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Activity-Based Ubiquitin-Protein Probes Reveal Target Protein Specificity of Deubiquitinating Enzymes

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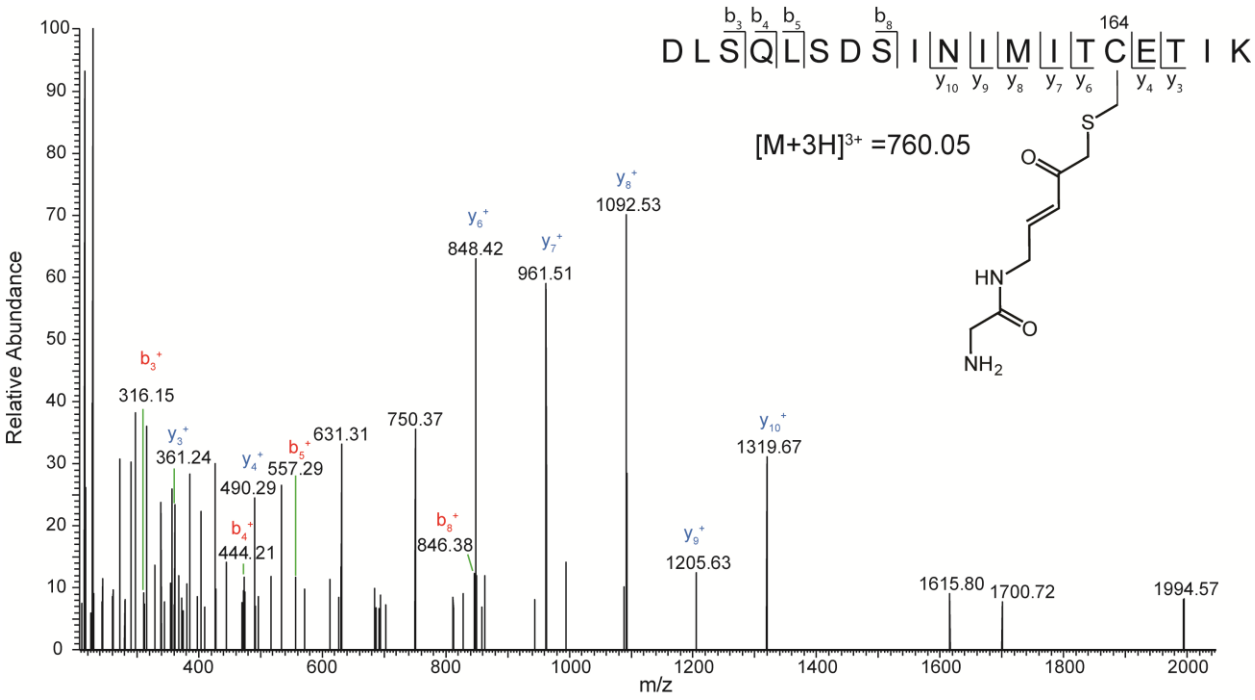

Figure S1. LC-MS/MS analysis of the linkage between PCNA and ubiquitin in K164C Ub-PCNA MAL probe. MS/MS spectra of the ion at m/z of 760.05 corresponding to the PCNA peptide flanking Cys164 (DLSQLSDSINIMITCETIK) with an attached peptide remnant as the result of tryptic digestion of ubiquitin$_{1-75}$-MAL.

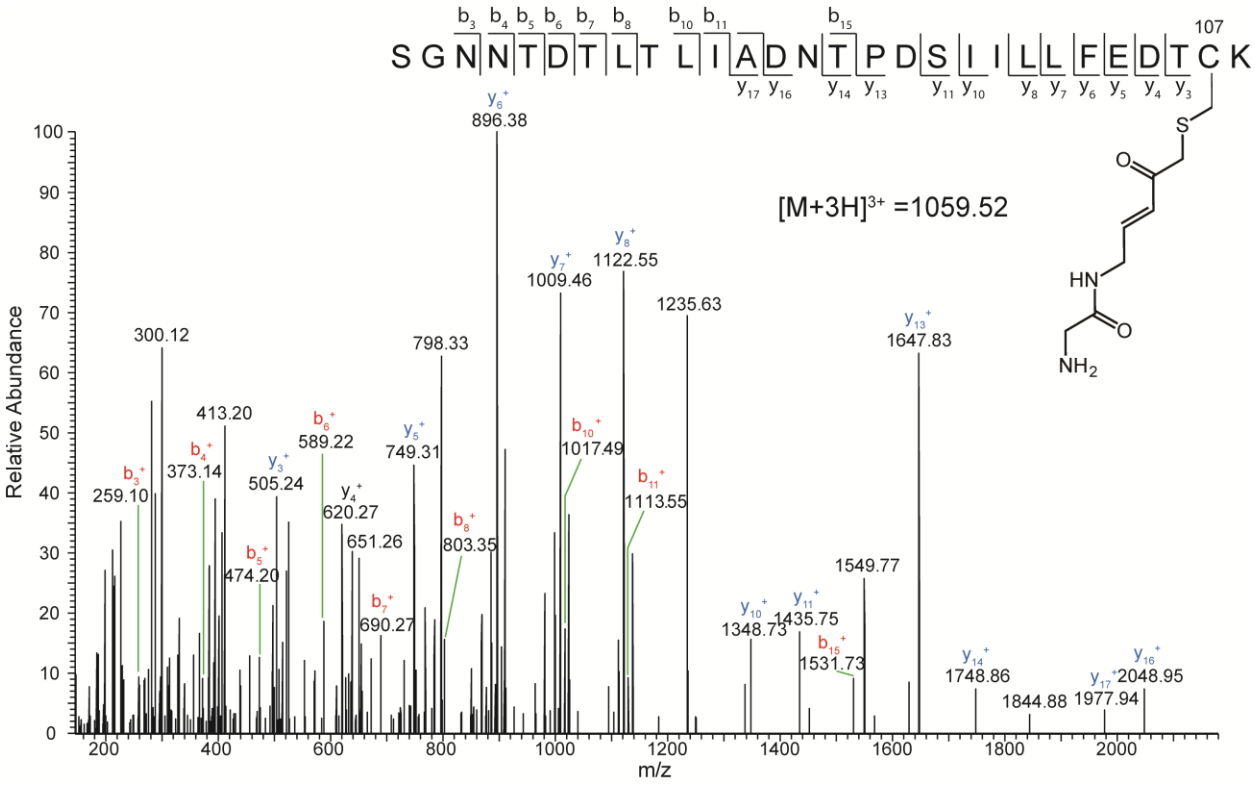

Figure S2.LC-MS/MS analysis of the linkage between PCNA and ubiquitin in K107C Ub-PCNA MAL probe. MS/MS spectra of the ion at m/z of 1059.52 corresponding to the PCNA peptide flanking Cys107 (SGNNTDTLTLIADNTPDSIILLFEDTCK) with an attached peptide remnant as the result of tryptic digestion of ubiquitin$_{1-75}$-MAL.

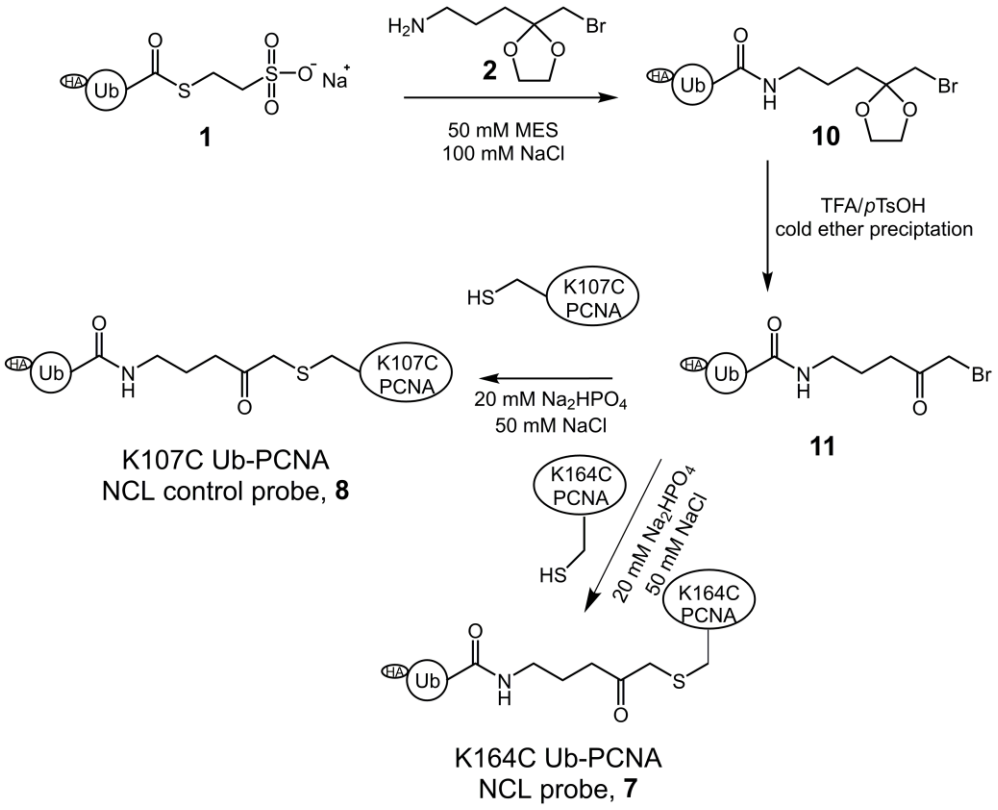

Figure S3. Generation of the K164C and K107C Ub-PCNA NCL probes.

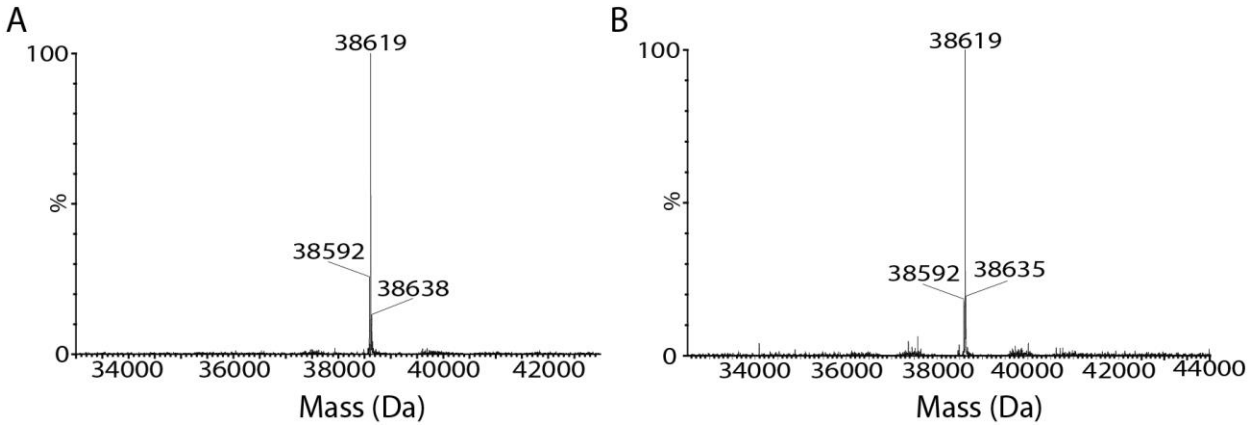

Figure S4. Mass spectrometry analysis of K164C Ub-PCNA NCL probe (panel A) and K107C Ub-PCNA NCL probe (panel B). The measured mass of 38,619 for the K164C Ub-PCNA NCL probe and 38,619 for the K107C Ub-PCNA NCL probe match the theoretical molecular weight of 38,620.

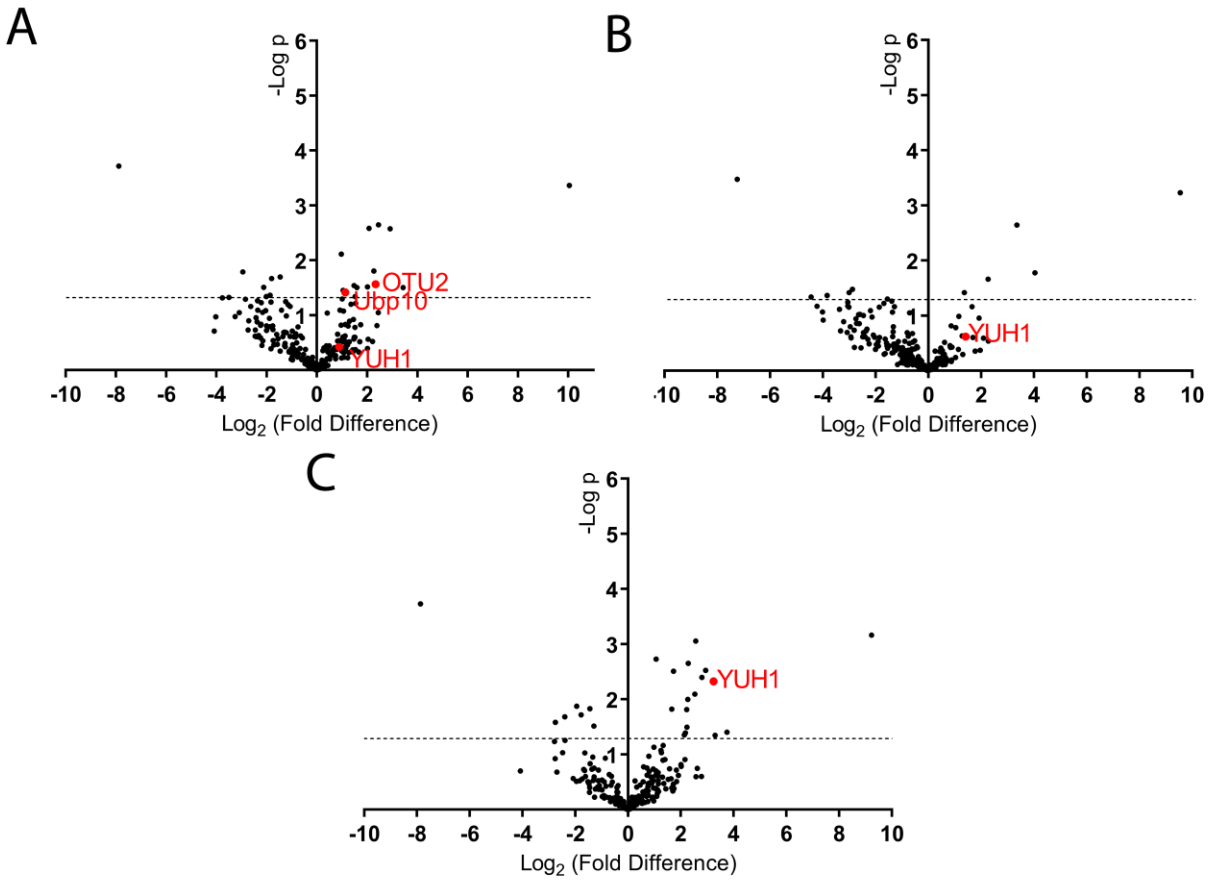

Figure S5. Volcano plot comparing the DUBs pulled down using (A) K164C Ub-PCNA NCL probe **7** versus beads control, (B) K107C Ub-PCNA MAL probe **6** versus beads control, and (C) K107C Ub-PCNA NCL probe **8** versus beads control.

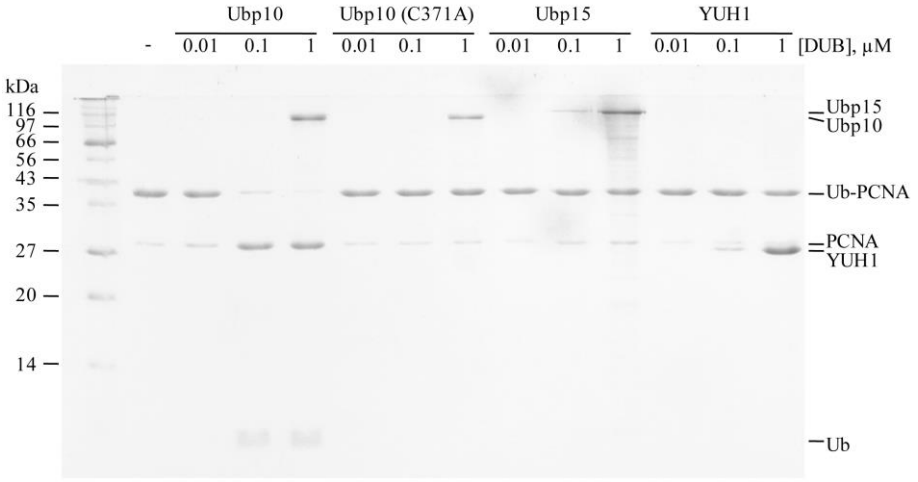

Figure S6. Gel-based assay assessing the cleavage of native yeast K164 Ub-PCNA by Ubp10, Ubp15 and YUH1. Ubp10(C371A) is a catalytically inactive mutant with the replacement of the catalytic Cys371 with alanine.

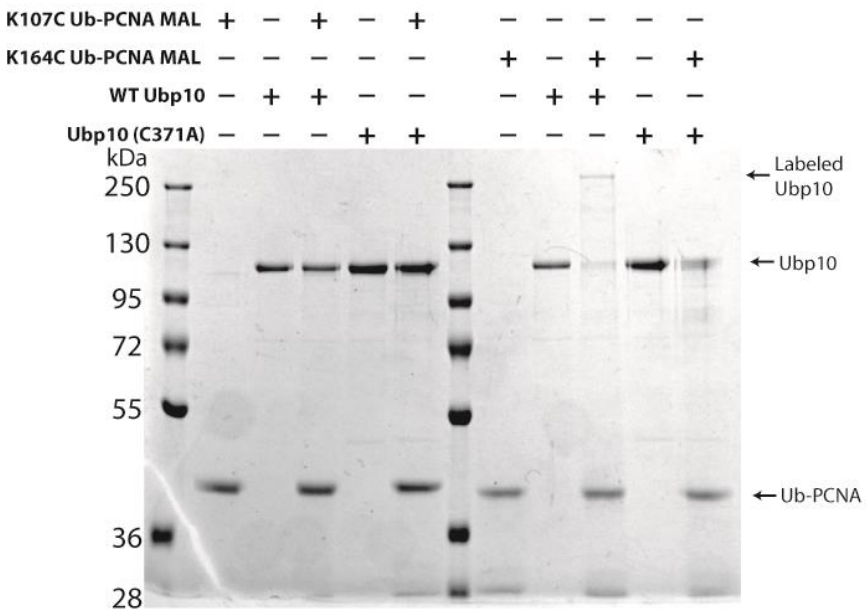

Figure S7. Labeling experiment of wild-type (WT) Ubp10 and active site mutant Ubp10(C371A) with K107C and K164C Ub-PCNA MAL probes. The SDS-PAGE gel was visualized by Coomassie blue stain.

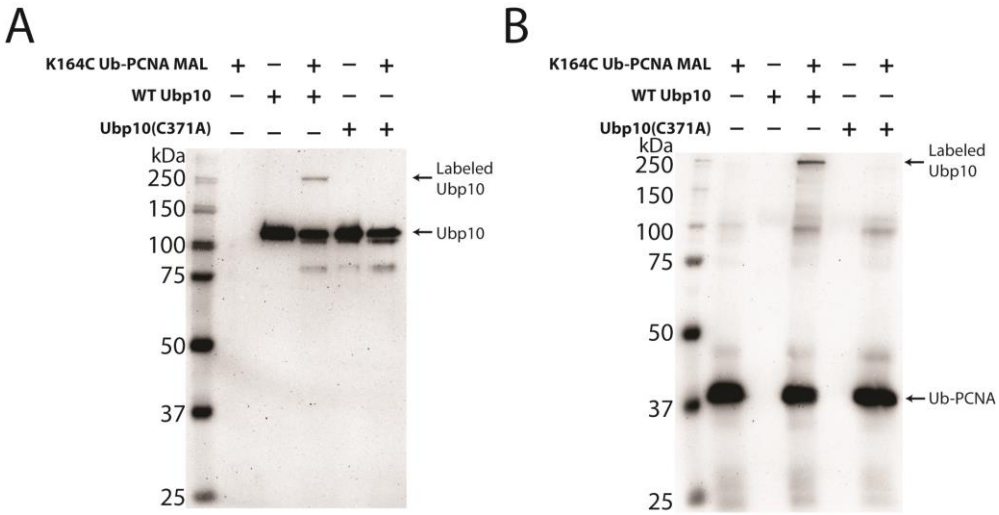

Figure S8. Labeling of wild-type (WT) Ubp10 and active site mutant Ubp10(C371A) with K164C Ub-PCNA MAL probe. (A) Anti-His tag Western blotting shows labeling of His-tagged WT Ubp10 by K164C Ub-PCNA MAL probe. Ubp10(C371A) mutant was not labeled by the probe. (B) Anti-HA Western blotting shows labeling of WT Ubp10 by K164C Ub-PCNA MAL probe that contains a HA-tag. Ubp10(C371A) mutant was not labeled by the probe.

General methods

Preparation of HA-Ub-MAL and HA-Ub-NCL probes

Yeast HA-tagged Ub_{1-75} gene was inserted into the pTYB1 vector (New England Biolabs) and transformed into BL21(DE3) competent cells. The cells were cultured in LB media at 37°C until OD_{600} reached 0.6-0.8. IPTG at 0.4 mM was used to induce protein expression. Cells were grown at 16 °C for 20 hours and harvested by centrifugation at 6,000 g at 4 °C for 10 min. Cell pellet was then resuspended in lysis buffer containing 20 mM Tris (pH 7.5), 200 mM NaCl, 1 mM EDTA and 10% glycerol. After sonication, the lysates were centrifuged at 15,000 g at 4 °C for 30 minutes. Cleared supernatant was incubated with chitin resin (New England Biolabs) at 4 °C for 4 hours under end-over-end rotation. The resins with bound proteins were loaded into a column and the unbound proteins were flushed out by gravity. The chitin resin was washed with 20 CV of high salt buffer containing 20 mM Tris (pH 7.5), 700 mM NaCl, 1 mM EDTA and 10% glycerol, and 5 CV of low salt buffer containing 50 mM MES (pH 6.5) and 100 mM NaCl. The resin was then incubated in a buffer containing 75 mM β-mercaptoethanesulfonic acid sodium salt (MESNA) in low salt buffer at room temperature for 12 hours before elution. Eluted protein was buffer exchanged into the same low salt buffer to remove excess amount of MESNA. The molecular weight of the HA-Ub-MESNA was determined by LC-MS to be 9,837 Da (theoretical MW is 9,835 Da).

Synthesis of Michael acceptor linker **2** (see Figure 1) was performed as described previously[1]. The reaction of HA-Ub-MESNA with **2** was carried out by mixing 0.3 mM ubiquitin in low salt buffer containing 50 mM MES (pH 6.5) and 100 mM NaCl with 450 mM of **2**. The addition of **2** increased pH of the solution to approximately 8. The reaction mixture was incubated at room temperature for 1.5 hours with end-over-end rotation, protected from light. Excess amount of **2** was removed by buffer exchange with the low salt buffer. The molecular weight of HA-Ub-MAL was determined by LC-MS to be 9,917 Da (theoretical MW is 9,916 Da).

HA-Ub-MAL at 3 mg/mL was treated with equal volume of TFA and 40 mM *p*TsOH. The carbonyl deprotection was carried out at room temperature for 6 hours with end-over-end rotation

under protection from light. After incubation, protein was precipitated with 10x volume of ice-cold ethyl ether and pelleted by centrifugation. Protein pellet was washed with additional 2 mL ice-cold ethyl ether and air dried at room temperature. The product was dissolved in buffer containing 20 mM Na_2HPO_4 (pH 6.0), 50 mM NaCl and 8 M urea. Protein solution was diluted to 15 mL with refolding buffer containing 20 mM Na_2HPO_4 (pH 6.0) and 50 mM NaCl. Buffer exchange was performed three times with refolding buffer to remove urea. The molecular weight of the deprotected ubiquitin species **4** (Figure 1) was determined by LC-MS to be 9,871 Da (theoretical MW is 9,872 Da).

Synthesis of non-cleavable linker **9** (see Figure S3) was carried out as described previously[2]. The HA-Ub-MESNA reaction with non-cleavable linker **9** (Figure S3) was carried out by mixing 10 mg/mL ubiquitin (1 mM) and 2 M **9,** to a final concentration of 0.26 mM ubiquitin and 400 mM **9** in low salt buffer containing 50 mM MES (pH 6.5) and 100 mM NaCl. The reaction was incubated for 1.5 hours with end-over-end rotation and protection from light. The reaction product was buffer exchanged into the low salt buffer. The molecular weight of HA-Ub-NCL was determined by LC-MS to be 9,917 Da (theoretical MW is 9,918 Da). HA-Ub-NCL at 0.12 mM was treated with an equal volume of TFA (700 µL) and 40 mM *p*TsOH for 12 to 15 hours. Post reaction, the protein was precipitated in ice-cold ethyl ether in a volume 10x that of the protein solution. The precipitate formed at the interphase after centrifugation and the TFA solution below the interphase was pipetted out. To completely remove the TFA this procedure was repeated two additional times. The pellet was then air dried for at least 30 minutes to remove the ethyl ether. The pellet was resuspended in a buffer containing 20 mM Na_2HPO_4 (pH 6.0), 50 mM NaCl and 6 M guanidine HCl (or 8 M urea as stated above). The dissolved pellet was diluted into 50 mL of the refolding buffer. The solution was then concentrated and buffer exchanged three times to remove the guanidine HCl. The recovered protein was analyzed by mass spectrometry and the molecular weight of the deprotected HA-Ub-NCL was determined to be 9,874 Da (theoretical MW is 9,874 Da).

Chemical ligation to generate HA-tagged Ub-PCNA DUB probes

Purification of K164C cysteine-light PCNA (C22S/C30S/C62S/C81S/K164C PCNA) and K107C cysteine-light PCNA (C22S/C30S/C62S/C81S/K107C PCNA) was described in our previous publication[3]. Purified K107C and K164C PCNA samples were both buffer exchanged into a buffer containing 20 mM Na_2HPO_4 (pH 6.0) and 50 mM NaCl to remove DTT and mixed with deprotected HA-Ub-MAL **4** in a 1:3 (PCNA monomer : Ub) molar ratio. The reaction was incubated at room temperature for 4 hours with end-over-end rotation and protection from light. The resulting HA-tagged K107C and K164C Ub-PCNA probes were purified with a HiTrap Q FF column (GE healthcare) connected to AKTA purifier (GE healthcare) with a linear gradient of 50 to 800 mM NaCl. The K107C NCL Ub-PCNA and K164C NCL Ub-PCNA were prepared by ligating deprotected HA-Ub-NHL **11** to PCNA at a molar ratio of 1:3 (PCNA monomer : Ub) for K107C PCNA and 1:6 (PCNA monomer : Ub) for K164C PCNA. Ligation was performed at room temperature for 4 hours with end-over-end rotation and protection from light. The non-cleavable Ub-PCNA was buffer exchanged using a buffer consisting of 50 mM Tris base (pH 7.5) and 50 mM NaCl. Protein was then purified using a 1 mL HiTrap Q FF column (GE healthcare) and AKTA purifier (GE healthcare). The K107C and K164C Ub-PCNA NCL probes were eluted from the column using a linear NaCl gradient (50 to 800 mM) in 50 mM Tris (pH 7.5) buffer.

Tryptic digestion and LC-MS/MS analysis

For tryptic digestion reactions, 10 μg of the Ub-PCNA species were heated at 90 °C for 10 min. After cooling down to room temperature, the pH of protein solution was adjusted to 8 by adding 1 M Tris (pH 8). Then 1 μg trypsin was added to the protein solution and the reaction was incubated at 37 °C for 10 hours. The digested peptides were desalted with ZipTip SCX (Millipore) following the instruction of the manufacture. The resulting peptide sample was loaded onto a Orbitrap Q-Exactive (Thermo Fisher Scientific) equipped with a HPLC for LC-MS/MS analysis to confirm the correct linkages between ubiquitin C-terminus and PCNA.

Yeast Cell Culture and Lysis

Saccharomyces cerevisiae was grown in YPD media comprised of 2% peptone, 1% yeast extract, and 2% glucose. Yeast stock was first grown on a YPD agar plate (YPD media with 2.4% agar) using sterile cell plating technique at 30˚C. A 20 mL solution of YPD media was inoculated using sterile technique with a colony from the YPD plate and grown at 30˚C in a large orbital shaker until solution was at an optical density (OD_{600}) of 1. A 1 L solution of YPD was then inoculated using sterile technique with the newly grown yeast cells diluted of roughly 10,000 fold. This was grown overnight under the same growing conditions. This culture was grown to an OD_{600} of 0.6-0.8 upon which the cells were pelleted by centrifugation. They were then washed with PBS buffer and centrifuged. This wash and centrifugation was repeated for a total of 2 times.

To prepare yeast lysate, 0.1-0.2 grams of harvested yeast cells were resuspended in 0.1-0.2 mL of yeast lysis buffer, 50 mM Tris base (pH 8), 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 1 mM TCEP, 1 mM PMSF, and 1x protease cocktail inhibitor (Bimake) keeping a 1:1 ratio (g:mL). Glass beads 425-600 µm in size were washed in yeast lysis buffer three times before added to the resuspended yeast cells in a 1.5-2 mL microcentrifuge tube so the mixture occupies half to two-thirds the volume of the tube. The yeast cells were then benchtop vortexed at 30 second intervals for 30 minutes, keeping the tube on ice when not being vortexed. The cell debris and intact cells were pelleted in a benchtop microcentrifuge at 4˚C for 10 minutes at 16,000 g. The supernatant above the beads was collected. Yeast lysis buffer with 500 mM NaCl (instead of 100 mM) was then added to the microcentrifuge tube using half the volume of buffer initially added, and mixed. The remaining cells were then vortexed and pelleted as before. The remaining supernatant was then collected and added to the first supernatant.

Cell lysate labeling using the Ub-PCNA DUB probes

For labeling the DUBs in yeast cell lysates, 0.2 µg of HA-Ub-PCNA probes were mixed with 10 µg of yeast cell lysates in PBS buffer. Solutions were incubated at 37˚C for 3 hours before adding gel loading dye and were analyzed using a 10% SDS-PAGE gel. SDS-PAGE was run at a

constant 200 volts, and western blotting membrane transfer was carried out for 14 hours at 4˚C at a constant 40 volts. The membrane was first washed in TBST buffer comprising of 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween20 and then blocked with 5% milk in TBST buffer for 1 hour. Primary anti-HA antibody (Sigma) was incubated with the membrane at a 10,000 fold dilution in 10 mL of 5% milk in TBST for 2 hours. The membrane was then washed 3 times with TBST for 10 minutes each. Anti-mouse secondary antibodies (Sigma) and StrepTactin-HRP Conjugate (BioRad) were diluted 25,000 and 50,000 fold respectively in 50 mL TBST buffer and were incubated with the membrane for 1 hour. This was followed by three washes with TBST buffer for 10 mins each. Lastly the Western blotting substrate 1 and 2 (Pierce ECL Western Blotting Substrate) was added and incubated with the membrane for 5 minutes before imaging.

Affinity pulldown of DUBs from yeast cell lysate

For pulldown 20 μg (500 pmol) Ub-PCNA probe or 5 μg (500 pmol) Ub-VME probe (generated as previously described)[4] was conjugated to 10 μL anti-HA magnetic beads in PBS buffer at room temperature for 30 minutes with end-over-end rotation. After washing out unbound probe with PBS buffer, 100 μg *S. Cerevisiae* lysate was added and incubated at room temperature for 2 hours with end-over-end rotation. Beads were then washed 3 times with PBS buffer before elution with 20 μL 50 mM NaOH, elution was repeated twice. Protein solutions eluted from beads were adjusted to pH 8 by addition of 1 M Tris (pH 8). The eluted proteins were then reduced with 10 mM DTT at 60 °C in a water bath for 1 hour, and subsequently were alkylated with 20 mM iodoacetamide at room temperature for 30 minutes under protection from light. The alkylation reaction was quenched by addition of 5 mM DTT before digestion with 1 μg trypsin at 37 °C for 20 hours. Tryptic digestion was quenched by 0.1% TFA and was centrifuged at 13,000 g at room temperature for 10 minutes to remove residual precipitation. The cleared sample was desalted with stage-tip made in house following a previous publication[5] and lyophilized before LC-MS/MS analysis. As a control experiment, anti-HA magnetic beads alone were incubated with 100 μg lysate and were processed with the same washing, elution and digestion procedure.

LC-MS/MS mass spectrometry analysis

Lyophilized samples following the tryptic digestion were dissolved in 10 μL 0.1% formic acid in water before LC-MS/MS analysis. Samples were analyzed by nanoflow ultrahigh performance liquid chromatography connected to Orbitrap Q-Exactive (Thermo Fisher Scientific) equipped with a nano-electrospray ionization source. A Thermo Fisher nano UHPLC column (15 cm long, 75 μm inner diameter column packed with 3 μm C18 resin) and a linear gradient from 5% to 60% acetonitrile in 0.1% formic acid was used to separate peptides for 150 minutes at a constant flow of 200 nL/min. The Orbitrap was set to positive polarity mode, high energy collision induced dissociation. The system was set to operate in a data-dependent mode with MS/MS scan of the six most abundant peaks from a full MS scan. Full scans were acquired between 300 to 1800 *m/z* with a resolution of 60000.

Data processing and analysis

Twenty-eight raw files were analyzed together using MaxQuant software version 1.5.4.1. Peak list was searched with built-in Andromeda search engine against *S. cerevisiae* proteome database downloaded from Uniprot website (http://www.uniprot.org/proteomes/UP000002311). Multiplicity was set to one since there was no isotope labeling. Trypsin was set to cleave after lysine and arginine, unless followed by a proline. A maximum of two missed cleavages were allowed and a maximum charge of peptide was set to 7. Minimum peptide length was set to seven amino acids. Cysteine carbamidomethylation was set as fixed modification. N-terminal acetylation, methionine oxidation, serine, threonine, and tyrosine phosphorylation, and the ubiquitin diglycine remnant were all set as variable modifications. Label-free quantification (LFQ) module within MaxQuant was enabled. The minimum ratio count was set to 1 and fast LFQ was not enabled. The second peptide search was enabled. The max number of modifications per peptide was set to 5. The option to require MS/MS for LFQ comparisons was also enabled.

The output result text file “protein groups” was imported to and analyzed by Perseus software version 1.5.4.1. Proteins belonging to “only identified by modified peptides” were

eliminated. Proteins identified with reversed decoy mode and common contaminant proteins were also excluded. A protein and peptide false discovery rate was set to 1%. Replicate experimental conditions were grouped. Proteins that had at least three valid LFQ values (LFQ intensity >0) in at least one group were kept. Missing values were then imputed from the normal distribution of the total matrix using default parameters of a 0.3 width and 1.8 down shift. Two sample unpaired *t*-test was then performed in Perseus using the default parameters. The $-\text{Log}_{10}$(*p*-value) and Log_2 (fold difference in LFQ value) were plotted in GraphPad Prism to generate the volcano plot.

Purification of yeast DUBs

Purification of yeast Ubp15 was described in previous publication[6]. UBP10 gene was amplified and cloned into pET28a vector using NdeI and XhoI restriction sites. Ubp10-pET28a was transformed to Rosetta (DE3) cells. The cells were cultured in LB media at 37°C until OD_{600} reached 0.6-0.8. IPTG at 0.4 mM was used to induce protein expression. Cells were harvested by centrifugation at 6,000 g at 4 °C for 10 min after 20 hours shaking at 16 °C. Cell pellet was then resuspended in lysis buffer containing 50 mM Tris (pH7.5), 500 mM NaCl, 10% glycerol, 10 mM imidazole and 10 mM β-ME. After sonication, the lysates were centrifuged at 15,000 g at 4 °C for 30 minutes. Cleared extract was incubated with Ni-NTA resin for 2 hours at 4 °C with end-over-end rotation. After extensive washing with lysis buffer, protein was eluted with 100 mM imidazole in lysis buffer. The eluted protein was buffer exchanged to 50 mM Tris (pH 7.5), 50 mM NaCl, 10% glycerol and 1 mM DTT. The protein was then loaded onto a SP column and eluted within a 50-700 mM NaCl gradient. The peak fractions were collected and exchanged to buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 10% glycerol and 1 mM DTT. The mutant Ubp10(C371A) was generated by Quikchange PCR using WT Ubp10-pET28a as a template. The mutant Ubp10 was purified following the same protocol described above for the wild-type Ubp10.

YUH1 in pET3a was a gift from Cecile Pickart (Addgene plasmid # 18895)[7] and transformed into BL21 (DE3) cells. For cell culture and protein purification of YUH1, we followed a previous publication[8]. Specifically, cells were cultured in LB media at 37°C until

OD_{600} reached 0.6-0.8. IPTG at 0.4 mM was used to induce protein expression. Cells were harvested by centrifugation at 6,000 g at 4 °C for 10 min after 20 hours shaking at 16 °C. Cell pellet was then resuspended in lysis buffer containing 50 mM Tris (pH8.0),100 mM NaCl, 2 mM DTT and 2.5% glycerol. After sonication, the lysate was centrifuged at 15,000 g at 4 °C for 30 minutes. Cleared cell extract was filtered with a 0.2 μm syringe filter and loaded onto a Q-Sepharose column connected to AKTA purifier FPLC. YUH1 was eluted y a 100-800 mM NaCl gradient. Peak fractions were pooled and buffer exchanged to a buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl and 2 mM DTT.

Deubiquitination assay using native Ub-PCNA as a substrate

Native Ub-PCNA (0.5 μM) was mixed with increasing concentration of purified DUB, including Ubp10, Ubp10(C371A), Ubp15 and YUH1 at 0, 0.01, 0.1 or 1 μM in a 10 μL buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol and 2 mM DTT. The reaction mixture was incubated at 37 °C for 10 min, quenched by 2 μL 6x SDS loading dye, and analyzed by a 20% SDS-PAGE gel and stained with Coomassie blue dye.

Ubp10 labeling experiment with Ub-PCNA MAL probes

WT Ubp10 or the active-site mutant Ubp10(C371A) (0.5 μM) was mixed with K164C or K107C Ub-PCNA MAL probes (4 μM) in a 20 μL solution containing 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10% glycerol and 1 mM DTT. The reaction mixture was incubated at room temperature for 1 hour, quenched with 4 μL 6x SDS loading dye. The reaction product was analyzed using a 10% SDS-PAGE gel and stained with Coomassie blue dye.

Western blotting analysis of Ubp10 labeling with Ub-PCNA MAL probes

WT Ubp10 or the active-site mutant Ubp10(C371A) (0.5 µM) was mixed with K164C or K107C Ub-PCNA MAL probes (2 µM) in a 20 µL solution containing 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10% glycerol and 1 mM DTT. The reaction mixture was incubated at room temperature for 1 hour, quenched by 4 µL 6x SDS loading dye. The reaction product was separated on a 10% SDS-PAGE gel and transferred to a PVDF blotting membrane at 4˚C overnight at 60 V. Membrane was washed in TBST buffer comprised of 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20, then blocked with 5% milk in 10 mL of TBST buffer for 1 hour. Primary antibody, anti-HA antibody (Sigma) or anti-His antibody (Thermo Fisher Scientific), was diluted in a solution of 5% milk in 10 mL TBST by 1:5,000 and 1:1,000 respectively and incubated for 2 hours. Membrane was then washed three times for ten minutes each with TBST. Secondary antibody, anti-mouse IgG (Sigma) was incubated with the membrane in 50 mL of TBST at a 1: 50,000 dilution for 1 hour. Membrane was washed three times in TBST for ten minutes each and then incubated with ECL Western blotting substrate for imaging.

Generate electrostatic surface of PCNA trimer

We utilized the Pymol 2.0.7 plugin "APBS Electrostatics"[9] to generate the electrostatic surface of yeast PCNA trimer with default parameters.

Reference

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