparative column. Purified peptides were characterized by RP-HPLC (20-60% H₂O/CH₃CN gradient over 30 min) over an analytical C18 column (Grace) and ESI-MS. Purified peptides were lyophilized to obtain the target peptides.

RP-HPLC and FPLC. All RP-HPLC were performed on Shimadzu Prominence HPLC (Prominence LC-20AT with SPD-20A UV/Vis detector). For peptide and reaction analysis, analytical Grace Vydac C4 (4.6×250 mm, 5 µm particle size) and C18 (4.6×150 mm, 5 µm particle size) columns were used at a flow rate of 1.0 mL/min. For peptide purifications, semi-preparative Grace Vydac C18 (10×250 mm, 10 µm particle size, flow rate of 3.4 mL/min) and semi-preparative Grace Vydac C18 (22×150 mm, 10 µm particle size, flow rate of 7.8 mL/min) were used. The UV absorption at 214 nm and 254 nm were monitored throughout the analysis and purifications. Mobile phase for RP-HPLC: buffer A (0.08-0.1% TFA in CH₃CN) and buffer B (0.1% TFA in water). The

solvents were sonicated for 25-30 min before use. FPLC was run on AKTA purifier 10 UPC-F920 with Superdex 75 column or Mono Q column. The injections were monitored at 280 nm. All the buffers were filtered through 0.22 μ m filter paper and sonicated for 10 min before use.

Hydrazide-based native chemical ligation. The hydrazide peptide (1 μ mol, 1 eq) was dissolved in 1 mL ligation buffer (6 M Gn-HCl, 200 mM NaH₂PO₄, pH 3.0) and then pre-cooled in an salt-ice bath (-8 ~ -10 °C). Then 10 μ L of 1 M NaNO₂ (1 M stock buffer) (10 μ mol, 10 eq) was added and incubated for 30 min in salt-ice bath to fully convert the hydrazide into the acyl azide. Next, MPAA (6.8 mg, 40 μ mol, 40 eq) was added and the pH was adjusted to 5.0 for 5 min to generate the thioester peptide. Finally, the N-terminal Cys peptide (1 μ mol, 1 eq) was added and the pH was adjusted to 6.5 to initiate standard NCL. The reaction was monitored by analytical RP-HPLC and the product was purified by semi-preparative RP-HPLC.

Protein expression and purification. All deubiquitinases were expressed from pGEX-6p-1 vectors. Protein was expressed in *E. coli* BL21 (DE3) cells that were grown to an OD600 of 0.8 at 37 °C and induced with 0.2 mM IPTG for 12-20 hr at 18 °C. Large-scale protein expression was performed in 1 L-8 L LB medium supplemented with amp. antibiotic. Protein purifications were performed at 4 °C. Cells were lysed by sonication in 20-160 mL lysis buffer for 1 hour (200 mM NaCl, 20 mM Tris [pH=8], 5 mM DTT, 1 mM PMSF), and cleared by centrifugation 18000 rpm for 30 min. The cleared lysates were then incubated with 2 mL equilibrated Glutathione Sepharose 4B resin for 2 hr, and subsequently washed with 500 mL buffer A (20 mM Tris [pH=8], 5 mM DTT) plus 500 mM NaCl, and 200 mL buffer A plus 50 mM NaCl. The GST tag was then cleaved on the resin with 50 μ g GST-tagged PreScission protease overnight. Cleaved protein was eluted with buffer A plus 50 mM NaCl to a final volume of 20 mL and subjected to anionS4 exchange chromatography (MonoQ, GE Healthcare) with a NaCl gradient from 50 mM to 1 M. Concentrated proteins were pooled and subjected to gel filtration (Superdex75) in buffer A plus 200 mM NaCl. Proteins were concentrated to 2-10 mg/mL using a 10 kDa MW cut-off concentrator and flash-frozen in liquid nitrogen.

Photocrosslinking reactions

The photocrosslinking reactions were carried out as previously report.¹ Photoaffinity probe was incubated with protein solutions or 293F cell lysates (2 mg/ml) on ice for 1 h. The mixtures were then irradiated at 254 nm using UVP CL-1000 UV Cross-Linker for 5 min at a distance of ~ 10 cm on ice. Then the protein solution were incubated with streptavidin agarose resin (Thermo Pierce) for 1.5 hour at 4°C. After thoroughly washing with PBS buffer with 0.2% SDS and PBS buffer for 3 times separately, 5× protein loading buffer was added to the resins and heated to 95°C for 10 min. The eluent were then analyzed by 15% SDS-PAGE.

LC-MS/MS based analysis of captured proteins

LC-MS/MS based analysis of captured proteins were carried out as previously report.¹ All SDS-PAGE gel bands were cut into small pieces respectively. Then, sample was reduced with 25 mM of dithiothreitol and alkylated with 55 mM iodoacetamide, then the gel bands were digested by trypsin at 37°C overnight. The digested peptides were extracted thrice with 1% formic acid in 50% acetonitrile aqueous solution, and dried to concentrated form by Speedvac.

For LC-MS/MS analysis, the digested peptides were separated by a 65-min gradient elution at a flow rate of 0.250 μ L/min with the EASY-nLCII integrated nano-HPLC system (Proxeon), which was directly interfaced with the Thermo LTQ-Orbitrap Velos mass spectrometer. The analytical column was a homemade fused silica capillary column (75 μ m internal diameter, 150 mm length; Upchurch) packed with C-18 resin (300A, 5 μ m, Varian). The LTQ-Orbitrap mass spectrometer was operated in the data-dependent acquisition mode using the Xcalibur 2.0.7 software and there was a single full-scan mass spectrum in the Orbitrap (400-1800 m/z, 30,000 resolution) followed by 20 data-dependent MS/MS scans in the ion trap at 35% normalized collision energy.

Then, LC-MS/MS Data were searched in the Human database from the NCBI by using the Proteome Discoverer 1.4 search engine to identify the captured proteins. The search parameters were: peptides mass tolerance of 20 ppm; MS/MS tolerance of 0.8 Da; two missed cleavages allowed; oxidation on Met and acetylation on any N-terminus as the dynamic modification, carbamidomethylation on Cys as static modification. The decoy database search was added with the criteria of FDR at 0.01.

Mass Spectrometry. All peptides and reaction products were characterized by ESI mass spectra on LC/MS 2020 (SHIMADZU).

2. Characterizations of peptide 1-3 and K29-diub photoaffinity probe



Figure S1. Characterization of synthetic peptide segment **1**, Ub(1-75)-NHNH₂. Analytical HPLC chromatogram ($\lambda = 214$ nm) of purified peptide **1**. HPLC condition: a linear gradient of 20%-60% acetonitrile (with 0.08-0.1% TFA) in water (with 0.1% TFA) over 30 min (5% for 2 min, then 20%-60% for 30 min) on a Vydac C18 column. ESI-MS spectrum of purified peptide **1**. The spectrum gave an observed mass of 8520.6 Da (calculated 8521.7 Da, average isotopes).



Figure S2. Characterization of synthetic peptide segment **2**, Ub(1-45)-K27Acm-NHNH₂. Analytical HPLC chromatogram ($\lambda = 214$ nm) of purified peptide **2**. HPLC condition: a linear gradient of 20%-60% acetonitrile (with 0.08-0.1% TFA) in water (with 0.1% TFA) over 30 min (5% for 2 min, then 20%-60% for 30 min) on a Vydac C18 column. ESI-MS spectrum of purified peptide **2**. The spectrum gave an observed mass of 5301.2 Da (calculated 5301.0 Da, average isotopes).



Figure S3. Characterization of synthetic peptide segment **3**, Ub(46-76)-AEEA-Biotin. Analytical HPLC chromatogram ($\lambda = 214$ nm) of purified peptide **3**. HPLC condition: a linear gradient of 20%-60% acetonitrile (with 0.08-0.1% TFA) in water (with 0.1% TFA) over 30 min (5% for 2 min, then 20%-60% for 30 min) on a Vydac C18 column. ESI-MS spectrum of purified peptide **3**. The spectrum gave an observed mass of 3998.9 Da (calculated 3998.6 Da, average isotopes).



Figure S4. Characterization of K29-diUb photoaffinity probe. Analytical HPLC chromatogram ($\lambda = 214 \text{ nm}$) of purified K29-diUb photoaffinity probe. HPLC condition: a linear gradient of 20%-60% acetonitrile (with 0.08-0.1% TFA) in water (with 0.1% TFA) over 30 min (5% for 2 min, then 20%-60% for 30 min) on a Vydac C18 column. ESI-MS spectrum of purified K29-diUb photoaffinity probe. The spectrum gave an observed mass of 17817.2 Da (calculated 17815.4 Da, average isotopes).

3. LC-MS/MS identification of captured proteins

Table S1. Proteins identified by the K27-diub photoaffinity probe. (Score: the sum of all peptide Xcorr values above the specified score threshold; PSMs: peptide spectrum matches, means total number of identified peptide sequences). These proteins are not present in the control (incubate streptavidin beads and cell lysates without probe).

Accession	Protein Description	Score	PSMs
F5H265	Polyubiquitin-C (Fragment) OS=Homo sapiens GN=UBC		1032
	PE=2 SV=1 - [F5H265_HUMAN]		
M0R2S1	Ubiquitin-60S ribosomal protein L40 (Fragment) OS=Homo	2133.86	827
	sapiens GN=UBA52 PE=2 SV=1 - [M0R2S1_HUMAN]		
Q06830	Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1	241.37	113
	SV=1 - [PRDX1_HUMAN]		
P31025	Lipocalin-1 OS=Homo sapiens GN=LCN1 PE=1 SV=1 -	208.09	75
	[LCN1_HUMAN]		
P61626	Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1 -	145.79	51
	[LYSC_HUMAN]		
Q86YZ3	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 -	113.82	43
	[HORN_HUMAN]		
P05165-2	Isoform 2 of Propionyl-CoA carboxylase alpha chain,	102.28	40
	mitochondrial OS=Homo sapiens GN=PCCA -		
	[PCCA_HUMAN]		
Q13085-3	Isoform 3 of Acetyl-CoA carboxylase 1 OS=Homo sapiens	99.14	36
	GN=ACACA - [ACACA_HUMAN]		
Q5VVQ6-	Isoform 2 of Ubiquitin thioesterase OTU1 OS=Homo	95.43	38
2	sapiens GN=YOD1 - [OTU1_HUMAN]		
E9PHF7	Methylcrotonoyl-CoA carboxylase subunit alpha,	94.87	33
	mitochondrial OS=Homo sapiens GN=MCCC1 PE=2 SV=1		
	- [E9PHF7_HUMAN]		
P10809	60 kDa heat shock protein, mitochondrial OS=Homo sapiens	94.49	35
	GN=HSPD1 PE=1 SV=2 - [CH60_HUMAN]		
Q92945	Far upstream element-binding protein 2 OS=Homo sapiens	88.50	32
	GN=KHSRP PE=1 SV=4 - [FUBP2_HUMAN]		
P11142	Heat shock cognate 71 kDa protein OS=Homo sapiens	87.66	32
	GN=HSPA8 PE=1 SV=1 - [HSP7C_HUMAN]		
P11498	Pyruvate carboxylase, mitochondrial OS=Homo sapiens	86.81	37
	GN=PC PE=1 SV=2 - [PYC_HUMAN]		
P08238	Heat shock protein HSP 90-beta OS=Homo sapiens	82.60	28
	GN=HSP90AB1 PE=1 SV=4 - [HS90B_HUMAN]		
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 -	80.49	24
	[DCD_HUMAN]		
Q5T6W5	Heterogeneous nuclear ribonucleoprotein K OS=Homo	78.15	25

	sapiens GN=HNRNPK PE=4 SV=1 - [Q5T6W5_HUMAN]		
E9PCY7	Heterogeneous nuclear ribonucleoprotein H OS=Homo	77.12	21
	sapiens GN=HNRNPH1 PE=2 SV=1 - [E9PCY7_HUMAN]		
P41250	Glycine-tRNA ligase OS=Homo sapiens GN=GARS PE=1	71.04	23
	SV=3 - [SYG_HUMAN]		
P32119	Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1	69.54	27
	SV=5 - [PRDX2_HUMAN]		
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens	68.97	28
	GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]		
D6RE83	Ubiquitin carboxyl-terminal hydrolase isozyme L1	68.35	27
	OS=Homo sapiens GN=UCHL1 PE=2 SV=1 -		
	[D6RE83_HUMAN]		
P22314	Ubiquitin-like modifier-activating enzyme 1 OS=Homo	67.69	20
	sapiens GN=UBA1 PE=1 SV=3 - [UBA1_HUMAN]		
P52597	Heterogeneous nuclear ribonucleoprotein F OS=Homo	60.01	16
	sapiens GN=HNRNPF PE=1 SV=3 - [HNRPF_HUMAN]		
C9JM50	Keratin, type I cytoskeletal 19 (Fragment) OS=Homo	58.90	25
	sapiens GN=KRT19 PE=4 SV=1 - [C9JM50_HUMAN]		
Q8NC51-	Isoform 4 of Plasminogen activator inhibitor 1 RNA-binding	58.34	19
4	protein OS=Homo sapiens GN=SERBP1 -		
	[PAIRB_HUMAN]		
P49411	Elongation factor Tu, mitochondrial OS=Homo sapiens	55.93	19
	GN=TUFM PE=1 SV=2 - [EFTU_HUMAN]		
Q9Y263	Phospholipase A-2-activating protein OS=Homo sapiens	49.40	17
	GN=PLAA PE=1 SV=2 - [PLAP_HUMAN]		
Q13162	Peroxiredoxin-4 OS=Homo sapiens GN=PRDX4 PE=1	46.88	28
	SV=1 - [PRDX4_HUMAN]		
P26641	Elongation factor 1-gamma OS=Homo sapiens GN=EEF1G	46.16	18
	PE=1 SV=3 - [EF1G_HUMAN]		
P07900	Heat shock protein HSP 90-alpha OS=Homo sapiens	45.89	13
	GN=HSP90AA1 PE=1 SV=5 - [HS90A_HUMAN]		
P04406	Glyceraldehyde-3-phosphate dehydrogenase OS=Homo	45.44	15
	sapiens GN=GAPDH PE=1 SV=3 - [G3P_HUMAN]		
Q5SZU1	D-3-phosphoglycerate dehydrogenase OS=Homo sapiens	39.82	12
	GN=PHGDH PE=2 SV=1 - [Q5SZU1_HUMAN]		
Q14974	Importin subunit beta-1 OS=Homo sapiens GN=KPNB1	38.46	11
	PE=1 SV=2 - [IMB1_HUMAN]		
Q7Z3Y8	Keratin, type I cytoskeletal 27 OS=Homo sapiens	38.40	18
	GN=KRT27 PE=1 SV=2 - [K1C27_HUMAN]		
P06753-3	Isoform 3 of Tropomyosin alpha-3 chain OS=Homo sapiens	35.93	20
	GN=TPM3 - [TPM3_HUMAN]		
P11586	C-1-tetrahydrofolate synthase, cytoplasmic OS=Homo	35.32	13
	sapiens GN=MTHFD1 PE=1 SV=3 - [C1TC_HUMAN]		

O00571-2	Isoform 2 of ATP-dependent RNA helicase DDX3X OS=Homo sapiens GN=DDX3X - [DDX3X_HUMAN]	35.08	12	
P01040	Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1 - [CYTA_HUMAN]	34.90	12	
B4E3P0	ATP-citrate synthase OS=Homo sapiens GN=ACLY PE=2 SV=1 - [B4E3P0_HUMAN]	31.93	11	
P11021	78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2 - [GRP78_HUMAN]	31.90	14	
P38646	Stress-70 protein, mitochondrial OS=Homo sapiens GN=HSPA9 PE=1 SV=2 - [GRP75_HUMAN]	30.41	14	
P63151	P63151 Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform OS=Homo sapiens GN=PPP2R2A PE=1 SV=1 - [2ABA HUMAN]			
P67936	Tropomyosin alpha-4 chain OS=Homo sapiens GN=TPM4 PE=1 SV=3 - [TPM4_HUMAN]	26.92	17	
P49327	Fatty acid synthase OS=Homo sapiens GN=FASN PE=1 SV=3 - [FAS_HUMAN]	26.54	16	
B0YJC4	Vimentin OS=Homo sapiens GN=VIM PE=3 SV=1 - [B0YJC4_HUMAN]	25.22	11	
P26599	Polypyrimidine tract-binding protein 1 OS=Homo sapiens GN=PTBP1 PE=1 SV=1 - [PTBP1_HUMAN]	24.24	10	
P12273	Prolactin-inducible protein OS=Homo sapiens GN=PIP PE=1 SV=1 - [PIP_HUMAN]	23.72	7	
P14618	Pyruvate kinase PKM OS=Homo sapiens GN=PKM PE=1 SV=4 - [KPYM_HUMAN]	23.63	11	
Q6UWP8	Suprabasin OS=Homo sapiens GN=SBSN PE=2 SV=2 - [SBSN_HUMAN]	23.34	7	
E9PG15	14-3-3 protein theta (Fragment) OS=Homo sapiens GN=YWHAQ PE=2 SV=1 - [E9PG15_HUMAN]	23.04	8	
B7Z6M1	Plastin-3 OS=Homo sapiens GN=PLS3 PE=2 SV=1 - [B7Z6M1_HUMAN]	22.12	9	
С9Ј9К3	40S ribosomal protein SA (Fragment) OS=Homo sapiens GN=RPSA PE=3 SV=2 - [C9J9K3_HUMAN]	21.97	7	
Q92995-2	Isoform 2 of Ubiquitin carboxyl-terminal hydrolase 13 OS=Homo sapiens GN=USP13 - [UBP13_HUMAN]	19.77	7	
P14866	Heterogeneous nuclear ribonucleoprotein L OS=Homo sapiens GN=HNRNPL PE=1 SV=2 - [HNRPL_HUMAN]	19.17	8	
I3L2G3	Ketosamine-3-kinase (Fragment) OS=Homo sapiens GN=FN3KRP PE=2 SV=1 - [I3L2G3_HUMAN]	19.02	9	

Table S2. Proteins identified by Dha based K27-diub probe.

Accession	Protein Description		PSMs
Q86YZ3	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 -		50
	[HORN_HUMAN]		
P02768	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 -	68.35	25
	[ALBU_HUMAN]		
P15924	Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3 -	65.70	28
	[DESP_HUMAN]		
P14923	Junction plakoglobin OS=Homo sapiens GN=JUP PE=1	53.76	20
	SV=3 - [PLAK_HUMAN]		
O60260	E3 ubiquitin-protein ligase parkin OS=Homo sapiens	46.09	18
	GN=PARK2 PE=1 SV=2 - [PRKN2_HUMAN]		
Q02413	Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 SV=2 -	45.70	21
	[DSG1_HUMAN]		
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 -	35.69	11
	[DCD_HUMAN]		
Q5D862	Filaggrin-2 OS=Homo sapiens GN=FLG2 PE=1 SV=1 -	34.26	16
	[FILA2_HUMAN]		
Q6UWP8	Suprabasin OS=Homo sapiens GN=SBSN PE=2 SV=2 -	29.13	12
	[SBSN_HUMAN]		
Q08554-2	Isoform 1B of Desmocollin-1 OS=Homo sapiens	26.65	12
	GN=DSC1 - [DSC1_HUMAN]		
P25311	Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1	26.02	11
	PE=1 SV=2 - [ZA2G_HUMAN]		
P29508	Serpin B3 OS=Homo sapiens GN=SERPINB3 PE=1 SV=2	24.94	11
	- [SPB3_HUMAN]		
P07355	Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2 -	24.94	10
	[ANXA2_HUMAN]		
P01040	Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1 -	21.78	9
	[CYTA_HUMAN]		
E9PHF7	Methylcrotonoyl-CoA carboxylase subunit alpha,	21.64	9
	mitochondrial OS=Homo sapiens GN=MCCC1 PE=2 SV=1		
	- [E9PHF7_HUMAN]		
Q5T749	Keratinocyte proline-rich protein OS=Homo sapiens	14.84	6
	GN=KPRP PE=1 SV=1 - [KPRP_HUMAN]		
P05109	Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1	14.46	7
	SV=1 - [S10A8_HUMAN]		
P06702	Protein S100-A9 OS=Homo sapiens GN=S100A9 PE=1	14.17	5
	SV=1 - [S10A9_HUMAN]		
E7EQB2	Kaliocin-1 (Fragment) OS=Homo sapiens GN=LTF PE=2	13.83	7
	SV=1 - [E7EQB2_HUMAN]		
P04406	Glyceraldehyde-3-phosphate dehydrogenase OS=Homo	13.70	7
	sapiens GN=GAPDH PE=1 SV=3 - [G3P_HUMAN]		
P31151	Protein S100-A7 OS=Homo sapiens GN=S100A7 PE=1	13.10	7

	SV=4 - [S10A7_HUMAN]		
Q13085-3	Isoform 3 of Acetyl-CoA carboxylase 1 OS=Homo sapiens		6
	GN=ACACA - [ACACA_HUMAN]		
Q96RL1-3	Isoform 3 of BRCA1-A complex subunit RAP80 OS=Homo	12.71	4
	sapiens GN=UIMC1 - [UIMC1_HUMAN]		
F8W9L1	Serpin B4 OS=Homo sapiens GN=SERPINB4 PE=2 SV=1	12.62	6
	- [F8W9L1_HUMAN]		
M0R1V7	Ubiquitin-60S ribosomal protein L40 (Fragment) OS=Homo	12.59	5
	sapiens GN=UBA52 PE=2 SV=1 - [M0R1V7_HUMAN]		
P31944	Caspase-14 OS=Homo sapiens GN=CASP14 PE=1 SV=2 -	12.17	6
	[CASPE_HUMAN]		
P01036	Cystatin-S OS=Homo sapiens GN=CST4 PE=1 SV=3 -	11.39	6
	[CYTS_HUMAN]		
M0QZK8	Gamma-glutamylcyclotransferase OS=Homo sapiens	11.10	5
	GN=GGCT PE=2 SV=1 - [M0QZK8_HUMAN]		
P04745	Alpha-amylase 1 OS=Homo sapiens GN=AMY1A PE=1	11.01	4
	SV=2 - [AMY1_HUMAN]		
Q08188	Protein-glutamine gamma-glutamyltransferase E OS=Homo	11.01	6
	sapiens GN=TGM3 PE=1 SV=4 - [TGM3_HUMAN]		
P05089	Arginase-1 OS=Homo sapiens GN=ARG1 PE=1 SV=2 -	10.45	5
	[ARGI1_HUMAN]		

 Table S3. Proteins identified by K29-diUb photoaffinity probe.

P02768	Serum albumin OS=Homo sapiens GN=ALB PE=1	130.33	48
	SV=2 - [ALBU_HUMAN]		
Q86YZ	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 -	84.86	31
3	[HORN_HUMAN]		
P15924	Desmoplakin OS=Homo sapiens GN=DSP PE=1	83.74	35
	SV=3 - [DESP_HUMAN]		
M0R1	Ubiquitin-60S ribosomal protein L40 (Fragment)	58.55	21
V7	OS=Homo sapiens GN=UBA52 PE=2 SV=1 -		
	[M0R1V7_HUMAN]		
P14923	Junction plakoglobin OS=Homo sapiens GN=JUP	52.85	20
	PE=1 SV=3 - [PLAK_HUMAN]		
Q0241	Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1	44.83	18
3	SV=2 - [DSG1_HUMAN]		
Q0855	Isoform 1B of Desmocollin-1 OS=Homo sapiens	37.31	14
4-2	GN=DSC1 - [DSC1_HUMAN]		
O6026	E3 ubiquitin-protein ligase parkin OS=Homo sapiens	36.90	15
0	GN=PARK2 PE=1 SV=2 - [PRKN2_HUMAN]		
Q5D86	Filaggrin-2 OS=Homo sapiens GN=FLG2 PE=1 SV=1	34.50	12

2	- [FILA2_HUMAN]		
P25311	Zinc-alpha-2-glycoprotein OS=Homo sapiens	33.19	13
	GN=AZGP1 PE=1 SV=2 - [ZA2G_HUMAN]		
H0YM	Annexin (Fragment) OS=Homo sapiens GN=ANXA2	25.47	11
D0	PE=2 SV=1 - [H0YMD0_HUMAN]		
Q6UW	Suprabasin OS=Homo sapiens GN=SBSN PE=2 SV=2	24.03	8
P8	- [SBSN_HUMAN]		
Q9UGI	Ubiquitin thioesterase ZRANB1 OS=Homo sapiens	22.64	10
0	GN=ZRANB1 PE=1 SV=2 - [ZRAN1_HUMAN]		
P05089	Arginase-1 OS=Homo sapiens GN=ARG1 PE=1 SV=2	22.58	9
	- [ARGI1_HUMAN]		
P04406	Glyceraldehyde-3-phosphate dehydrogenase	22.05	10
	OS=Homo sapiens GN=GAPDH PE=1 SV=3 -		
	[G3P_HUMAN]		
P01036	Cystatin-S OS=Homo sapiens GN=CST4 PE=1 SV=3	20.73	8
	- [CYTS_HUMAN]		
P31151	Protein S100-A7 OS=Homo sapiens GN=S100A7	18.63	8
	PE=1 SV=4 - [S10A7_HUMAN]		
P29508	Serpin B3 OS=Homo sapiens GN=SERPINB3 PE=1	18.47	8
	SV=2 - [SPB3_HUMAN]		
P31944	Caspase-14 OS=Homo sapiens GN=CASP14 PE=1	18.27	8
	SV=2 - [CASPE_HUMAN]		
P01040	Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1	16.93	6
	- [CYTA_HUMAN]		
Q5VV	Isoform 2 of Ubiquitin thioesterase OTU1 OS=Homo	16.11	7
Q6-2	sapiens GN=YOD1 - [OTU1_HUMAN]		
F5H8K	Keratin, type II cytoskeletal 4 OS=Homo sapiens	15.87	8
9	GN=KRT4 PE=2 SV=1 - [F5H8K9_HUMAN]		
G5E9X	Methylcrotonoyl-CoA carboxylase subunit alpha,	15.33	7
5	mitochondrial OS=Homo sapiens GN=MCCC1 PE=4		
	SV=1 - [G5E9X5_HUMAN]		
Q1308	Isoform 3 of Acetyl-CoA carboxylase 1 OS=Homo	15.26	6
5-3	sapiens GN=ACACA - [ACACA_HUMAN]		
P06702	Protein S100-A9 OS=Homo sapiens GN=S100A9	14.02	5
	PE=1 SV=1 - [S10A9_HUMAN]		
Q5T74	Keratinocyte proline-rich protein OS=Homo sapiens	13.97	5
9	GN=KPRP PE=1 SV=1 - [KPRP_HUMAN]		
B0YJC	Vimentin OS=Homo sapiens GN=VIM PE=3 SV=1 -	13.67	6
4	[B0YJC4_HUMAN]		
P05109	Protein S100-A8 OS=Homo sapiens GN=S100A8	13.43	5
	PE=1 SV=1 - [S10A8_HUMAN]		
P06733	Alpha-enolase OS=Homo sapiens GN=ENO1 PE=1	13.22	6
	SV=2 - [ENOA_HUMAN]		

E9PI65	Heat shock cognate 71 kDa protein (Fragment)	12.97	6
	OS=Homo sapiens GN=HSPA8 PE=2 SV=1 -		
	[E9PI65_HUMAN]		
P12273	Prolactin-inducible protein OS=Homo sapiens	12.64	6
	GN=PIP PE=1 SV=1 - [PIP_HUMAN]		
Q96P6	Serpin B12 OS=Homo sapiens GN=SERPINB12 PE=1	12.55	6
3	SV=1 - [SPB12_HUMAN]		
P04745	Alpha-amylase 1 OS=Homo sapiens GN=AMY1A	11.89	4
	PE=1 SV=2 - [AMY1_HUMAN]		
Q0146	Fatty acid-binding protein, epidermal OS=Homo	11.43	5
9	sapiens GN=FABP5 PE=1 SV=3 - [FABP5_HUMAN]		
P11021	78 kDa glucose-regulated protein OS=Homo sapiens	10.52	6
	GN=HSPA5 PE=1 SV=2 - [GRP78_HUMAN]		

Table S4. The comparison of proteins identified by K27-diUb and K29-diUb photoaffinity probe.

	K27 Photoa Pro	K27 diUb Photoaffinity Probe		diUb affinity obe
	Score PSMs Score		Score	PSMs
OTUD2	95.43	38	16.11	7
USP13	19.77	7	0	0
ZRANB1	0	0	22.64	10

4. References

1. J. Liang, L. Zhang, X. L. Tan, Y. K. Qi, S. Feng, H. Deng, Y. Yan, J. S. Zheng, L. Liu and C. L. Tian, *Angew. Chem. Int. Ed.*, 2017, **56**, 2744-2748.