Identify ubiquitination sites for a protein of interest by selective enrichment of ubiquitin remnant peptides

Experiment design

A flow chart of the protocol is shown in Fig. 1. The detailed experiment design is discussed in each section below and in the PROCEDURE section.

Sample preparation. While cell lysates can be directly treated with trypsin and URPs are isolated through an immobilized anti-GGεK antibody, this can be problematic. Several mature Ub-like modifiers such as ISG15 and NEDD8 also terminate with Arg-Gly-Gly at their C-termini. The last Gly can be conjugated to lysine residues in a manner similar to protein ubiquitination[1] and after trypsin digestion, these conjugates can generate peptides containing diglycine-modified lysines that are indistinguishable from URPs. Although these modifications are rare compared to ubiquitination[1], to reduce the possibility of misinterpretation of these peptides as URPs, a purification step that enriches for Ub conjugates prior to trypsin digestion and the anti-diglycyl-lysine purification is recommended.

In our approach, we express hexahistidine-tagged Ub (His_6-Ub) in human embryo kidney (HEK) 293 cells and purify the ubiquitinated proteins under denaturing conditions using immobilized metal affinity chromatography (IMAC) to enrich the ubiquitinated proteins prior to digestion. To avoid significant alterations of the protein ubiquitination pathway, His_6-Ub is expressed at low levels and accounts for only a small fraction of the total cellular Ub. Under these conditions, the expression of His_6-Ub does not significantly affect the levels of protein ubiquitination, as demonstrated by the comparable signal for cell lysates from non-transfected and His_6-Ub-transfected cells in the high molecular weight region of the anti-Ub western blot. In general, any tagged Ub can be used for the expression and purification of ubiquitinated

proteins. Alternatively, Ub-interacting proteins[2] or anti-Ub antibodies[3,4] can also be used for the initial purification of ubiquitinated proteins.

For the identification of ubiquitination sites for a single protein, we chose RhoA as an example since RhoA has been previously shown to be ubiquitinated in epithelial cells[5] and the E3 ligase that mediates the ubiquitination is known[5]. We used a modified RhoA that bears a biotin acceptor peptide (BAP, amino acid sequence: GLNDIFEAQKIEWHE)[6] and a short linker (SGR) at the N-terminus. In the presence of BirA from *Escherichia Coli* and exogenously supplied biotin, the lysine residue in BAP is modified with biotin[6], which can be used as a handle for the purification of ubiquitinated RhoA under denaturing conditions using avidin affinity chromatography. In addition to the co-expression of His_6-Ub, BAP-RhoA and BirA, the RhoA E3 ligase, Smurf1[5], can also be expressed. To identify Ub sites on other proteins of interest, the appropriate E3 ligase, if known, can be expressed to increase the yield of URPs. Alternatively, certain stimuli[7] that induce ubiquitination of the target protein may also be employed to increase the abundance of URPs from the protein of interest. Finally, proteasome inhibitors may be added to the cells to prevent rapid degradation of ubiquitinated proteins, which will also result in a higher yield of URPs.

The samples are purified by IMAC, for example on a Ni-NTA column, and eluted under denaturing conditions. The ubiquitinated RhoA is further purified by avidin agarose beads and eluted with LDS sample buffer at elevated temperature.

Protein digestion. We use the in-gel trypsin digestion protocol[8] to digest ubiquitinated proteins because this procedure is compatible with small amounts of detergent in the purified Ub conjugates and produces peptide samples with little salt and denaturant, which are suitable for the downstream immunopurification of URPs and MS analysis. The purified ubiquitinated proteins are first separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the bands above 25 kDa are excised, disulfide bonds are reduced and thiols are alkylated. In the alkylation step, iodoacetamide used in the majority of proteomics studies has been replaced by chloroacetamide to avoid potential introducing two $–CH_2CONH_2$ modification on one lysine residue, which generates a modification with exactly the same chemical formula as a diglycine modification on lysines[9]. This artifact would be indistinguishable from the diglycine modification on lysine in MS/MS spectra and thus could be misinterpreted as a diglycine modification[9]. All other steps are similar to other in-gel digestion protocols[8]. Alternative digestion approaches, such as in-solution digestion[10] and on-bead digestion[11] can also be adapted to this protocol. In our experiments, we find that in-gel digestion gives better

results than the other two approaches in terms of the number of ubiquitination sites identified by tandem MS. If urea is used during the in-solution or on-bead trypsin digestion, a C18 purification step, such as a zip-tip[12] or StageTip[13] purification, is recommended prior to the immunopurification of URPs. The presence of urea in the peptide sample may slightly reduce the binding affinity of the anti-GGεK antibody to URPs.

Because some ubiquitination sites are located in regions with multiple lysines, trypsin digestion may generate peptides which are too short to be detected by MS. In this case, other enzymes, such as Arg-C or a combination of Arg-C and Glu-C, may be used to generate URPs which are suitable for MS identification.

Antibody coupling and immunopurification of URPs. In this protocol, the anti-GGεK antibody is coupled to the Affi-gel 10 resin for immunopurification of URPs. Alternatively, protein G beads can be used to bind and couple the monoclonal antibody by dimethyl pimelimidate (DMP)[14] or bis(sulfosuccinimidyl) suberate (BS^3)[15] under slightly basic conditions. Our experiments show that protein G retains more nonspecific peptides than Affi-gel 10 resin.

The digest obtained from trypsinolysis of ubiquitinated proteins is lyophilized and resuspended in a small volume, such as 300 μL, of resuspension buffer. Large volume of the resuspension buffer reduces the concentration of URPs and may adversely affect the efficiency of the immunoprecipitation of these peptides from the digest. The digest is then incubated at 4 °C with Affi-gel 10 or protein G beads with immobilized anti-GGεK antibody. The beads are washed and URPs are eluted with a low pH solution for MS identification.

MS analysis. The introduction of a post-translational modification (PTM) in peptides can significantly increase the false positive rate if a low-resolution MS instrument is used[16]. Therefore, high-resolution MS instruments such as Q-TOF and OrbiTrap are recommended for sample analysis in order to provide accurate mass-to-charge ratio (m/z) of precursor ions.

Since trypsin does not efficiently cleave peptide bonds after diglycine-modified lysines, URPs are generally longer and have higher charge state than regular tryptic peptides[17]. It has been demonstrated that electron transfer dissociation (ETD) can provide more confident identification for long, highly charged or modified peptides[18]. Therefore, it is suggested that ETD be employed, if possible, during the MS analysis of URPs.

Database search. Database search can be performed with commercial software from the MS instrument vendor, such as Sequest[19] and Spectrum Mill[20]. Online open source or commercial

available search programs, such as X!Tandem[21], OMSSA[22], Mascot[23], or Phenyx[24], can also be used for peptide identification. In some cases, it may be necessary to convert the original MS data file to a suitable format recognized by the search program[25]. During the database search, a fixed modification is carbamidomethylation for cysteines and variable modifications are diglycine modification for lysines and oxidation for methionines. The number of trypsin missed-cleavages is set up to four since trypsin does not efficiently cleave lysines after diglycine modification. Partial trypsin cleavage may be set as the digestion condition during the database search, which could be helpful in identifying multiple ubiquitination sites, if they exist, for a protein of interest, due to the presence of trace amounts of active chymotrypsin in trypsin[26] and potential internal protein processing events in the proximity of the ubiquitination sites[27].

Based on the Delta Mass website (http://www.abrf.org/index.cfm/dm.home; a database of protein PTMs), two modifications, asparagyl- and hydroxyprolyl-, are isobaric to the diglycine-modification using a 0.02 Da mass error window. These rare modifications could be mistaken for the diglycine modification on lysines by the search program. Alternatively, if these residues are adjacent to a lysine residue, the MS/MS fragments could be misinterpreted to contain a diglycine-modified lysine by the search program. Therefore a manual validation is required for any peptide containing diglycine-modified lysine residues. Alternatively, the false positive rate can be estimated using a target-decoy search strategy[28] for large scale proteomic studies.

Manual validation. Due to the presence of isobaric modifications to diglycine modification (see above), the URPs identified by search program need to be manually validated. The five highest-ranked peptides, if they exist, for each MS/MS spectrum that are obtained from search programs should be carefully examined to eliminate the possibility of an alternative match in which the diglycyl-modified lysine is replaced with an asparagine or hydroxyproline which is in the proximity of the lysine residue identified with a diglycine modification. Manual verification of fragment ions, such as *b*- and *y*-ions, in MS/MS spectra should be performed.

For peptides that are difficult to resolve due to mass ambiguities and MS/MS assignment, we suggest the ubiquitinated form of these proteins of interest be purified using a bimolecular affinity purification approach[29], described in this protocol, followed by the anti-GGεK antibody purification of the tryptic peptides. Multiple ubiquitination sites for a protein, if they exist, can be confidently identified using this approach due to the reduction of sample complexity. The MS-identified ubiquitination sites should be verified by conventional mutation, immunoprecipitation, and western blotting analysis to assess the contribution of the identified lysines on protein ubiquitination.

REAGENT SETUP

Lysis buffer 8 M urea, 0.3 M NaCl, 50 mM phosphate, 0.5 % NP-40 (vol/vol), pH 8.0 with 5 mM chloroacetamide (freshly made).

Ni-NTA washing buffer A 8 M urea, 0.3 M NaCl, 50 mM phosphate, 0.5 % NP-40 (vol/vol), pH 8.0.

Ni-NTA washing buffer B 8 M urea, 0.3 M NaCl, 50 mM phosphate, 0.5 % NP-40 (vol/vol), pH 6.5.

Ni-NTA elution buffer 8 M urea, 0.3 M NaCl, 50 mM phosphate, 250 mM imidazole, 0.5 % NP-40 (vol/vol), pH 8.0.

Neutravidin washing buffer A 8 M urea, 0.2 M NaCl, 2 % SDS (wt/vol), 0.5 % NP-40 (vol/vol), 100 mM Tris-HCl, pH 8.0.

Neutravidin washing buffer B 8 M urea, 1.2 M NaCl, 0.2 % SDS (wt/vol), 0.5 % NP-40 (vol/vol), 100 mM Tris-HCl, 10 % ethanol (vol/vol), 10 % 2-propanol (vol/vol), pH 8.0.

Gel washing buffer 50 % acetonitrile (vol/vol) in 25 mM ammonium bicarbonate (freshly made).

Protein reduction solution 10 mM dithiothreitol in 25 mM ammonium bicarbonate (freshly made).

Protein alkylation solution 55 mM chloroacetamide in 25 mM ammonium bicarbonate (freshly made).

Protein digestion solution 12.5 μg/mL trypsin solution in 25 mM ammonium bicarbonate and 1 mM $CaCl_2$ (aliquot and store at -20 °C; minimize freeze/thaw cycles up to three times).

Peptide extraction solvent 5 % formic acid (vol/vol) /50 % acetonitrile (vol/vol) (can be stored at room temperature for 1 month).

Peptide elution buffer 10 mM sodium citrate, pH 3.0 (adjust pH with 5 M HCl in distilled H_2O).

Phosphate buffered saline (PBS) Make 1 L PBS with 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, 0.262 g of $NaH_2PO_4 \cdot H_2O$, adjust pH to 7.4 and sterilize by autoclaving or filtering through a 0.45 μm filter.

Buffers for liquid chromatography (LC) MS/MS

LC Buffer A 0.1 % formic acid (vol/vol) in LC-MS grade water (can be stored at room temperature for 1-4 months).

LC Buffer B 90 % acetonitrile (vol/vol) /0.1 % formic acid (vol/vol) in LC-MS grade water (can be stored at room temperature for 1-4 months).

LC-MS/MS equipment The Agilent 1100 HPLC system connected to the Q-TOF mass spectrometry is equipped with a chip cube (Agilent). LC gradient: Buffer B, 3 % to 40 % from 0 min to 20 min; 40 % to 90 % from 20 min to 23 min; 90 % to 3 % from 23 min to 24 min; 3 % from 24 min to 35 min. Alternatively, a longer gradient can be used for complex peptide mixtures. Other high resolution MS instruments, such as Orbitrap, can also be used for MS/MS analysis.

PROCEDURE

Cell culture and transfection

1 | Transfect twenty 10-cm Petri dishes of HEK293 cells.

(i) Grow HEK293 cells in DMEM supplemented with 10 % FBS, 100 unit/mL penicillin G and 100 μg/mL streptomycin in a 5 % CO_2 cell culture incubator in 95 % humidity atmosphere. Split HEK293 cells the day before transfection to twenty 10-cm Petri dishes. Adjust the splitting ratio so that the cells are approximately 50 % confluent on the day of transfection.

(ii) Replace media with pre-warmed media 1 ~ 3 h before transfection.

(iii) Transfect cells with 10 μg His_6-Ub plasmid for each Petri dish using the calcium phosphate transfection method[30].

Note: To identify the ubiquitination sites for a protein of interest such as RhoA, co-transfect 10 μg His_6-Ub plasmid with 10 μg pCMV BAP-RhoA, 5 μg pCMV Smurf1, 5 μg pCMV BirA and supply 1 mM biotin in the next step.

▲ CRITICAL STEP Ensure that the pH of the 2XHBS reagent is ~ 7.2. Check cells after transfection under microscope and make sure that calcium phosphate precipitates in the form of small particles are visible over the cells in the Petri dishes. For other type of cells, appropriate transfection methods must be used in order to reach satisfactory transfection efficiency.

(iv) Replace media with fresh, pre-warmed media between 3 and 24 h post transfection.

(v) (optional) In the evening of the 1st d post transfection, treat cells with 25 μM LLnL and incubate cells for 16 h to accumulate ubiquitinated proteins which are targeted for proteasome degradation.

Purification of ubiquitinated proteins

2 | Cell harvest and enrichment of ubiquitinated proteins.

(i) Wash cells twice with 5 mL ice-cold PBS per dish.

(ii) Detach cells with 5 mL ice-cold PBS per dish by pipetting up and down several times and transfer cells to four 50 mL conical tubes.

(iii) Centrifuge at 2000 ×*g* for 5 min at 4 °C in a swing-bucket centrifuge.

(iv) Decant PBS and transfer cells to a 1.6 mL Eppendorf tube with 1 mL ice-cold PBS for each 50 mL conical tube. Centrifuge at 2000 ×*g* for 5 min at 4 °C in a benchtop centrifuge and decant PBS.

■ Cells can be frozen at – 80 °C for several weeks.

(v) Lyse cells with 1 mL lysis buffer per tube.

▲ Make sure no DTT or EDTA are present in the lysis buffer as they can interfere with IMAC during the purification of ubiquitinated proteins.

(vi) Sonicate samples on ice at 40 % output in a Branson sonifier four times for 10 s. Alternatively, lyse cells by grinding in liquid nitrogen to maximally preserve protein ubiquitination[31].

▲ Avoid sample heating during sonication.

(vii) Centrifuge sample at 21,000 ×*g* for 10 min at 25 °C. It is recommended to save 5 μL of sample to run an anti-Ub western blotting after 2X dilution of the sample with distilled H_2O to reduce the concentration of salt and urea in the elution buffer because high concentration of salt and urea can adversely interfere protein mobility in SDS-PAGE. His_6-Ub has a slower mobility than the endogenous Ub due to the increase in molecular weight and charge. In an anti-Ub western blotting, two bands at about ~8.5 kDa and ~10 kDa are visible, which confirms the expression of His_6-Ub (~10 kDa band). Make sure that His_6-Ub is expressed in a low level compared with endogenous Ub.

(viii) Transfer 500 μL of a 50 % slurry of Ni-NTA agarose beads to a 1.6 mL Eppendorf tube.

(ix) Wash Ni-NTA agarose beads three times with 5 column bed volumes (CV) of lysis buffer.

(x) Transfer 500 μL Ni-NTA beads to a 15 mL conical tube.

(xi) Filter the lysate from step (vii) through a 0.45 μm filter and transfer the lysate to the 15 mL conical tube with Ni-NTA beads.

(xii) Incubate the lysate with Ni-NTA agarose beads on an end-over-end rotator at 25 °C for 2 h. Alternatively, run the lysate over Ni-NTA agarose beads in a 5″ chromatography column 3-5 times.

(xiii) Transfer the lysate to the beads to a 5″ chromatography column and let the lysate drain.

▲ Do not let the column dry.

(xiv) Wash the beads twice with 5 CVs of Ni-NTA washing buffer A (pH 8.0).

(xv) Wash the beads twice with 5 CVs of Ni-NTA washing buffer B (pH 6.5).

(xvi) Elute His_6-Ub and ubiquitinated proteins in five 250 μL fractions of Ni-NTA elution buffer and combine the eluate in a 1.6 mL Eppendorf tube. Save 5 μL of sample for anti-Ub western blotting analysis to ensure that the ubiquitinated proteins are eluted. Alternatively, measure protein concentration using the Bradford protein assay. The ubiquitinated proteins can also be eluted in 1XLDS sample buffer at elevated temperature, such as 100 °C for 5 min.

TALON resin has much lower nonspecific binding to proteins without hexahistidine tags than Ni-NTA resin[32]. Additionally, it binds strongly to hexahistidine-tagged proteins, in our case, free His_6-Ub and His_6-tagged ubiquitinated proteins. The downstream immunoaffinity purification of ubiquitin remnant peptides may be improved if TALON resin is used for the purification of ubiquitinated proteins. It should be noted that the washing step (xv) should be skipped if TALON resin is used for the purification of ubiquitinated proteins because Ni-NTA washing buffer B (pH 6.5) can elute ubiquitinated proteins from TALON resin.

Steps from (xvii) to (xxiv) are additional steps for bimolecular affinity purification of the ubiquitinated form for a protein of interest expressed with a biotin acceptor peptide tag, such as BAP-RhoA.

(xvii) Transfer 100 μL of a 50 % slurry of Neutravidin agarose beads to a 1.6 mL Eppendorf tube.

(xviii) Wash beads three times with 1 mL of Ni-NTA elution buffer.

(xix) Incubate the eluate from step 2 (xvi) with Neutravidin beads on an end-over-end rotator at 25 °C for 2 h.

(xx) Wash Neutravidin beads twice by incubating with 1 mL of Neutravidin washing buffer A for 10 min.

(xxi) Wash Neutravidin beads twice by incubating with 1 mL of Neutravidin washing buffer B for 10 min.

(xxii) Wash Neutravidin beads twice by incubating with 1 mL of 25 mM ammonium bicarbonate for 10 min.

(xxiii) Incubate beads with 50 μL 1XLDS sample buffer with 2.5 % β-mercaptoethanol at 100 °C for 5 min.

(xxiv) Repeat the above step twice and combine the eluate.

■ The eluate can be kept at – 80 °C for more than a week.

Coomassie stain, anti-RhoA and anti-Ub western blotting of the purified ubiquitinated RhoA are shown in Fig. 2. Three bands with strong signals in Coomassie stained gel are excised for in-gel trypsin digestion and the identification of lysine ubiquitination sites.

Trypsin digestion of ubiquitinated proteins

3 | In-gel trypsin digestion of ubiquitinated proteins

(i) Run SDS-PAGE and separate proteins on a NuPAGE 4-12 % Tris-Glycine mini-gel (1.5 mm in thickness).

▲ If the sample volume is too large, concentrate the sample with an Amicon filter (10 kDa molecular weight cutoff) or use multiple lanes of a mini-gel for each sample. Dilute the eluate 2X with distilled water before running SDS-PAGE to reduce the concentration of salt and urea. Otherwise, the sample runs irregularly due to the presence of high concentration of salts.

(ii) (optional) Stain gel with SimplyBlue™ SafeStain for 1 h and destain gel with distilled H_2O. Alternatively, gel can be stained by silver stain. This step is optional and is used to confirm that the ubiquitinated proteins are entered the polyacrylamide gel.

(iii) Cut the gel above 10 kDa into ~ 1 mm × 1 mm gel pieces.

(iv) Destain or wash gel pieces with gel washing buffer with constant shaking in a thermomixer (1000 RPM) at 25 °C.

▲ If the gel is stained with SimplyBlue, change gel washing buffer every 30 min until the gel is destained.

■ Gel pieces can be stored in gel washing buffer overnight at 4 °C.

(v) Dehydrate gel pieces with 200 μL of 100 % acetonitrile for 5 min and carefully remove acetonitrile using a gel loading tip.

(vi) Dry gel pieces completely by SpeedVac at 25 °C for 5 min.

(vii) Add 100 μL (or suitable amount) of protein reduction solution to wet and cover the gel pieces and incubate at 50 °C for 30 min with constant shaking in a thermomixer (1000 RPM).

(viii) Wash the gel pieces once with 25 mM ammonium bicarbonate.

(ix) Add 100 μL (or suitable amount) of protein alkylation solution to cover gel pieces and incubate at 25 °C for 45 min with constant shaking in a thermomixer (1000 RPM) in the dark.

(x) Remove alkylation solution from gel pieces using a gel loading tip.

(xi) Incubate gel pieces twice with gel washing buffer at 25 °C for 20 min with constant shaking in a thermomixer (1000 RPM) and carefully remove gel washing buffer using a gel loading tip.

(xii) Dehydrate gel pieces with 200 μL of 100 % acetonitrile at 25 °C for 5 min with constant shaking in a thermomixer (1000 RPM) and carefully remove acetonitrile using a gel loading tip.

(xiii) Dry gel pieces completely by SpeedVac at 25 °C for 5 min.

(xiv) Add three gel volumes of protein digestion solution to gel pieces and wait for 10 min to ensure that gel pieces are covered by protein digestion solution.

(xv) Perform digestion at 37 °C for 16 h with constant shaking in a thermomixer (600 RPM).

(xvi) Transfer digestion solution to a 15-mL conical tube.

(xvii) Extract tryptic peptides from the gel pieces with peptide extraction solution (200 μL) at 25 °C by constant shaking in a thermomixer (1000 RPM) for 30 min and sonicate in a water bath sonicator for 20 min.

(xviii) Repeat peptide extraction step once.

(xix) Extract peptides with 200 μL of 100 % acetonitrile once at 25 °C by constant shaking in a thermomixer (1000 RPM) for 5 min.

(xx) Combine the extracted peptide solution and lyophilize overnight.
Alternatively, any in-gel digestion protocol can be used here.

Conjugation of anti-GGεK antibody

Transfer 50 μL of 50 % Affi-gel 10 resin to a 1.6 mL Eppendorf tube.

4. Wash Affi-gel 10 resin twice with 1 mL of ice-cold 1 mM HCl.
5. Adjust pH of 500 μL of anti-GGεK antibody (> 1 mg/mL) to ~ 8.5 with 1 M HEPES (pH 9).
   ▲ The antibody used in this experiment is stored in PBS. If the antibody solution contains bovine serum albumin or free amines, such as Tris and Glycine, protein A/G should be used to immobilize antibody[33].
6. Add antibody solution to Affi-gel 10 resin and incubate at 25 °C for 4 h or at 4 °C overnight.

7. Wash beads with 500 μL of 1 M Tris-HCl (pH 8.0) and incubate with 500 μL of 1 M Tris-HCl solution on an end-over-end rotator at 25 °C for 2 h or at 4 °C overnight to block the remaining NHS active groups.
8. Briefly wash beads twice with 500 μL of 0.1 M glycine (pH 2.7) to remove the unbinding antibodies, and twice with 0.1 M Tris-HCl (pH 8.0) to neutralize pH.
9. Resuspend Affi-gel 10 resin with PBS containing 0.02 % sodium azide and store at 4 °C. Alternatively, protocols of coupling antibody to protein G beads can be used here[33].

   ■ The immobilized antibody can be kept at 4 °C for more than four weeks.

Immunoprecipitation of URPs

Dissolve lyophilized peptides in 300 μL of 150 mM NaCl, 50 mM Tris-HCl (pH 7.4) and 2 mM EDTA. Make sure that the pH of the solution is ~ 7.4.

10. Incubate the sample in boiling water for 10 min to inactivate residual trypsin. Cool sample to 4 °C on ice.
11. Incubate peptide sample with 20 μL of Affi-gel 10 resin coupled with anti-GGεK antibody for at least 4 h at 4 °C on an end-over-end rotator. Alternatively, the incubation can be extended to overnight at 4 °C.
12. Transfer beads to a Pierce micro-spin column. Wash beads three times with 0.5 mL 2XPBS and three times with 0.5 mL 1XPBS.
13. Elute URPs six times with 20 μL of 10 mM sodium citrate solution (pH 3.0). Alternatively, 0.1 % TFA can be used to elute the URPs, which can improve the yield of URPs up to 20 %.
14. Reduce the volume of the eluate to ~ 20 μl by SpeedVac at 25 °C.

■ PAUSE POINT Samples can be stored overnight at - 20 °C.

Mass spectrometry and data analysis

Centrifuge the sample(s) at 21,000 ×*g* in a benchtop centrifuge for 10 min at 4 °C.

15. Transfer 10 μL of sample to an LC-MS/MS sample vial and load 8 μL sample to LC-MS/MS (Q-TOF or Orbitrap) instruments with a 35 min or 1 h gradient.

    ▲ The length of the LC gradient depends on the complexity of the sample. Longer gradients are required for very complex samples. Repeated runs with an exclusion list during MS/MS fragmentation may be required to identify more URPs.

16. Perform database search of MS/MS spectra using Mascot[23], X!Tandem[21], OMSSA[22], Sequest[19], Spectrum Mill[20] or other database search program with appropriate protein sequence database, such as Swiss-Prot[34], NCBI[35], IPI[36] protein databases. Select fixed modification for cysteines (carbamidomethylation, the same as chloroacetamide modification), and variable modifications for lysines (Gly-Gly-) and methionines (oxidation). Set appropriate mass accuracy for precursor ions (20 p.p.m. for Q-TOF) and fragment ions (40 p.p.m. for Q-TOF). Note: It may be required to convert LC-MS/MS data to a format, such as mzXML and MGF, which a search program recognizes.
17. Manual validation of URPs. Make sure that no peptide with a sequence containing isobaric modification to the diglycine modification matches MS/MS spectra of URPs. Confirm all the major ions in the MS/MS spectra match the theoretical masses of fragment ions, such as *b*- and *y*-ions. Confirm the fragmentation of identified peptides fits the common rules, such as preferred cleavages at the proline residues.

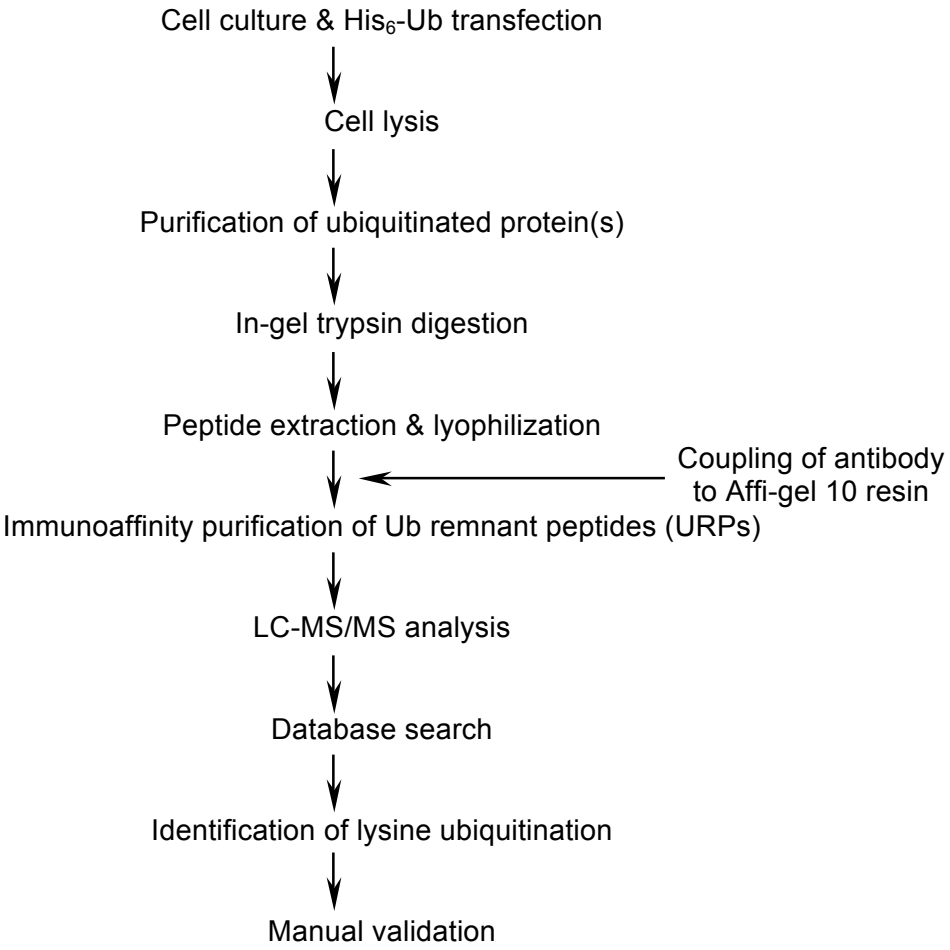

Figure 1. A flow chart for the protocol to identify lysine ubiquitination sites.

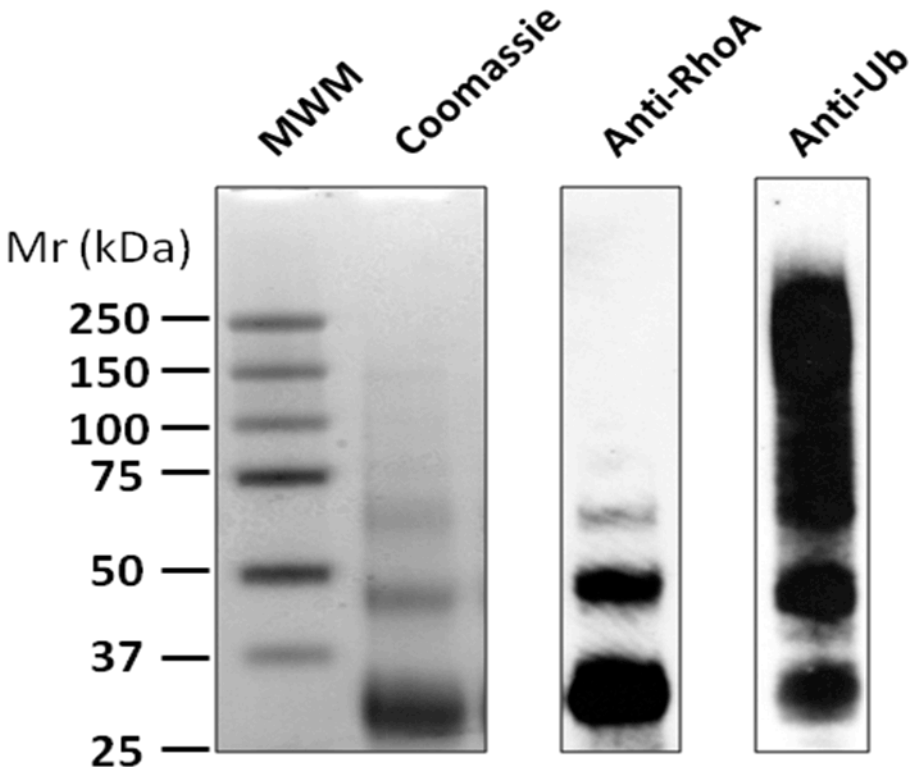

Figure 2. Bimolecular affinity purification under denaturing conditions obtained highly enriched ubiquitinated RhoA.

The ubiquitinated RhoA from HEK 293 cells, expressing BAP-RhoA, His_6-Ub, Smurf1, and BirA in the presence of exogenously supplied biotin and after a proteasome inhibitor treatment (LLnL: 25 μM for 16 h), was sequentially purified by Ni-NTA resin and Neutravidin beads under denaturing conditions. The purified proteins were stained by Coomassie brilliant blue and western blotted with anti-RhoA and anti-Ub antibodies. The higher molecular weight bands contained high anti-Ub immunoreactivity relative to anti-RhoA immunoreactivity, consistent with the high molecular weight species containing exclusively ubiquitinated RhoA. The three bands at lower molecular weights with strong signal in Coomassie stain were excised and digested by in-gel trypsinolysis followed by anti-diglycine antibody immunoaffinity purification and mass spectrometry analysis.

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