Supporting Information

Specific and Unbiased Detection of Polyubiquitination via a Sensitive Non-Antibody Approach

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SUPPLEMENTAL MATERIALS AND METHODS

Cell culture and protein extraction. *E. coli* cell (BL21) for western blotting was grown in medium at 37 ℃ to an OD_{600} of 1.0, and then collected by centrifugation at 5 000 g for 10 minutes at 4 ℃. The collected cells were resuspended in lysis buffer (50 mM Tris-HCl, pH=8.0, 150 mM NaCl, 8 M urea, 1% NP-40, 1 × protease inhibitor cocktail) and lysed by ultrasonication on ice for 6 minutes. The cell lysate was centrifuged at 13 000 g at 4 ℃ for 10 minutes, and the supernatant was collected. The protein concentration was determined through a bicinchoninic acid (BCA) protein assay.

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. For MG132 or PS341 treatment, cells were grown to 50% of the plate, then the culture was replaced by DMEM medium supplemented with 10 nM of MG132 or 1 nM of PS341 for 1 hour. The collected cells were broken in the same lysis buffer and handled as mentioned above.

Lsb1 purification and LC-MS analysis. The Lsb1 with 6 ×histidine and biotin (HB) tags on C-terminal was inserted into the pUB221 vector and then transformed into the *Saccharomyces cerevisiae* SUB592 strain[1]. Cells were grown at 30 ℃ to an OD_{600} of 1.5, and then harvested by centrifugation at 5 000 g at 4 ℃ for 10 minutes. The harvested cells were suspended in lysis buffer A (50 mM Tris pH=8.0, 150 mM NaCl, 8 M urea, 5 mM IAA, 5 mM imidazole, 0.5% NP-40, 1 mM NEM, 1 mM PMSF), then lysed by the glass beads beating method. The HB tagged Lsb1 was firstly purified with Ni-NTA beads and then eluted by elution buffer B (50 mM Tris pH=8.0, 150 mM NaCl, 8 M urea, 5 mM imidazole). The eluted sample was termed as Elution 1. The Elution 1 sample was further purified by streptavidin beads, and eluted by boiling in 1x SDS loading buffer (50 mM Tris-HCl, pH = 8.0, 2% SDS, 10% glycerol, 10 mM DTT, 0.04% bromophenol), which was termed as Elution 2. The Lsb1 proteins were separated by SDS-PAGE, cut into 6 fractions and digested with trypsin for LC-MS/MS analysis as described before.[2-3]

ThUBD purification. The ThUBD was cloned with a glutathione S-transferase (GST) tag on the C-terminal and expressed in *E. coli* BL21 (*DE3*). Cells were grown at 37 ℃ to an OD_{600} of 0.6 and then induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 8 hours at 20 ℃. The harvested cells were lysed by sonication in lysis buffer (20 mM Tris pH=8.0, 150 mM NaCl, 5% glycerol, 1 mM DTT). The ThUBD tagged with GST were purified from cell lysates using glutathione-Sepharose (GSH) 4B beads (QIAGEN, Valencia, CA) as previously described . The purified protein was completely eluted by 20 mM reduced glutathione. Protein buffer was changed into 20% glycerol in PBS through ultrafiltration. Protein concentration was determined through a bicinchoninic acid (BCA) protein assay, and then stored at -30 ℃ prior to use.

Western blotting. The regular western blotting experiment was performed routinely. Protein samples were separated by SDS-PAGE and transferred to nitrocellulose (NC) or PVDF membrane

using the Bio-Rad Trans-Blot SD Semi-Dry Transfer System for 20-45 minutes at 15 V. The Ponceau S staining or silver staining were set as loading controls. Membranes were blocked with 10% nonfat milk in TBST (20 mM Tris, 150 mM NaCl, containing 0.1% Tween-20, pH=7.4) for 60 minutes. Then the membranes were then incubated with primary antibodies to detect the signal from ubiquitin (Ab-1, sc-8017 Santa Cruz, California, USA; Ab-2, ab139101, Abcam, Cambridge, UK) or biotin (anti-streptavidin, ab191338, Abcam, Cambridge, UK) in TBST with 5% nonfat milk at room temperature for 2 hours or at 4℃ overnight, and the antibodies were used at a dilution ratio of 1:1000. Removal of excess primary antibody was carried out by washing the membranes in TBST three times for 10 minutes each. The secondary antibody (peroxidase-conjugated anti-mouse, A9044, Sigma-Aldrich, Darmstadt, Germany) diluted with 1:10 000 was incubated with the membrane in TBST with 5% nonfat milk for 60 minutes at room temperature. Excess secondary antibody was removed by washing the membranes in TBST three times for 10 minutes each. Membranes were exposed to SuperSignal West Pico chemiluminescence substrate (34580, Thermo Scientific, Massachusetts, USA) for 1 minute at room temperature and visualized using Tanon 5200 Chemiluminescence imaging analysis system (Tanon, Shanghai, China). Detection and quantification of the band intensities was conducted using ImageJ 1.48v software (National Institutes of Health, USA).

Abbreviations:

FBS: Fetal Bovine Serum

DTT: DL-Dithiothreitol

SDS: Sodium DodecylSulfate

LC-MS/MS: Liquid Chromatography-tandem Mass spectrometry

PVDF: PolyVinyliDene Fluoride

NC: Nitrocellulose

PBS: Phosphate Buffer Saline

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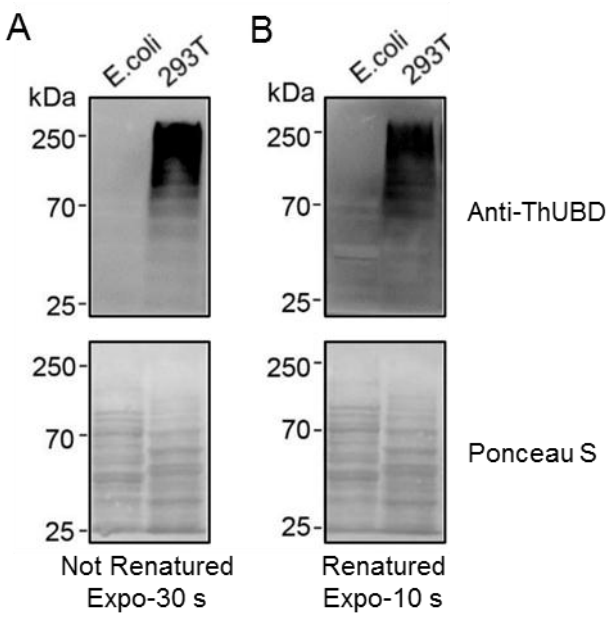

Figure S1. Non- and renaturation validation of the far-western blotting experiment. The 2 far-western workflows without (A) or with (B) denaturation and renaturation steps were used to analyze the cell total lysate of *Escherichia coli* (*E. coli*) and human embryonic kidney 293T cells (293T). The denaturation and renaturation procedure strengthened the sensitivity of far WB, but the signal-to-noise ratio was lower than that of the not renatured membrane.

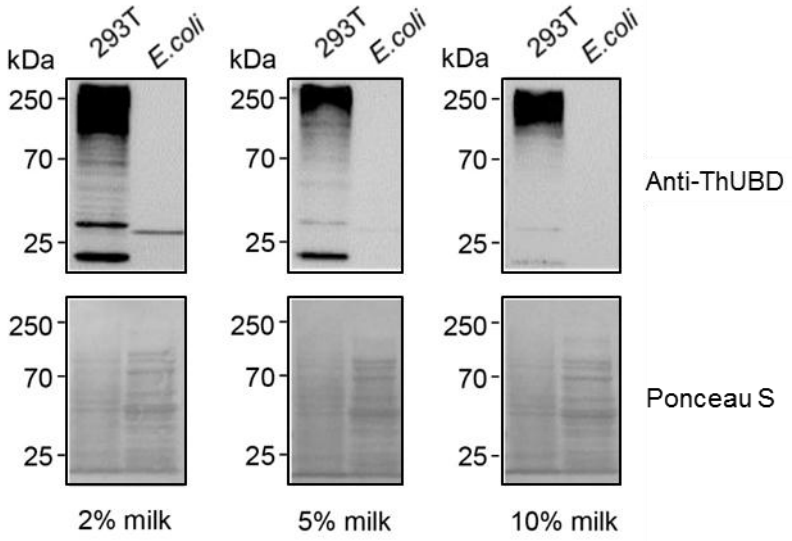

Figure S2. Optimizing the blocking concentration of milk. Membranes were blocked with 2%, 5% and 10% of milk and then incubated with ThUBD. After washing with TBST, the membranes were incubated with GST antibody and sequentially detected. The nonspecific binding decreased along with the increasing concentration of milk.

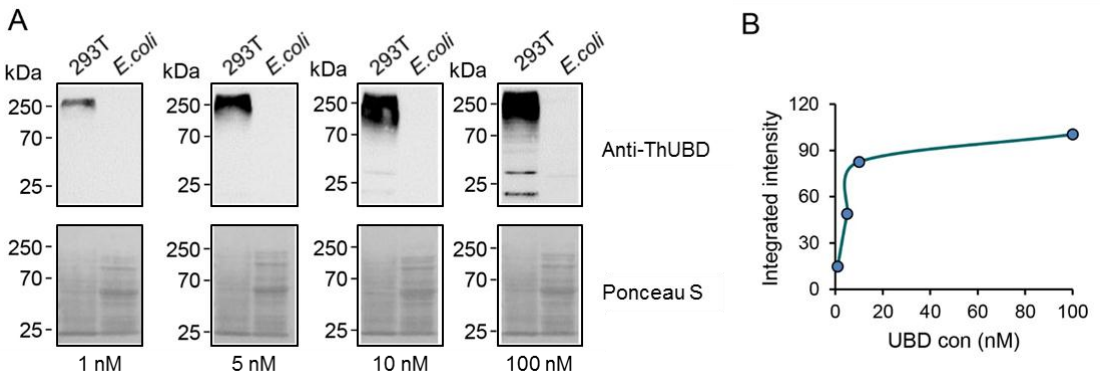

Figure S3. Optimizing the concentration of ThUBD used in the TUF-WB method. (A) The membranes were incubated with different concentrations of ThUBD, ranging from 1 nM to 100 nM. (B) The saturation curve indicated that the 10 nM concentration nearly reached the saturation point.

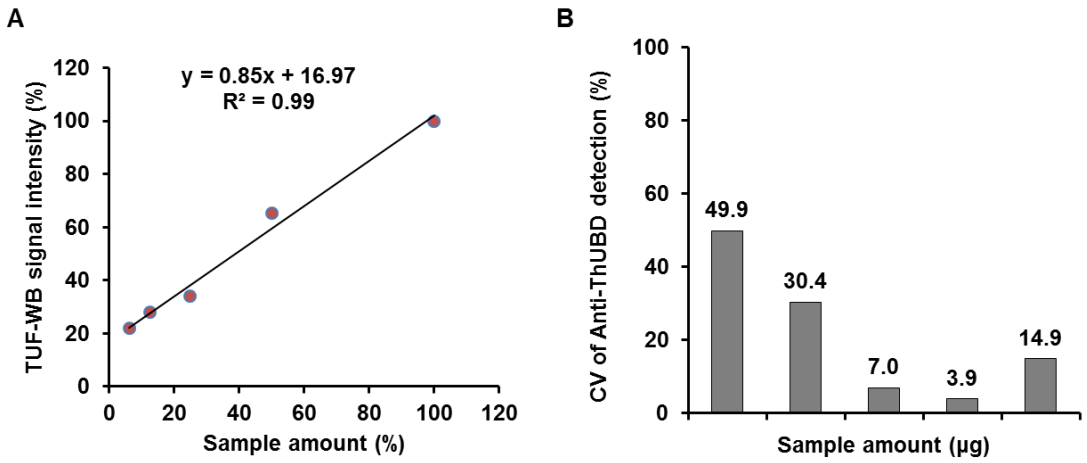

Figure S4. Quantification accuracy of TUF-WB with complex sample. (A) The linear regression between sample amount and TUF-WB signal intensity. The sample amount and signal intensity was normalized based on the 20 μg and its corresponding intensity in figure 2C. (B) The Coefficient of Variation (CV) between ThUBD detected signals in figure 2C and their theoretical values.

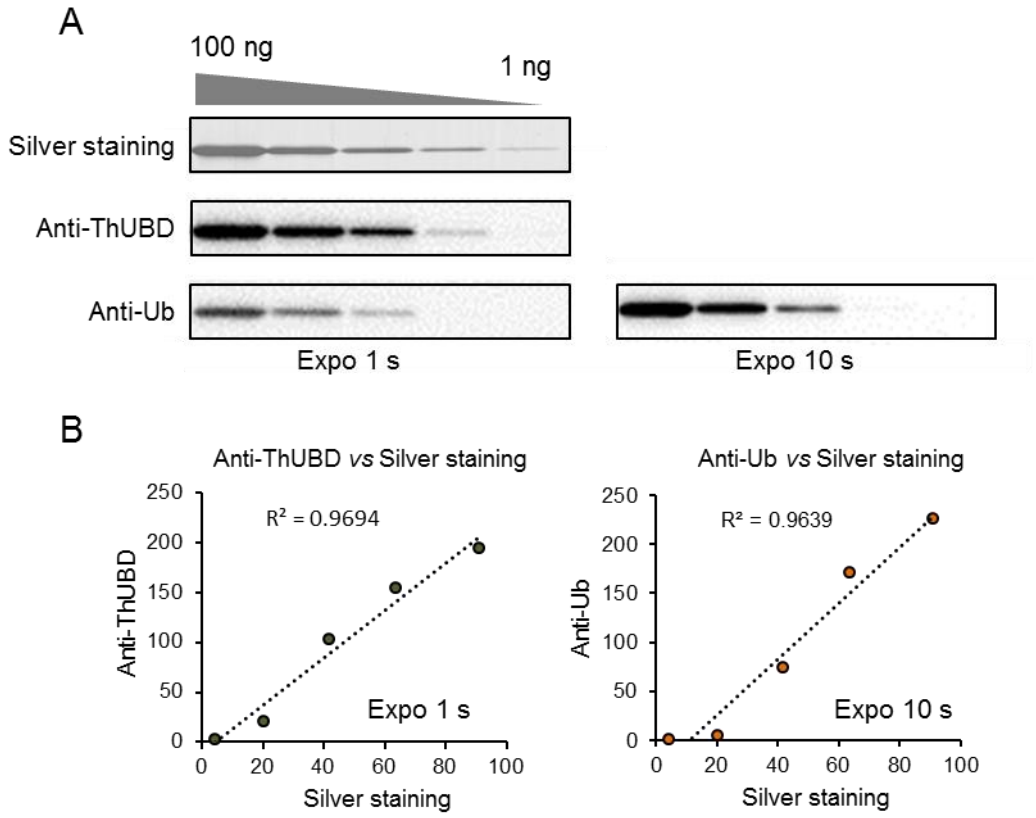

Figure S5. The TUF-WB with higher accuracy for Ub chain detection. (A) The tetra linear ubiquitin chain was loaded in different amounts, ranging from 1 ng to 100 ng, and analyzed by ThUBD and the ubiquitin antibody in parallel. (B) The quantification curve of ThUBD detected at 1 s and anti-ubiquitin detected at 10 s exposure showed good linearity between the signal and the amount of ubiquitin chain. However, the ThUBD detected the ubiquitin chain over a broader range of ubiquitin amounts than ubiquitin antibody.

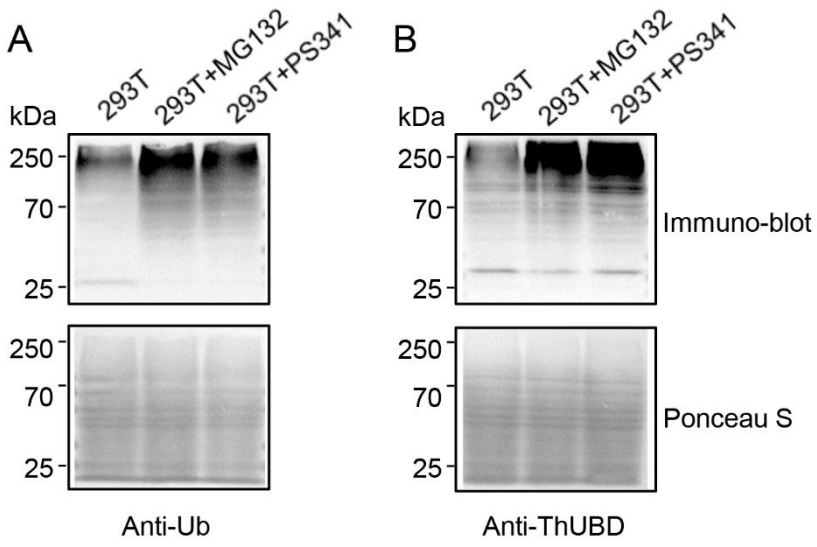

Figure S6. Ubiquitination detection after proteasome inhibitor treatment by TUF-WB. The ThUBD (B) and ubiquitin antibody (A) were both used to monitor the ubiquitin accumulation of human embryonic kidney 293T (293T) total cell lysate under MG132 and PS341 treatments. A significant accumulation of ubiquitin was observed by both methods.

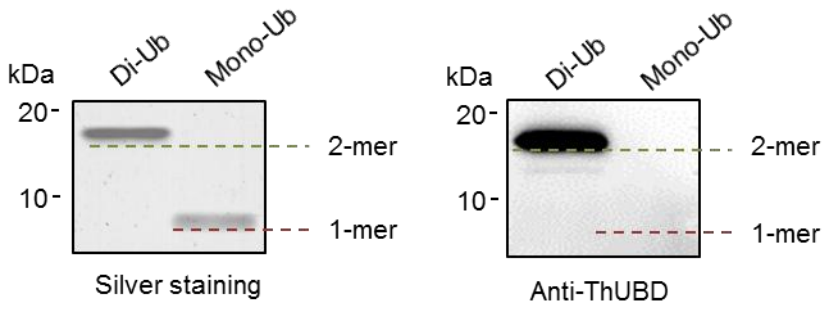

Figure S7. Ubiquitin chains and mono ubiquitin detection by TUF-WB. The ThUBD displayed higher sensitivity to ubiquitin chains than mono-ubiquitin. K63 linked di-Ub and mono-Ub were analyzed by silver staining (left) and ThUBD (right) in parallel with the same sample amount. The ThUBD-based method could specifically recognize the ubiquitin chain but showed poor affinity to mono-ubiquitin.

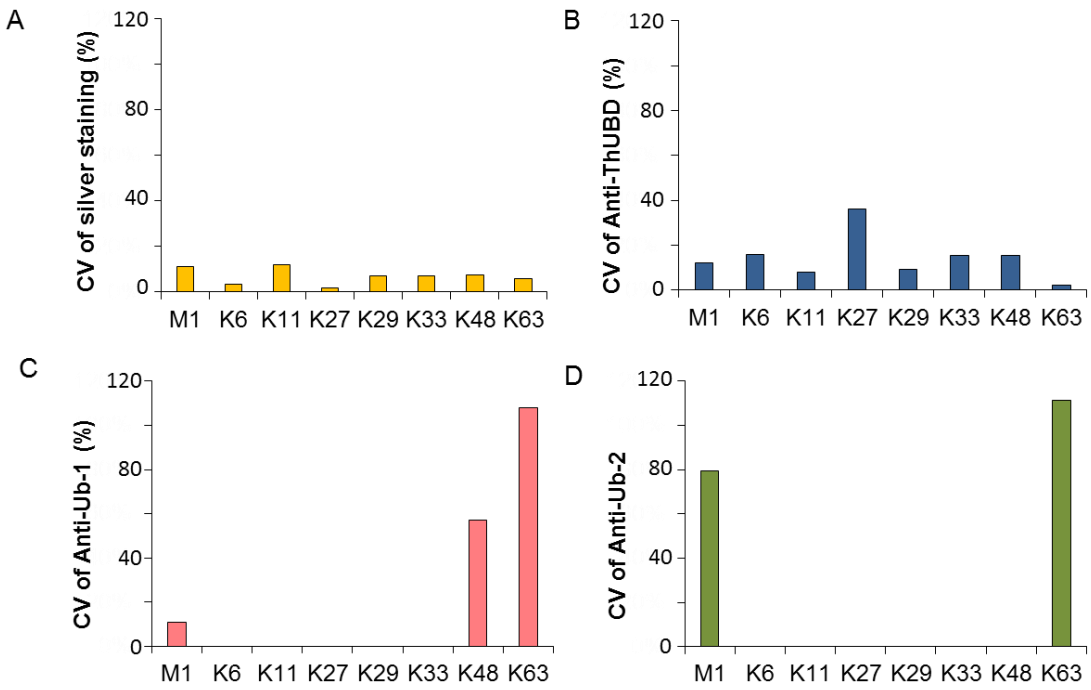

Figure S8. CVs of the eight Ub chains detection. (A) The CV between silver staining signals of the eight ubiquitin chains and their theoretical value (12.5%). (B) The CV between ThUBD detected signals of the eight ubiquitin chains and the silver staining detected signals. (C, D) The CV between commercial ubiquitin antibodies (Ub-1 and Ub-2) detected signals of the eight ubiquitin chains and the silver staining detected signals.

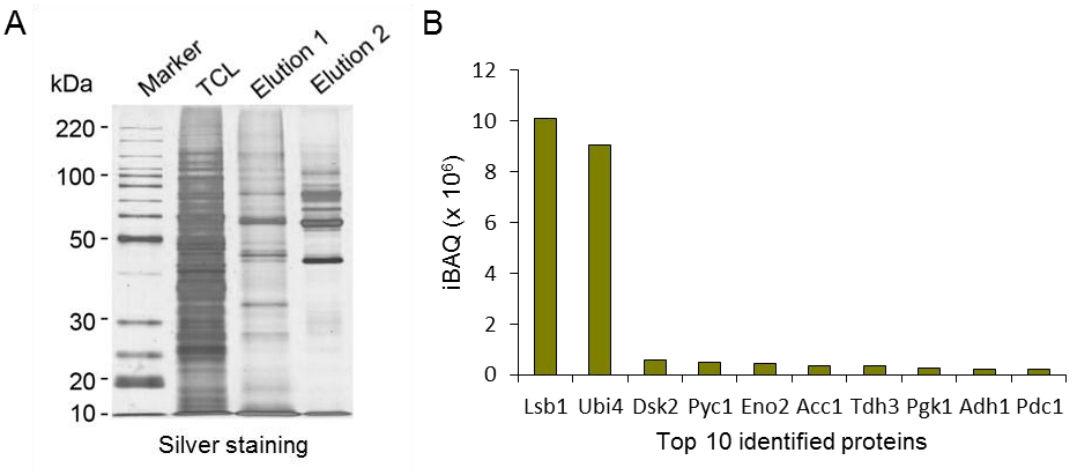

Figure S9. Purification and LC-MS/MS analysis of Lsb1. (A) The his- and biotin-tagged Lsb1 was purified from the total cell lysate (TCL) through tandem purification of Ni-NTA (Elution 1), followed by streptavidin beads (Elution 2). (B) The purified Lsb1 (Elution 2) was analyzed by LC-MS/MS, and the most high abundant protein in the purified sample were Lsb1 and ubiquitin.

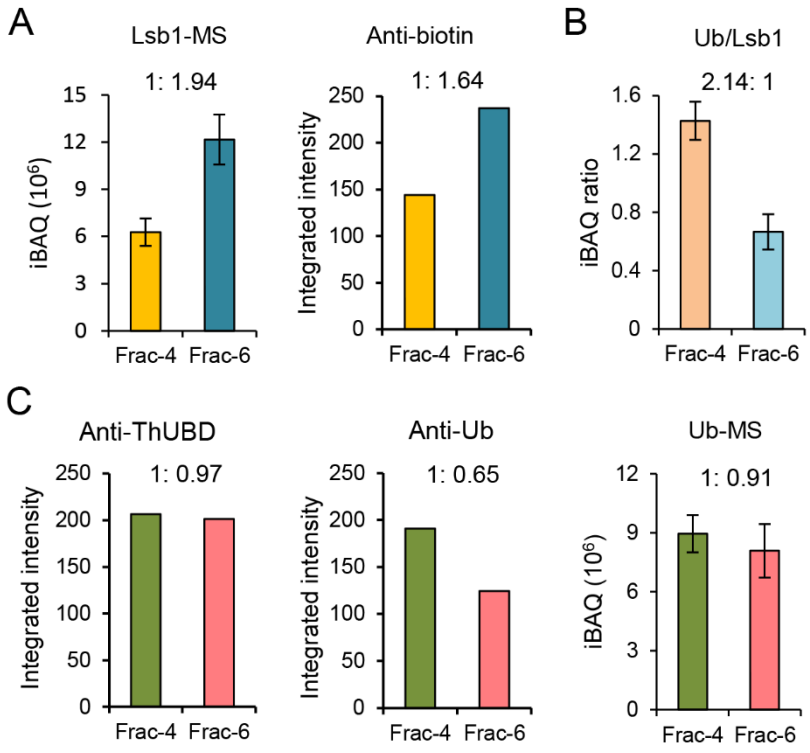

Figure S10. Quantification of fraction 4 and fraction 6 by LC-MS/MS and immunoblotting. (A) The signal ratio of Lsb1 between fraction 4 (Frac-4) and fraction 6 (Frac-6) in Figure 4C was calculated by both LC-MS/MS (left) and anti-biotin western blotting (right). (B) The Ub/Lsb1 ratios in these two fractions detected by LC-MS/MS were also calculated. (C) The signal ratio of ubiquitin between fraction 4 and fraction 6 was calculated by LC-MS/MS, ThUBD (left) and ubiquitin antibody (middle) in parallel, and the results of LC-MS/MS (right) and ThUBD were consistent.