# A diubiquitin-based photoaffinity probe for profiling 

## K27-linkage targeting deubiquitinases

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## 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 $\alpha, \alpha^{\prime \prime}$-di-bromo-adipyl(bis)amide reagent was purchased from Adamasbeta (Shanghai, China). N,N-Diisopropyl-carbodiimide (DIC), 1,2-ethanedithiol, N,Ndiisopropylethylamine (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). $\mathrm{Na}_{2} \mathrm{HPO}_{4} \cdot 12 \mathrm{H}_{2} \mathrm{O}, \mathrm{CH}_{3} \mathrm{OH}$, guanidine hydrochloride $(\mathrm{Gn} \cdot \mathrm{HCl})$, $\mathrm{Et}_{2} \mathrm{O}$, 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). $\mathrm{CH}_{2} \mathrm{Cl}_{2}(\mathrm{DCM})$ and $\mathrm{NaNO}_{2}$ 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 $75^{\circ} \mathrm{C}$ and $50^{\circ} \mathrm{C}$ (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 $90^{\circ} \mathrm{C}$. While the peptides synthesis completed, the resins were transferred into customized Sand core funnel, and incubating with cleavage cocktail (thioanisole: water: 1,2Ethanedithiol: trifluoroacetic $\operatorname{acid}=5: 5: 3: 87$ ) for 3 h at room temperature. The eluent was concentrated by blowing with $\mathrm{N}_{2}$. The crude peptides were obtained by precipitating with cold ether and centrifuge at 4500 rpm . The crude peptides were purified by RP-HPLC $\left(20-60 \% \mathrm{H}_{2} \mathrm{O} / \mathrm{CH}_{3} \mathrm{CN}\right.$ over 30 min ) with welch $\mathrm{xb}-\mathrm{C} 4$ or $\mathrm{xb}-\mathrm{C} 18$ semi-preparative column. Purified peptides were characterized by RP-HPLC $\left(20-60 \% \mathrm{H}_{2} \mathrm{O} / \mathrm{CH}_{3} \mathrm{CN}\right.$ gradient over 30 min$)$ over an analytical C 18 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 ( $4.6 \times 250 \mathrm{~mm}, 5 \mu \mathrm{~m}$ particle size $)$ and $\mathrm{C} 18(4.6 \times 150 \mathrm{~mm}, 5 \mu \mathrm{~m}$ particle size) columns were used at a flow rate of $1.0 \mathrm{~mL} / \mathrm{min}$. For peptide purifications, semi-preparative Grace Vydac C18 ( $10 \times 250 \mathrm{~mm}, 10 \mu \mathrm{~m}$ particle size, flow rate of $3-4 \mathrm{~mL} / \mathrm{min})$ and semi-preparative Grace Vydac C18 ( $22 \times 150 \mathrm{~mm}, 10 \mu \mathrm{~m}$ particle size, flow rate of $7-8 \mathrm{~mL} / \mathrm{min}$ ) 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 $\left(0.08-0.1 \% \mathrm{TFA}\right.$ in $\left.\mathrm{CH}_{3} \mathrm{CN}\right)$ and buffer $\mathrm{B}(0.1 \%$ 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 $0.22 \mu \mathrm{~m}$ filter paper and sonicated for 10 min before use.

Hydrazide-based native chemical ligation. The hydrazide peptide ( $1 \mu \mathrm{~mol}, 1 \mathrm{eq}$ ) was dissolved in 1 mL ligation buffer ( $6 \mathrm{M} \mathrm{Gn}-\mathrm{HCl}, 200 \mathrm{mM} \mathrm{NaH}_{2} \mathrm{PO}_{4}, \mathrm{pH} 3.0$ ) and then pre-cooled in an salt-ice bath $\left(-8 \sim-10^{\circ} \mathrm{C}\right)$. Then $10 \mu \mathrm{~L}$ of $1 \mathrm{M} \mathrm{NaNO}_{2}(1 \mathrm{M}$ stock buffer) $(10 \mu \mathrm{~mol}, 10 \mathrm{eq})$ was added and incubated for 30 min in salt-ice bath to fully convert the hydrazide into the acyl azide. Next, MPAA ( $6.8 \mathrm{mg}, 40 \mu \mathrm{~mol}, 40 \mathrm{eq}$ ) was added and the pH was adjusted to 5.0 for 5 min to generate the thioester peptide. Finally, the N -terminal Cys peptide ( $1 \mu \mathrm{~mol}, 1 \mathrm{eq}$ ) was added and the pH was adjusted to 6.5 to initiate standard NCL. The reaction was monitored by analytical RPHPLC 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 $37{ }^{\circ} \mathrm{C}$ and induced with 0.2 mM IPTG for $12-20 \mathrm{hr}$ at $18^{\circ} \mathrm{C}$. Large-scale protein expression was performed in 1 L-8 L LB medium supplemented with amp. antibiotic. Protein purifications were performed at $4^{\circ} \mathrm{C}$. Cells were lysed by sonication in 20-160 mL lysis buffer for 1 hour ( 200 mM $\mathrm{NaCl}, 20 \mathrm{mM}$ Tris [ $\mathrm{pH}=8$ ], 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 [ $\mathrm{pH}=8$ ], 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 $50 \mu \mathrm{~g}$ 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 $2-10 \mathrm{mg} / \mathrm{mL}$ 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 293 F cell lysates ( $2 \mathrm{mg} / \mathrm{ml}$ ) 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 $\sim 10 \mathrm{~cm}$ on ice. Then the protein solution were incubated with streptavidin agarose resin (Thermo Pierce) for 1.5 hour at $4^{\circ} \mathrm{C}$. After thoroughly washing with PBS buffer with $0.2 \%$ SDS and PBS buffer for 3 times separately, $5 \times$ protein loading buffer was added to the resins and heated to $95^{\circ} \mathrm{C}$ 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 SDSPAGE 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 $37^{\circ} \mathrm{C}$ 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 $65-\mathrm{min}$ gradient elution at a flow rate of $0.250 \mu \mathrm{~L} / \mathrm{min}$ 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 ( $75 \mu \mathrm{~m}$ internal diameter, 150 mm length; Upchurch) packed with C-18 resin (300A, $5 \mu \mathrm{~m}$, 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 $20 \mathrm{ppm} ; \mathrm{MS} / \mathrm{MS}$ 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)- $\mathrm{NHNH}_{2}$. Analytical HPLC chromatogram $(\lambda=214 \mathrm{~nm})$ of purified peptide 1 . HPLC condition: a linear gradient of $20 \%-60 \%$ acetonitrile (with $0.08-0.1 \% \mathrm{TFA}$ ) in water (with $0.1 \% \mathrm{TFA}$ ) over $30 \mathrm{~min}(5 \%$ 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 $(\lambda=214 \mathrm{~nm})$ 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 $30 \mathrm{~min}(5 \%$ 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 ( $\lambda=214 \mathrm{~nm}$ ) of purified peptide 3. HPLC condition: a linear gradient of $20 \%-$ $60 \%$ acetonitrile (with $0.08-0.1 \% \mathrm{TFA}$ ) in water (with $0.1 \% \mathrm{TFA}$ ) over $30 \mathrm{~min}(5 \%$ 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 nm ) of purified K29-diUb photoaffinity probe. HPLC condition: a linear gradient of 20\%-60\% acetonitrile (with $0.08-0.1 \% \mathrm{TFA}$ ) in water (with $0.1 \% \mathrm{TFA}$ ) over $30 \mathrm{~min}(5 \%$ 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 $\mathrm{PE}=2 \mathrm{SV}=1-[\mathrm{F} 5 \mathrm{H} 265$ HUMAN] | 2847.42 | 1032 |
| M0R2S1 | Ubiquitin-60S ribosomal protein L40 (Fragment) OS=Homo sapiens GN=UBA52 PE=2 SV=1 - [M0R2S1_HUMAN] | 2133.86 | 827 |
| Q06830 | Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1 SV=1 - [PRDX1_HUMAN] | 241.37 | 113 |
| P31025 | Lipocalin-1 OS=Homo sapiens GN=LCN1 PE=1 SV=1 [LCN1_HUMAN] | 208.09 | 75 |
| P61626 | Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1 [LYSC_HUMAN] | 145.79 | 51 |
| Q86YZ3 | Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 [HORN_HUMAN] | 113.82 | 43 |
| P05165-2 | Isoform 2 of Propionyl-CoA carboxylase alpha chain, mitochondrial OS=Homo sapiens GN=PCCA [PCCA_HUMAN] | 102.28 | 40 |
| Q13085-3 | Isoform 3 of Acetyl-CoA carboxylase 1 OS=Homo sapiens GN=ACACA - [ACACA_HUMAN] | 99.14 | 36 |
| $\begin{aligned} & \text { Q5VVQ6- } \\ & 2 \end{aligned}$ | Isoform 2 of Ubiquitin thioesterase OTU1 OS=Homo sapiens GN=YOD1 - [OTU1_HUMAN] | 95.43 | 38 |
| E9PHF7 | Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial OS=Homo sapiens $\mathrm{GN}=\mathrm{MCCC} 1 \mathrm{PE}=2 \mathrm{SV}=1$ - [E9PHF7_HUMAN] | 94.87 | 33 |
| P10809 | 60 kDa heat shock protein, mitochondrial OS=Homo sapiens GN=HSPD1 PE=1 SV=2 - [CH60_HUMAN] | 94.49 | 35 |
| Q92945 | Far upstream element-binding protein 2 OS=Homo sapiens GN=KHSRP PE=1 SV=4 - [FUBP2_HUMAN] | 88.50 | 32 |
| P11142 | Heat shock cognate 71 kDa protein $\mathrm{OS}=\mathrm{Homo}$ sapiens GN=HSPA8 PE=1 SV=1 - [HSP7C_HUMAN] | 87.66 | 32 |
| P11498 | Pyruvate carboxylase, mitochondrial OS=Homo sapiens $\mathrm{GN}=\mathrm{PC}$ PE=1 SV=2 - [PYC_HUMAN] | 86.81 | 37 |
| P08238 | Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4 - [HS90B_HUMAN] | 82.60 | 28 |
| P81605 | Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 [DCD_HUMAN] | 80.49 | 24 |
| 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 sapiens GN=HNRNPH1 PE=2 SV=1 - [E9PCY7_HUMAN] | 77.12 | 21 |
| P41250 | Glycine-tRNA ligase OS=Homo sapiens GN=GARS PE=1 SV=3 - [SYG_HUMAN] | 71.04 | 23 |
| P32119 | Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5 - [PRDX2_HUMAN] | 69.54 | 27 |
| P13647 | Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3-[K2C5 HUMAN] | 68.97 | 28 |
| D6RE83 | Ubiquitin carboxyl-terminal hydrolase isozyme L1 OS=Homo sapiens GN=UCHL1 PE=2 SV=1 [D6RE83_HUMAN] | 68.35 | 27 |
| P22314 | Ubiquitin-like modifier-activating enzyme 1 OS=Homo sapiens GN=UBA1 PE=1 SV=3 - [UBA1_HUMAN] | 67.69 | 20 |
| P52597 | Heterogeneous nuclear ribonucleoprotein F OS=Homo sapiens GN=HNRNPF PE=1 SV=3 - [HNRPF_HUMAN] | 60.01 | 16 |
| C9JM50 | Keratin, type I cytoskeletal 19 (Fragment) OS=Homo sapiens GN=KRT19 PE=4 SV=1 - [C9JM50_HUMAN] | 58.90 | 25 |
| $\begin{aligned} & \text { Q8NC51- } \\ & 4 \end{aligned}$ | Isoform 4 of Plasminogen activator inhibitor 1 RNA-binding protein OS=Homo sapiens [PAIRB_HUMAN] | 58.34 | 19 |
| P49411 | Elongation factor Tu , mitochondrial $\mathrm{OS}=$ Homo sapiens GN=TUFM PE=1 SV=2 - [EFTU_HUMAN] | 55.93 | 19 |
| Q9Y263 | Phospholipase A-2-activating protein OS=Homo sapiens $\mathrm{GN}=\mathrm{PLAA} \mathrm{PE}=1 \mathrm{SV}=2$ - [PLAP_HUMAN] | 49.40 | 17 |
| Q13162 | Peroxiredoxin-4 OS=Homo sapiens GN=PRDX4 PE=1 SV=1 - [PRDX4_HUMAN] | 46.88 | 28 |
| P26641 | Elongation factor 1-gamma OS=Homo sapiens GN=EEF1G $\mathrm{PE}=1 \mathrm{SV}=3$ - [EF1G_HUMAN] | 46.16 | 18 |
| P07900 | Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5 - [HS90A_HUMAN] | 45.89 | 13 |
| P04406 | Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3 - [G3P_HUMAN] | 45.44 | 15 |
| Q5SZU1 | D-3-phosphoglycerate dehydrogenase $\mathrm{OS}=\mathrm{Homo}$ sapiens GN=PHGDH PE=2 SV=1 - [Q5SZU1_HUMAN] | 39.82 | 12 |
| Q14974 | Importin subunit beta-1 OS=Homo sapiens GN=KPNB1 $\mathrm{PE}=1 \mathrm{SV}=2$ - [IMB1_HUMAN] | 38.46 | 11 |
| Q7Z3Y8 | Keratin, type I cytoskeletal 27 OS=Homo sapiens $\mathrm{GN}=\mathrm{KRT} 27 \mathrm{PE}=1 \mathrm{SV}=2-[\mathrm{K} 1 \mathrm{C} 27$ HUMAN] | 38.40 | 18 |
| P06753-3 | Isoform 3 of Tropomyosin alpha-3 chain OS=Homo sapiens GN=TPM3 - [TPM3_HUMAN] | 35.93 | 20 |
| P11586 | C-1-tetrahydrofolate synthase, cytoplasmic OS=Homo sapiens GN=MTHFD1 PE=1 SV=3 - [C1TC_HUMAN] | 35.32 | 13 |


| 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 $\mathrm{SV}=1-$ [B4E3P0_HUMAN] | 31.93 | 11 |
| P11021 | 78 kDa glucose-regulated protein $\mathrm{OS}=\mathrm{Homo}$ 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 | Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform $\mathrm{OS}=$ Homo sapiens $\mathrm{GN}=\mathrm{PPP} 2 \mathrm{R} 2 \mathrm{~A}$ $\mathrm{PE}=1 \mathrm{SV}=1-[2 \mathrm{ABA}$ _HUMAN] | 30.15 | 17 |
| 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 $\mathrm{OS}=\mathrm{Homo}$ sapiens $\mathrm{GN}=\mathrm{PIP}$ $\mathrm{PE}=1 \mathrm{SV}=1$ - [PIP_HUMAN] | 23.72 | 7 |
| P14618 | Pyruvate kinase PKM OS=Homo sapiens $\mathrm{GN}=\mathrm{PKM}$ 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 $\mathrm{GN}=\mathrm{YWHAQ}$ 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 |
| C9J9K3 | 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 $\mathrm{GN}=\mathrm{FN} 3 \mathrm{KRP}$ PE=2 SV=1 - [I3L2G3_HUMAN] | 19.02 | 9 |

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

| Accession | Protein Description | Score | PSMs |
| :---: | :---: | :---: | :---: |
| Q86YZ3 | Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 [HORN_HUMAN] | 134.21 | 50 |
| P02768 | Serum albumin $\mathrm{OS}=$ Homo sapiens $\mathrm{GN}=\mathrm{ALB} \mathrm{PE}=1 \mathrm{SV}=2$ [ALBU_HUMAN] | 68.35 | 25 |
| P15924 | Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3 [DESP_HUMAN] | 65.70 | 28 |
| P14923 | Junction plakoglobin OS=Homo sapiens GN=JUP PE=1 SV=3 - [PLAK_HUMAN] | 53.76 | 20 |
| O60260 | E3 ubiquitin-protein ligase parkin $\mathrm{OS}=\mathrm{Homo}$ sapiens GN=PARK2 PE=1 SV=2-[PRKN2_HUMAN] | 46.09 | 18 |
| Q02413 | Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 SV=2 [DSG1_HUMAN] | 45.70 | 21 |
| P81605 | Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 [DCD_HUMAN] | 35.69 | 11 |
| Q5D862 | Filaggrin-2 OS=Homo sapiens GN=FLG2 PE=1 SV=1 [FILA2_HUMAN] | 34.26 | 16 |
| Q6UWP8 | Suprabasin OS=Homo sapiens GN=SBSN PE=2 SV=2 [SBSN_HUMAN] | 29.13 | 12 |
| Q08554-2 | Isoform 1B of Desmocollin-1 OS=Homo sapiens GN=DSC1 - [DSC1_HUMAN] | 26.65 | 12 |
| P25311 | Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1 $\mathrm{PE}=1 \mathrm{SV}=2$ - [ZA2G_HUMAN] | 26.02 | 11 |
| P29508 | Serpin B3 OS=Homo sapiens GN=SERPINB3 PE=1 SV=2 - [SPB3_HUMAN] | 24.94 | 11 |
| P07355 | Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2 [ANXA2_HUMAN] | 24.94 | 10 |
| P01040 | Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1 [CYTA_HUMAN] | 21.78 | 9 |
| E9PHF7 | Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial OS=Homo sapiens $\mathrm{GN}=\mathrm{MCCC} 1 \mathrm{PE}=2 \mathrm{SV}=1$ - [E9PHF7_HUMAN] | 21.64 | 9 |
| Q5T749 | Keratinocyte proline-rich protein OS=Homo sapiens GN=KPRP PE=1 SV=1-[KPRP_HUMAN] | 14.84 | 6 |
| P05109 | Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1 - [S10A8_HUMAN] | 14.46 | 7 |
| P06702 | Protein S100-A9 OS=Homo sapiens GN=S100A9 PE=1 SV=1 - [S10A9_HUMAN] | 14.17 | 5 |
| E7EQB2 | Kaliocin-1 (Fragment) OS=Homo sapiens GN=LTF PE=2 SV=1 - [E7EQB2_HUMAN] | 13.83 | 7 |
| P04406 | Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3 - [G3P_HUMAN] | 13.70 | 7 |
| 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 <br> GN=ACACA - [ACACA_HUMAN] | 13.05 | 6 |
| Q96RL1-3 | Isoform 3 of BRCA1-A complex subunit RAP80 OS=Homo <br> sapiens GN=UIMC1 - [UIMC1_HUMAN] | 12.71 | 4 |
| F8W9L1 | Serpin B4 OS=Homo sapiens GN=SERPINB4 PE=2 SV=1 <br> - [F8W9L1_HUMAN] | 12.62 | 6 |
| M0R1V7 | Ubiquitin-60S ribosomal protein L40 (Fragment) OS=Homo <br> sapiens GN=UBA52 PE=2 SV=1 - [M0R1V7_HUMAN] | 12.59 | 5 |
| P31944 | Caspase-14 OS=Homo sapiens GN=CASP14 PE=1 SV=2 - <br> [CASPE_HUMAN] | 12.17 | 6 |
| P01036 | Cystatin-S OS=Homo sapiens GN=CST4 PE=1 SV=3 - <br> [CYTS_HUMAN] | 11.39 | 6 |
| M0QZK8 | Gamma-glutamylcyclotransferase OS=Homo _ sapiens <br> GN=GGCT PE=2 SV=1 - [M0QZK8_HUMAN] | 11.10 | 5 |
| P04745 | Alpha-amylase 1 OS=Homo sapiens GN=AMY1A PE=1 <br> SV=2 - [AMY1_HUMAN] | 11.01 | 4 |
| Q08188 | Protein-glutamine gamma-glutamyltransferase E OS=Homo <br> sapiens GN=TGM3 PE=1 SV=4 - [TGM3_HUMAN] | 11.01 | 6 |
| P05089 | Arginase-1 OS=Homo sapiens GN=ARG1 PE=1 SV=2 - <br> [ARG1_HUMAN] | 10.45 | 5 |

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

| P02768 | Serum albumin OS=Homo sapiens GN=ALB PE=1 <br> SV=2 - [ALBU_HUMAN] | 130.33 | 48 |
| :--- | :--- | :--- | :--- |
| Q86YZ <br> 3 | Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 - <br> [HORN_HUMAN] | 84.86 | 31 |
| P15924 | Desmoplakin OS=Homo sapiens GN=DSP PE=1 <br> SV=3-[DESP_HUMAN] | 83.74 | 35 |
| M0R1 <br> V7 | Ubiquitin-60S ribosomal protein L40 (Fragment) <br> OS=Homo sapiens GN=UBA52 PE=2 SV=1 - <br> [M0R1V7_HUMAN] | 58.55 | 21 |
| P14923 | Junction plakoglobin OS=Homo sapiens GN=JUP <br> PE=1 SV=3 - [PLAK_HUMAN] | 52.85 | 20 |
| Q0241 <br> 3 | Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 <br> SV=2 - [DSG1_HUMAN] | 44.83 | 18 |
| Q0855 <br> 4-2 | Isoform 1B of Desmocollin-1 OS=Homo sapiens <br> GN=DSC1 - [DSC1_HUMAN] | 37.31 | 14 |
| O6026 <br> 0 | E3 ubiquitin-protein ligase parkin OS=Homo sapiens <br> GN=PARK2 PE=1 SV=2 - [PRKN2_HUMAN] | 36.90 | 15 |
| 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 GN=AZGP1 PE=1 SV=2-[ZA2G_HUMAN] | 33.19 | 13 |
| $\begin{aligned} & \text { H0YM } \\ & \text { D0 } \end{aligned}$ | Annexin (Fragment) OS=Homo sapiens GN=ANXA2 PE=2 SV=1 - [H0YMD0_HUMAN] | 25.47 | 11 |
| $\begin{aligned} & \text { Q6UW } \\ & \text { P8 } \end{aligned}$ | Suprabasin OS=Homo sapiens GN=SBSN PE=2 SV=2 <br> - [SBSN_HUMAN] | 24.03 | 8 |
| $\begin{aligned} & \hline \text { Q9UGI } \\ & 0 \end{aligned}$ | Ubiquitin thioesterase ZRANB1 OS=Homo sapiens GN=ZRANB1 PE=1 SV=2 - [ZRAN1_HUMAN] | 22.64 | 10 |
| P05089 | Arginase-1 OS=Homo sapiens GN=ARG1 PE=1 SV=2 <br> - [ARGI1_HUMAN] | 22.58 | 9 |
| P04406 | Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3 [G3P_HUMAN] | 22.05 | 10 |
| P01036 | Cystatin-S OS=Homo sapiens GN=CST4 PE=1 SV=3 <br> - [CYTS_HUMAN] | 20.73 | 8 |
| P31151 | Protein S100-A7 OS=Homo sapiens GN=S100A7 $\mathrm{PE}=1 \mathrm{SV}=4$ - [S10A7_HUMAN] | 18.63 | 8 |
| P29508 | Serpin B3 OS=Homo sapiens GN=SERPINB3 PE=1 SV=2 - [SPB3_HUMAN] | 18.47 | 8 |
| P31944 | Caspase-14 OS=Homo sapiens GN=CASP14 PE=1 SV=2 - [CASPE_HUMAN] | 18.27 | 8 |
| P01040 | Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1 <br> - [CYTA_HUMAN] | 16.93 | 6 |
| $\begin{aligned} & \text { Q5VV } \\ & \text { Q6-2 } \end{aligned}$ | Isoform 2 of Ubiquitin thioesterase OTU1 OS=Homo sapiens GN=YOD1-[OTU1_HUMAN] | 16.11 | 7 |
| $\begin{aligned} & \text { F5H8K } \\ & 9 \end{aligned}$ | Keratin, type II cytoskeletal 4 OS=Homo sapiens GN=KRT4 PE=2 SV=1 - [F5H8K9_HUMAN] | 15.87 | 8 |
| $\begin{aligned} & \text { G5E9X } \\ & 5 \end{aligned}$ | Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial OS=Homo sapiens $\mathrm{GN}=\mathrm{MCCC} 1 \mathrm{PE}=4$ SV=1-[G5E9X5_HUMAN] | 15.33 | 7 |
| $\begin{aligned} & \text { Q1308 } \\ & 5-3 \end{aligned}$ | Isoform 3 of Acetyl-CoA carboxylase 1 OS=Homo sapiens GN=ACACA - [ACACA_HUMAN] | 15.26 | 6 |
| P06702 | Protein S100-A9 OS=Homo sapiens GN=S100A9 $\mathrm{PE}=1 \mathrm{SV}=1$ - [S10A9_HUMAN] | 14.02 | 5 |
| $\begin{aligned} & \text { Q5T74 } \\ & 9 \\ & \hline \end{aligned}$ | Keratinocyte proline-rich protein OS=Homo sapiens GN=KPRP PE=1 SV=1-[KPRP_HUMAN] | 13.97 | 5 |
| $\begin{aligned} & \text { B0YJC } \\ & 4 \end{aligned}$ | Vimentin OS=Homo sapiens GN=VIM PE=3 SV=1 [B0YJC4_HUMAN] | 13.67 | 6 |
| P05109 | Protein S100-A8 OS=Homo sapiens GN=S100A8 $\mathrm{PE}=1 \mathrm{SV}=1$ - [S10A8_HUMAN] | 13.43 | 5 |
| P06733 | Alpha-enolase OS=Homo sapiens GN=ENO1 PE=1 SV=2 - [ENOA_HUMAN] | 13.22 | 6 |


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

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

|  | K27 diUb <br> Photoaffinity <br> Probe |  | K29 diUb <br> Photoaffinity <br> Probe |  |
| :---: | :---: | :---: | :---: | :---: |
|  | Score | PSMs | 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.
