Published in final edited form as:
Methods Mol Biol. 2012 ; 893: 417–429. doi:10.1007/978-1-61779-885-6_26.

Analysis of Ubiquitinated Proteome by Quantitative Mass Spectrometry

Chan Hyun Na[1] and **Junmin Peng**[1,2,3]

[1]Department of Human Genetics, Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, GA, 30322

[2]Emory Proteomics Service Center, Emory University School of Medicine, Atlanta, GA, 30322

Abstract

Protein modification by ubiquitin is one of the most common posttranslational events in eukaryotic cells. Ubiquitinated proteins are destined to various fates such as proteasomal degradation, protein trafficking, DNA repair, and immune response. In the last decade, vast improvements of mass spectrometry make it feasible to analyze the minute amount of ubiquitinated components *in vivo*. When combined with quantitative strategies, such as stable isotope labeling with amino acids in cell culture (SILAC), it is capable of profiling ubiquitinated proteome under different experimental conditions. Here we describe a procedure to perform such a study, including differential protein labeling by the SILAC method, enrichment of ubiquitinated species, mass spectrometric analysis, and quality control to reduce false positives. The potential challenges and limitations of the procedure are also discussed.

Keywords

Ubiquitin; proteomics; mass spectrometry; SILAC

1. Introduction

Ubiquitination is one of the most versatile modifications in eukaryotic cells, evidenced by the presence of several hundreds of ubiquitin (Ub) enzymes in the proteome (~800 in human) (1), including Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), Ub ligase (E3), and deubiquitinating enzymes (DUBs) (2,3). Ubiquitin is a small protein of 76 amino acids, which modifies proteins on lysine residues or alternatively on the N-terminal amino group or even cysteine residues. In general, this modification results in the formation of an isopeptide bond between the C-terminal carboxyl group of ubiquitin and the ε-amino group of lysine residues on target proteins (Fig. 1A). Moreover, ubiqutin is attached to substrates in the forms of monomer or polymers, and the polyUb chains are assembled through all eight possible amino groups in ubiqutin (4,5). The function of ubiquitination is modulated by the length and topologies of the chains on the substrates (6). For instance, conventional

[3]Correspondence to Dr. Peng (jpeng@emory.edu).

[12]Quantitative comparison of protein samples from negative control (e.g. cells expressing untagged ubiquitin) with cells expressing tagged ubiquitin is an alternative method to differentiate contaminants and Ub-conjugates. While the contaminants are proposed to be isolated from both sources at equal efficiency, the real Ub-conjugates are only enriched from cells carrying the tagged ubiquitin. This strategy has been successfully used for mapping SUMOylated proteins (45) (*see* also **Chapter 25**).

[13]Development of specific antibodies to GG-tagged ubiquitinated peptides provides an independent method for enriching ubiquitinated species, reported by Cell Signaling Technology (www.cellsignal.com/services/ubiquitination.html) and another academic group (46). This method allows the enrichment of ubiquitinated peptides instead of Ub-conjugates.

K48 polyUb chains are degradation signals in the ubiquitin-proteasome system (2,3), whereas mono-ubiquitination and K63-linked polyUb chains function in proteasome-independent pathways, such as protein sorting, DNA repair, inflammation, and virus budding (7). More recently, unconventional polyUb linkages (e.g. K6, K11, K27, K29, and K33) are also suggested to direct the protein conjugates to proteasomal degradation (8,9). Linear ubiquitin chains and free ubiqutin polymers are newly recognized regulators in immune responses (10). In addition to ubiqutin, a family of ubiquitin-like proteins (e.g. SUMO, Nedd8, and ISG15) adopts analogous biochemical mechanisms to control cellular activities in eukaryotes (11), and this scenario of protein-protein modification is also revealed in prokaryotes by the discovery of protein pupylation (12).

The large-scale analysis of protein ubiquitination (13, 14, Wang, 2007 #1544) is greatly promoted by rapid development in mass spectrometry (MS)-based proteomics that enables the analysis of thousands proteins with sub-femtomolar sensitivity (15–17) (for summary *see* **Chapter 6**). The challenges for analyzing protein ubiquitination are largely due to low stoichiometry of ubiquitinated species in cells and further deubiquitination by persistent DUB activities during purification (18). To overcome these limitations, it is essential to enrich for ubiquitinated proteins prior to MS analysis. Epitope-tagged ubiquitin (e.g. FLAG, HA, Myc, His, and biotin) (13, 14, 19–21), ubiquitin binding antibody (22–24), and ubiquitin binding domains (25–29) have been used for this purpose. After purification, the Ub-conjugates are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the identities as well as the ubiquitination sites. The identification of ubiquitination sites is based on the di-glycine tag (GG, 114.043 Da) on a Ub-modified lysine residue after tryptic digestion (4, 30, 31), and missed tryptic cleavage at the modified site (32). The strategy also allows the identification of polyUb linkages in polyUb chains (Fig. 1B). Furthermore, quantitative MS strategies, such as stable isotope labeling with amino acids in cell culture (SILAC) (17) (see also **Chapters 11, 12**), have been successfully used for profiling ubiquitinated proteome in yeast (8, 33) and HeLa cells (21).

In this article, we describe a modified procedure to compare ubiquitinated proteome in *S. cerevesiae* strains by SILAC (Fig. 2A), including yeast differential labeling by light or heavy amino acids (i.e. Lys and Arg), isolation of ubiquitinated proteins, MS analysis, and removal of false positives that are co-purified with Ub-conjugates. As an example, we present the detailed protocols to compare two strains expressing wild type and K11R ubiquitin, respectively (8). Cautions and alternative methods are also discussed.

2. Materials

2.1 Yeast differential labeling by light or heavy amino acids

1. Two yeast strains: one strain expressing only wild type His-tagged ubiquitin, and the other expressing His-tagged K11R ubiquitin (8). Both *LYS2* and *ARG4* genes are deleted in these auxotrophic strains.
2. YPD media (Difco™, BD).
3. Synthetic media without amino acids: 0.7% Difco yeast nitrogen base (Difco™, BD), 2% dextrose (Sigma), adenine (20 mg/liter) (Sigma), and uracil (20 mg/liter) (Sigma).
4. Amino acid cocktail (no Lys/Arg, 100 X): L-tryptophan (2 g/liter), L-histidine (2 g/liter), L-methionine (2 g/liter), L-tyrosine (3 g/liter), L-leucine (10 g/liter), L-isoleucine (3 g/liter), L-phenylalanine (5 g/liter), L-glutamic acid (10 g/liter), L-aspartic acid (10 g/liter), L-valine (15 g/liter), L-threonine (20 g/liter), and L-serine (40 g/liter) (all from Sigma).

5. L-arginine and L-lysine (Sigma).
6. Heavy stable isotope labeled L-type amino acids: [$^{13}C_6$ $^{15}N_4$] arginine (+10.0083 Da) and [$^{13}C_6$ $^{15}N_2$] lysine (+8.0142 Da) (Cambridge isotope laboratories).
7. SILAC light media: mix the synthetic media, the amino acid cocktail and the regular L-arginine (12 mg/liter) and L-lysine (18 mg/liter).
8. SILAC heavy media: similar to the light media except equal molar concentration of the heavy stable isotope labeled L-arginine and L-lysine (*see* **Note** 1 and 2).
9. 10 mM sodium azide.

2.2 Enrichment of ubiquitinated proteins from yeast lysate

1. Lysis buffer: 10 mM Tris, pH 8.0, 0.1 M NaH_2PO_4, 8 M urea, 10 mM β-mercaptoethanol (all from Sigma).
2. 500 mM iodoacetamide (IAA) (Sigma): light sensitive.
3. BeadBeater and glass beads (BioSpec Products, 0.5 mm diameter).
4. Ni-NTA agarose (Qiagen).
5. Empty Poly-Prep chromatography column (2 ml, BioRad).
6. Washing buffer A: 10 mM Tris, pH 8.0, 0.1 M NaH_2PO_4, 8 M urea, 10 mM IAA.
7. Washing buffer B: 10 mM Tris, pH 6.3, 0.1 M NaH_2PO_4, 8 M urea, 10 mM IAA.
8. Elution buffer: 10 mM Tris, pH 4.5, 0.1 M NaH_2PO_4, 8 M urea, and 10 mM IAA.

2.3 Identification of enriched ubiquitinated proteins by MS

2.3.1. Multidimensional liquid chromatography-tandem mass spectrometry (LC/LC-MS/MS) and 1D SDS gel coupled with LC-MS/MS (GeLC-MS/MS)

1. IAA (Sigma): light sensitive.
2. 5 mM Tris-HCl, pH 7.5, 2 M urea (all from Sigma).
3. Trypsin (Promega).
4. Trifluoroacetic acid (TFA) (B&J).
5. SCX column (2.1 mm X 20 cm Polysulfoethyl A, Poly LC Inc., Columbia, MD).
6. SCX solvent A: 5 mM phosphate buffer and 25% acetonitrile (ACN), pH 3.0.
7. SCX solvent B: 5 mM phosphate buffer, 25% ACN, pH 3.0, and 350 mM KCl.
8. MS sample loading buffer: 6% acetic acid, 0.005% heptafluorobutyric acid, 5% ACN, and 0.1% TFA.
9. Acetone (Sigma).

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[1]The growth of yeast strains is dependent on the media used. The yeast in general grows faster in the YPD (doubling time: ~90 min) media than in the SILAC media (doubling time: ~140 min).

[2]The heavy stable isotope labeled amino acid used in the SILAC media varies in different labs (for other protocols *see* **Chapters 11, 12, 23**). Although the yeast standard synthetic media use higher concentration of L-lysine (30 mg/liter) and L-arginine (20 mg/liter), we found that the yeast strains could grow well in slighter lower concentration of lysine (18 mg/liter) and arginine (12 mg/liter) prior to OD_{600} of 0.8 (8). No significant heavy isotope-labeled Arg-Pro conversion was observed under this culture condition. We also noticed that in a current report (40), the concentration of lysine was 20 mg/liter but the arginine level was reduced to 5 mg/liter in the media. More recently, The SILAC strategy has also been applied to other model systems, including fly (41), and mouse (42). It will be possible to perform similar analysis of ubiquitinated proteins in higher organism.

10. SDS sample loading buffer: 10 mM Tris-HCl, pH 8.0, 4% Ficoll, 2% SDS, 0.02% bromophenol blue and 10 mM DTT (all from Sigma).
11. 6–12% gradient SDS-polyacrylamide gel.
12. Coomassie Blue G-250 staining buffer: 0.2% Brilliant Blue G250 (Sigma), 0.5% acetic acid (J.T. Baker) and 20% methanol (Sigma).
13. Methanol (Sigma).
14. Razor blade.
15. Gel washing buffer: 50% ACN (Sigma) and 50% 50 mM ammonium bicarbonate (ABC) (Sigma).
16. Extraction buffer: 5% formic acid (FA) (Fisher Scientific) and 50% ACN (Sigma).
17. 100 μm i.d. × 12 cm fused-silica capillary C_{18} column (Magic C18AQ; particle size, 5 μm; pore size, 200 Å; Michrom Bioresources, Auburn, CA).
18. Buffer A: 0.4% acetic acid (J.T. Baker), 0.005% heptafluorobutyric acid (Sigma), and 5% ACN (Sigma).
19. Buffer B: 0.4% acetic acid (J.T Baker), 0.005% heptafluorobutyric acid (Sigma), and 95% ACN (Sigma).
20. LTQ-Orbitrap mass spectrometer (Thermo Finnigan, San Jose, CA).
21. Sequest-Sorcerer algorithm (Sage-N-Research, Inc., San Jose, CA).
22. An in-house program for SILAC quantification analysis.

3. Methods

3.1 Yeast differential labeling by light or heavy amino acids

The cell strains are typically maintained on YPD plates and inoculated in the YPD media to obtain fresh culture before SILAC labeling. After labeling, the cells are mixed equally for further analysis.

1. Grow both yeast strains in the YPD media (~20 ml) until OD_{600} 0.7.
2. Harvest cells by centrifuging at 4000 × g at 4°C for 10 min.
3. Wash the cells with ice-cold water twice.
4. Transfer 10% of the cells to the SILAC media (2 liters of light or heavy media, ~1000 fold dilution, *see* **Note** 3).
5. When OD_{600} reaches 0.7, harvest cells by centrifuging at 4000 × g at 4°C for 10 min.
6. Wash with ice-cold 10 mM sodium azide twice.
7. Mix the differentially labeled cells in equal amount (the same ODML).

[3]In principle, five generations of yeast culture enable ~97% of labeling efficiency of proteins. We, however, grow the cells for 8–10 generations to ensure almost complete incorporation of heavy isotopes. As the purchased materials of heavy isotope labeled amino acids contain impurities, we recommend a testing experiment to monitor the labeling process during SILAC analysis. Because of the impurities in amino acids, SILAC labeling may generate unexpected variants in proteins that are not relevant to designed experimental conditions. Therefore, a replicating experiment is often performed simultaneously by reversing the labeling order of heavy and light isotopes.

3.2 Enrichment of ubiquitinated proteins from yeast lysate

1. Lyse the mixed yeast cells by glass beads in a BeadBeater using buffer/cells ratio of 3:1 (v/v).
2. Add glass beads up to 30% of total volume.
3. Vortex vigorously for 20 sec with 40 sec interval, and repeat the cycle 20 times.
4. Add IAA to 30 mM to quench β-mercaptoethanol and alkylate Cys residues (*see* **Note** 4 and 5).
5. Centrifuge the cells at 70,000 × g for 30 min at 4 °C.
6. Take the supernatant and measure the protein concentration (*see* **Note** 6).
7. Prepare the Ni-NTA agarose resin by pre-conditioning with lysis buffer.
8. Mix with the Ni-NTA agarose resin and incubated at room temperature for 30 min with gentle rotation (*see* **Note** 7).
9. Spin down the beads at 1000 × g for 1 min and remove the supernatant.
10. Resuspend the beads in Washing buffer A and transfer the beads to an empty column.
11. Wash the beads with 50 bed volumes of washing buffer A.
12. Wash the beads with 4 bed volumes of washing buffer B.
13. Elute with 4 bed volumes of elution buffer.
14. Make aliquots and freeze samples on dry ice and store at −80°C (*see* **Note** 8).
15. Examine the purity and yield of the enriched Ub-conjugates by a SDS gel and silver staining (Fig. 2B).

3.3 Identification of enriched ubiquitinated proteins by MS

3.3.1. Multidimensional liquid chromatography-tandem mass spectrometry (LC/LC-MS/MS)

1. Prepare the SCX column by pre-conditioning with 50% SCX solvent A with 50% SCX solvent B, and then equilibrating with SCX solvent A. Perform a testing run to examine the performance of the column.
2. Adjust the pH of eluted Ub-conjugate sample to 7.5.
3. Reduce urea concentration to 2 M by dialysis or dilution (*see* **Note** 9).

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[4]Iodoacetamide (IAA) is a commonly used Cys-alkylation reagent that inhibits most of DUB activities. At high temperature (e.g. heating in SDS gel loading buffer), IAA modifies a fraction of Lys residues twice to form a tag of 114.0429 Da, the same mass of a GG tag generated by tryptic digestion of ubiquitin (43). To avoid the introduction of the pseudo-GG peptides, it is important to keep samples at room temperature or lower and to use reduced level of IAA (e.g. 10 mM) (8). An alternative approach is to use chloroacetamide (CAA) to replace IAA (43), due to less reactivity of CAA than that of IAA.

[5]To examine if pseudo-GG peptides are present in digested, ubiquitinated samples, one may ask if the ubiquitin K48-pseudo-GG peptide is detectable using two criteria: (i) the pseudo peptide and the genuine K48-GG peptide have ~0.5 min retention time difference during a 30-min reverse phase HPLC gradient; (ii) the pseudo peptide but not the K48-GG peptide produce a strong MS/MS ion from neutral loss of water in collision-induced dissociation (8).

[6]The protein concentration may be critical to allow efficient binding of Ub-conjugates to the Ni-NTA resin. A concentration of at least 5 mg/ml is anticipated, while the lower concentration usually leads to low yield of Ub-conjugates.

[7]The ratio of Ni-NTA agarose to total protein amount needs optimization to reduce co-purified contaminants. A pilot purification of small scale is recommended for this step. For instance, various levels of proteins (e.g. 0.1–5 mg) are used to perform the purification on 10 μl of resin.

[8]It is possible that some active DUBs are co-purified with Ub-conjugates by binding to Ub chains, so that we still keep 10 mM IAA in the elution buffer and freeze the protein samples immediately after elution.

4. Digest the sample with trypsin (enzyme/substrates ratio of 1/20) overnight at 37°C.
5. Supplement the sample with ACN to 25% and acidify with TFA to 0.5%.
6. Load the sample on the SCX column and wash with 10 V of solvent A.
7. Elute with 5~20% gradient of solvent B in 70 min and 20~100% solvent B gradient in 10 min with flow rate at 0.2 ml/min.
8. Concentrate the eluted samples by drying.
9. Dissolve the dried peptide in the MS sample loading buffer.
10. Load the sample on LC-MS system (e.g. LTQ-Orbitrap) using an optimized protocol (34). Briefly, the peptides are loaded onto a C_{18} column and eluted during a 10–30% gradient. The eluted peptides are detected by Orbitrap (350–1500 m/z, 1,000,000 AGC target, 1,000 ms maximum ion time, resolution 60,000 fwhm) followed by ten data-dependent MS/MS scans in LTQ (2 m/z isolation width, 35% collision energy, 5,000 AGC target, 200 ms maximum ion time).
11. MS/MS spectra are searched against yeast database using the SEQUEST Sorcerer algorithm (version 2.0, SAGE-N) (35). Searching parameters included mass tolerance of precursor ions (±50 ppm) and product ion (±0.5 m/z), partially tryptic restriction, fixed modification of carboxyamidomethylated Cys (+57.0215 Da), dynamic modifications for oxidized Met (+15.9949), stable isotope labeled Lys (+8.0142) and Arg (+10.0083), five maximal modification sites and three maximal missed cleavages.
12. The target-decoy strategy is used to evaluate false discovery rate (36,37). In general, the protein false discovery rate is controlled less than 1% after filtering (*see* also **Chapter 29**).
13. The SILAC quantification and bioinformatics analysis are performed as previously reported (8, 38) (*see* also **Chapters 11, 12**).

3.3.2. One dimensional SDS gel (1 D gel) coupled with LC-MS/MS

1. Concentrate eluted Ub-conjugates by acetone precipitation by adding four times cold acetone (−20°C) of the sample volume, and then incubate at −20°C for 1 hr (*see* **Note** 10).
2. Centrifuge the precipitated proteins at 15,000 × g for 10 min.
3. Discard the supernatant and dry the pellet by careful evaporation.
4. Dissolve the pellet in the SDS sample loading buffer by heating at 65°C for 5 min.
5. Run the purified Ub-conjugates on 6–12% gradient gel and stain with Coomassie Blue (*see* **Chapter 3**).
6. Excise the gel lanes into multiple slices for in-gel digestion (39).
7. Cut the each gel slices into as small pieces as 1 mm^3.
8. Wash the gel with gel washing buffer briefly and then dehydrate the gel with 100% ACN.
9. Remove ACN and completely dry the gel pieces in speedvac for 15 min.

[9] 2 M urea is an optimized concentration to maintain the solubility of Ub-conjugates and not to severely inhibit trypsin activity (44).
[10] Sample loss may occur during the concentration step by acetone precipitation.

10. Cover the gel pieces with trypsin solution on ice (12.5 ng/μl trypsin in 50 mM ammonium bicarbonate) to allow the gel rehydrate.
11. Incubate the sample at 37°C overnight and extract the digested peptides.
12. Dry the sample in speedvac and then resuspend into MS sample loading buffer.
13. Analyze the peptides by LC-MS/MS.
14. Validate the identified Ub-conjugates by western blotting (*see* **Notes** 11–14).

Acknowledgments

This work was partially supported by the National Institutes of Health grants (RR025822, and NS055077), and the American Cancer Society grant (RSG-09-181).

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[11]Proteins that are not modified by ubiquitin are often co-purified during the enrichment of ubiquitinated proteome, raising a main issue in this type of profiling: how to remove these false positives. The first effective approach is to reduce the contaminants during purification by introducing highly stringent buffers (e.g. 8 M urea), the denaturing condition is useful for not only minimizing contaminants but also inhibiting DUB activities. Only two types of tags (6xHis and biotin) on ubiquitin are suitable for the denatured conditions (4, 20). Even under such a stringent condition, the contaminants may still contribute to ~50% of the identified ubiquitinated proteome, since many contaminants exist in a minute amount but are still detectable by highly sensitive mass spectrometry (32). Moreover, the status of protein ubiquitination may be verified by traditional western blotting, virtual western blotting images reconstructed based on 1D gel and LC-MS/MS (32), and the ubiquitinated sites. The caveats of analyzing ubiquitinated lysine residues are discussed in another review paper (18).

[14]In addition to SILAC, one may use iTRAQ (*see* **Chapter 7**) or TMT method (*see* **Chapter 8**) to perform multiplex comparison of ubiquitinated species. The iTRAQ method uses up to eight isobaric tags to label primary amine groups of peptides. During MS/MS analysis, the tags are fragmented into report ions to represent the intensity of the corresponding peptides/proteins in the initial samples (47). Furthermore, targeted proteomics technique termed selective reaction monitoring (SRM) is usually used to quantify the level of known proteins or modifications (*see* **Chapter 16**), in this case of ubiquitination, to measure the abundance of different polyUb linkages (8, 48).

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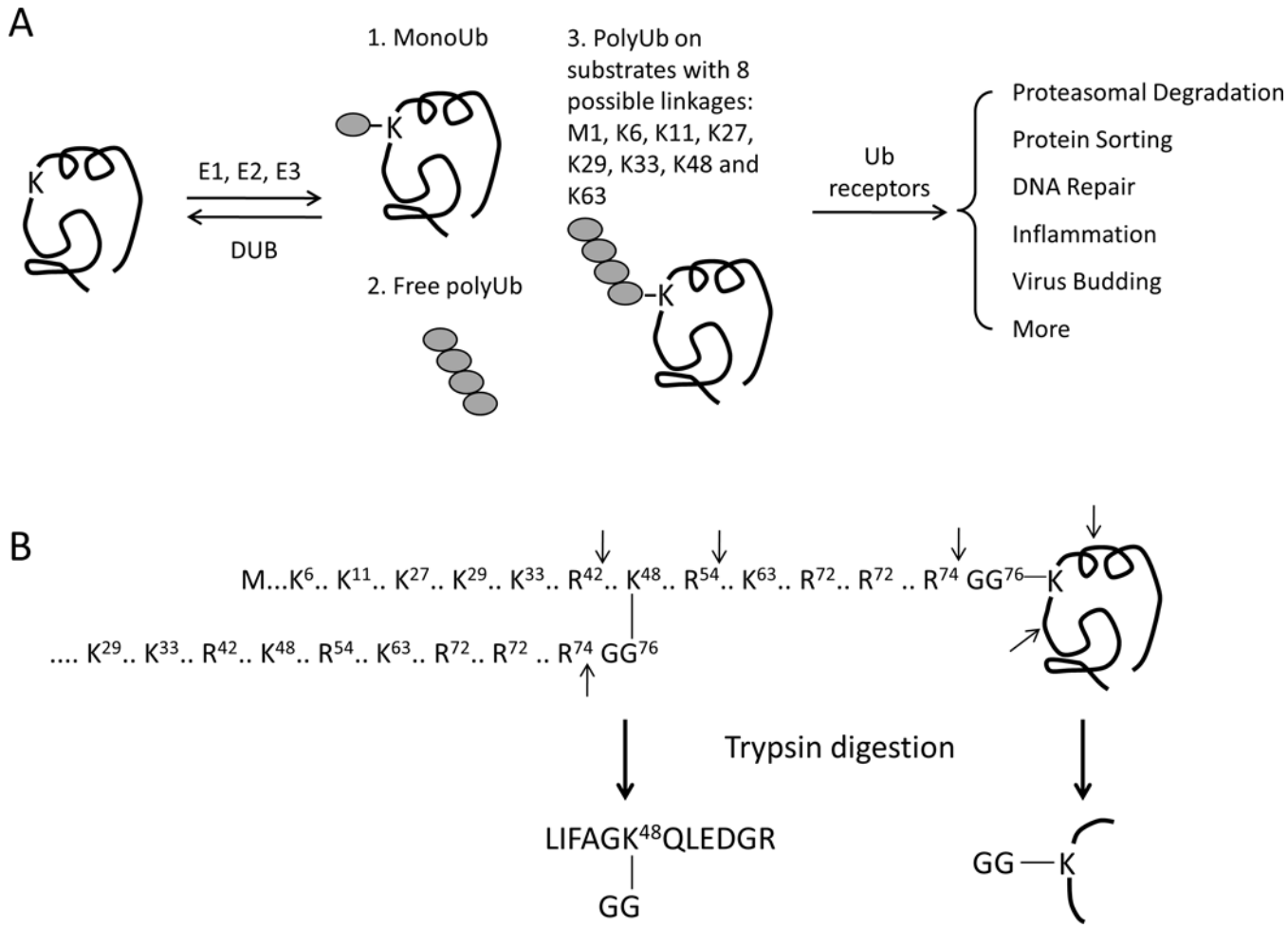

Fig. 1. The chemistry and function of protein ubiquitination

(A) Protein ubiquitination is carried out by an enzymatic cascade by E1, E2 and E3 enzymes. This ubiquitination reaction is reversible by the activity of DUB. The ubiquitination reactions result in the formation of monoubiquitinated proteins, free ubiquitin polymers and polyubiquitinated proteins with diverse chain topologies. These ubiquitinated species are recognized by a range of Ub receptors that contain Ub-binding domains, leading to different functional consequences.

(B) Trypsin digestion of ubiquitinated proteins generates a small tag of two amino acids (Gly-Gly) on the ubiquitinated sites in the sequences of modified proteins and ubiquitin itself. These signature peptides enable the identification of these sites by mass spectrometry.

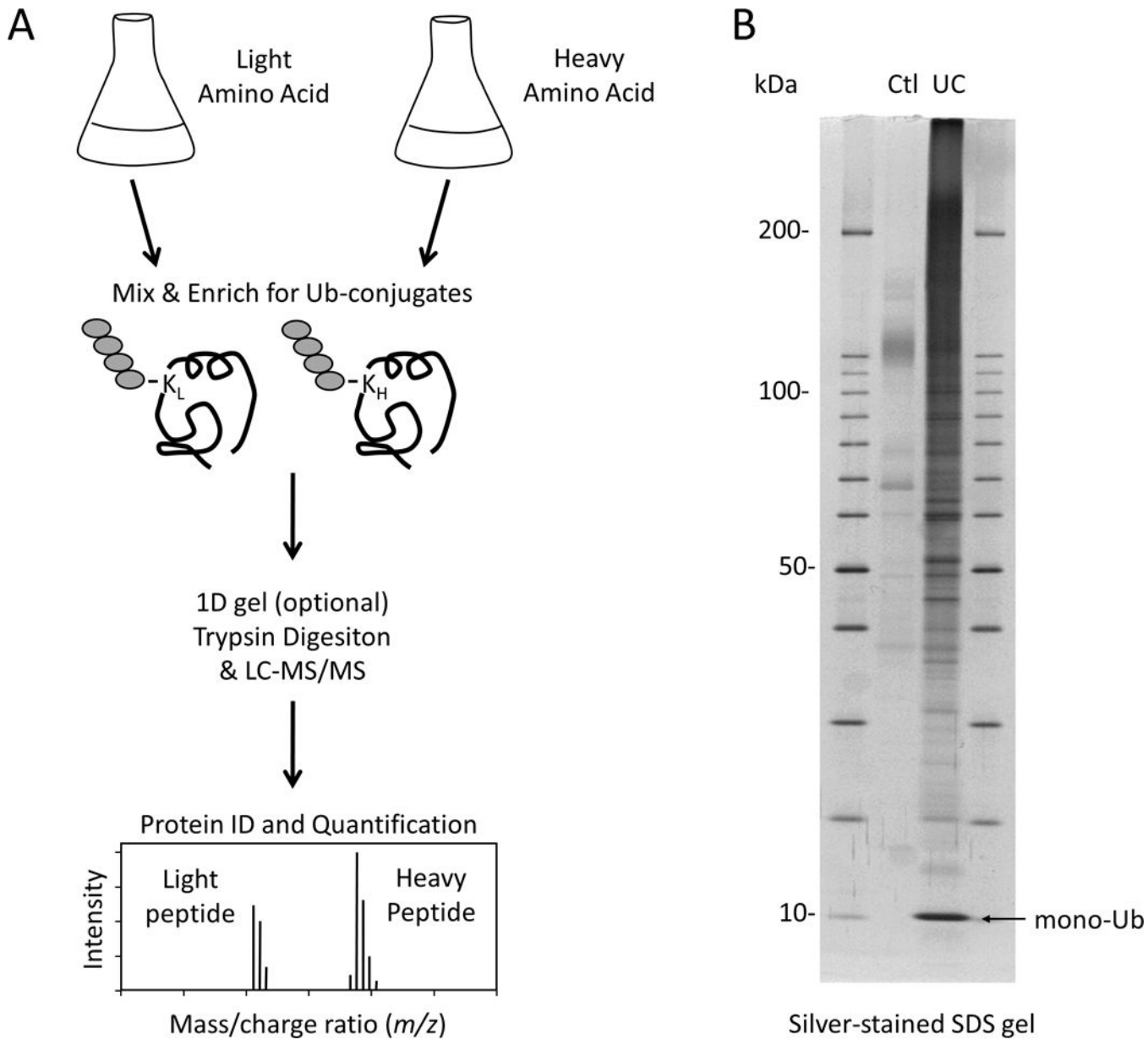

Fig. 2. Quantification of ubiquitinated proteome by the SILAC strategy

(A) The scheme of SILAC. Two yeast strains are differentially labeled and mixed. Ubiquitinated proteins are then isolated and analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Proteins are identified by the MS/MS spectra and are quantified by the corresponding ion peaks in the MS spectra.

(B) A stained SDS gel example to show the purity of Ub-conjugates (UC) from cells expressing His-tagged ubiquitin. The control cells (Ctl) expresses native ubiquitin without tag (modified from ref. 4, with permission from Nature Publishing Group).