BMB
reports

Mini Review

Evaluation of proteomic strategies for analyzing ubiquitinated proteins

*Junmin Peng**

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

Ubiquitin is an essential, highly-conserved small regulatory protein in eukaryotic cells. It covalently modifies a wide variety of targeted proteins in the forms of monomer and polymers, altering the conformation and binding properties of the proteins and thus regulating proteasomal delivery, protein activities and localization. Mass spectrometry has emerged as an indispensable tool for in-depth characterization of protein ubiquitination. Ubiquitinated proteins in cell lysates are usually enriched by affinity chromatography and subsequently analyzed by mass spectrometry for identification and quantification. Ubiquitin-conjugated amino acid residues can be determined by unique mass shift caused by the modification. Moreover, the complex structure of polyubiquitin chains on substrates can be dissected by bottom-up and middle-down mass spectrometric approaches, revealing potential novel functions of polyubiquitin linkages. Here I review the advances and caveats of these strategies, emphasizing caution in the validation of ubiquitinated proteins and in the interpretation of raw data. [BMB reports 2008; 41(3): 177-183]

INTRODUCTION

In almost all cellular events, cells generate and relay versatile signals through post-translational protein modifications, such as phosphorylation and ubiquitination. Ubiquitin (Ub) is a highly conserved protein of 76 amino acids, which modifies substrates by a cascade of enzymatic reactions through Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub ligase (E3) (1-3). As a result, the carboxyl group of Gly76 of ubiquitin forms an isopeptide bond with, most typically, the ε-amine group of a lysine residue within substrates. In some cases, the N-terminal amine groups (4) or even Cys residues (5) in proteins are used as alternative docking sites for ubiquitination. In addition to monoubiquitination, polyubiquitin (polyUb) chains with various lengths are often assembled by successive attachment of ubiquitin to any of the seven lysine residues of previously conjugated ubiquitin (6). On the other side, protein ubiquitination is a reversible process through the action of a large number of deubiquitinating enzymes (DUBs) (7, 8). In the human proteome, whereas only a few E1 enzymes and ~50 different E2s are present, more than 500 E3 ligases and ~150 DUBs are proposed to modify thousands of substrates under pathophysiological conditions (9).

A central question in ubiquitination is how to generate, recognize and eliminate ubiquitin signals under certain cellular conditions. The high specificity is primarily achieved by substrate recognition by E3 ligases, interaction between ubiquitin moieties (monoUb or polyUb) and Ub receptors (10), and possibly ubiquitin removal by specific DUBs (8), as well as subcellular localization. The Ub signal is recognized and transmitted through Ub-binding proteins. At least 15 families of ubiquitin-binding domains (UBD) have been characterized in the context of different sequences of at least 250 human proteins (11, 12), suggesting of the capacity and complexity of ubiquitin-based network in cells.

Large-scale characterization of endogenous ubiquitinated species has been virtually impossible, until recent development of mass spectrometry-based sequencing technologies with femtomolar to even subfemtomolar sensitivity (13, 14). The level of ubiquitinated proteins is usually too low to be detected without enrichment. In recent years, a number of strategies have been developed to isolate Ub-conjugates, including ubiquitin antibodies, ubiquitin-binding proteins, and epitope-tagged ubiquitin (e.g., FLAG, HA, Myc, His, and biotin). Ubiquitination sites in modified proteins are identifiable by tandem mass spectrometry according to related mass shift (6, 15, 16). Moreover, activity-based proteomics methods have been used to identify novel DUBs and to investigate mechanisms regulating the DUB activity (17, 18). The strategies have been applied to multiple organisms and greatly expanded the scope of protein ubiquitination. As this topic has been extensively reviewed (19-22), I update recent implementation in the methods, discuss the advances and caveats in the approaches and underscore caution in data analysis.

*Corresponding author. Tel: 404-712-8510; Fax: 404-727-3728; E-mail: jpeng@genetics.emory.edu

Received 12 March 2008

Keywords: Affinity chromatography, Mass spectrometry, Middle-down, Proteomics, Ubiquitin

Identification, validation and quantification of ubiquitinated proteome

In general, endogenous ubiquitinated proteins in cells have been isolated from cell lysate by affinity chromatography, and then analyzed by selected mass spectrometric platforms. The most reliable method is to purify ubiquitinated substrates under denaturing conditions that inhibit the activities of DUBs and reduce protein binding. The first large-scale analysis was performed using a yeast strain expressing only His-tagged ubiquitin, revealing ~1,000 ubiquitinated candidates (6, 23). To further increase the purity of enrichment, a tandem tag containing double 6xHis motif and a biotin domain was designed to isolate yeast ubiquitinated proteins in two denaturing steps, leading to the identification of ~250 candidates (24). More recently, a transgenic mouse line expressing 8xHis-FLAG-Ub was generated for capturing and identifying ~120 Ub-modified candidates in the heart (25). However, the tag-based approach is not compatible with biological samples without genetic manipulation (e.g. clinical specimen). To alleviate this problem, ubiquitin antibodies (26, 27) and Ub-binding proteins (28-31) were adapted for affinity purification with some success. The combination of Ub-binding proteins and His tag was also reported for isolating ubiquitinated components in yeast (32).

One of the challenges in purifying native Ub-conjugates is to restrain the cleavage activities of DUBs during purification, because DUBs are associated with ubiquitin molecules and it is difficult to fully inactivate DUBs even under denaturing conditions (e.g. 8 M urea). It is critical to optimize experimental conditions, including the amount of resins and input, and the conditions for loading, washing and eluting. The parameters are adjusted to balance the purity and yield of Ub-conjugates, and to minimize co-purified contaminants. In addition, it is important to carry out the experiments rapidly and to freeze purified samples intermediately.

No matter which types of affinity matrix are selected for purification, a difficult and recurring problem is that some contaminants that are not conjugated by ubiquitin are co-purified in the process and also identified by mass spectrometry. Although the concentration of contaminants may be reduced to low level, the number of contaminants could be large, especially when mass spectrometry is highly sensitive to detect minute amount of proteins. Theoretically, there are two forms of contaminants found in the procedure of affinity chromatography (Fig. 1A): (i) matrix-associated contaminants, such as His-rich proteins during nickel purification (6); (ii) Ub-associated contaminants that interact with the moieties of Ub-conjugates bound to the affinity matrix. The matrix-associated contaminants could be determined by performing negative control experiments (e.g. cell lysate without tagged ubiquitin) and then removed from the list of ubiquitinated candidates. In contrast, the Ub-associated contaminants are more challenging to distinguish. This form of contaminants is greatly reduced under denaturing conditions in which proteins are unfolded to limit protein-protein interactions, but the denaturing conditions cannot completely eliminate Ub-associated contaminants. Therefore, it is essential to validate whether MS-identified proteins are genuine Ub-conjugates or co-isolated contaminants, prior to subsequent functional analysis that often demands considerable efforts.

The status of ubiquitination can be validated by direct mapping of ubiquitination sites through MS/MS or by Western blotting analysis of individual proteins (6). However, complete mapping of modification sites requires almost 100% coverage of peptides "sequenced" by MS/MS, which is not possible in "shotgun" proteomics. In the yeast large-scale analysis, only a

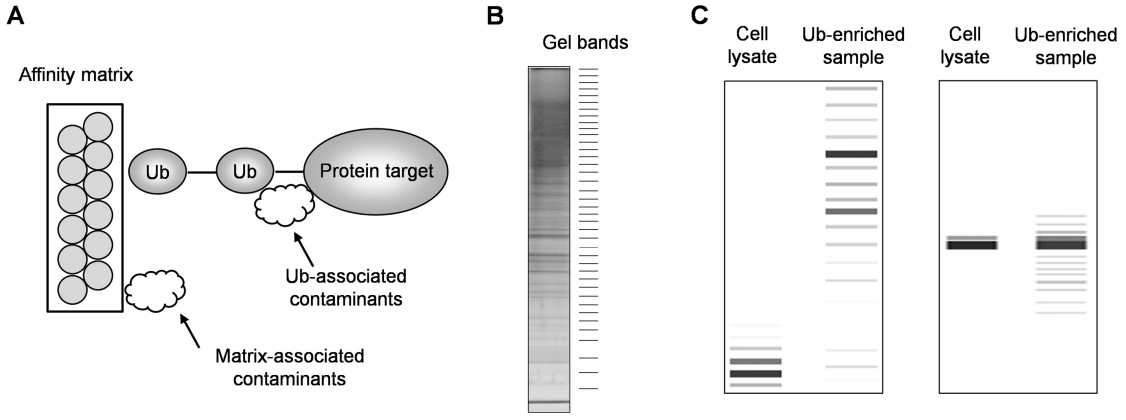

Fig. 1. Enrichment and validation strategies for ubiquitinated proteins. (A) Two types of contaminated proteins that are co-purified with Ub-conjugates during affinity chromatography. Whereas one type of contaminants binds to affinity matrix nonspecifically, the other type of impurities is resulted from specific association with Ub-conjugates. (B) The analysis of a purified Ub-conjugate sample by geLC-MS/MS. (C) Virtual Western blotting images of a genuine Ub-conjugate and a co-isolated unmodified protein. The images were reconstituted from the data of geLC-MS/MS. The relative abundance of proteins in every gel band was accessed by spectral counts and indicated by the darkness of protein bands.

small fraction of ubiquitination sites were identified, matching to less than 10% of identified proteins. During Western blotting, immunoprecipitated Ub-conjugates are confirmed by two principles: (i) ubiquitination causes dramatic increase in apparent molecular weight (MW), as Ub-conjugates display an increase of approximately 8 kDa after mono-ubiquitination and an even more precipitous increase after poly-Ub events; (ii) ubiquitination often generates heterogeneous modified substrates that display as a ladder on the Western blot. To minimize co-immunoprecipitation of other ubiquitinated proteins, highly stringent condition (e.g. SDS) should be included. However, immunoprecipitation and Western blotting become impractical for the validation of large-scale studies in which hundreds to thousands of candidates are identified.

More recently, the principles of Western blotting have been used to develop a simple strategy for large-scale confirmation of ubiquitination by computing virtual Western blot for every identified protein (33). The protein sample was analyzed by one-dimensional SDS gel followed by liquid chromatography-tandem mass spectrometry (GeLC-MS/MS, Fig. 1B). Experimental molecular weight of each Ub-conjugate candidate was reconstructed from spectral count intensity and distribution in the gel using a Gaussian curve fitting approach, whereas the unmodified counterpart in cell lysate was also analyzed. Comparison of images before and after affinity purification enabled the discrimination of Ub-conjugates from contaminants (Fig. 1C). The optimized filtering cutoff incorporated the mass of ubiquitin and experimental variances. Surprisingly, only ~30% of > 1,000 identified proteins in the Ub-conjugate sample were accepted after the filtering, despite that the purification was carried out under denaturing condition. Compared with the validation method based on identification of ubiquitinated sites, ~95% of proteins with modification sites displayed an obvious shift on the virtual Western blots, indicating the consistency of both methods. However, the virtual Western blot strategy has certain bias toward polyubiquitinated proteins, and some mono-ubiquitinated species may not be affirmed due to limited mass resolution of the SDS gel. Nevertheless, the results emphasized the importance of validation to control data quality, and suggested that false discovery rate in previously published datasets of ubiquitinated proteome may be underestimated.

Beyond the identification of Ub-conjugates, the availability of such technologies makes it possible to compare ubiquitinated proteome under different treatments. For example, numerous membrane-associated ubiquitin substrates in endoplasmic reticulum-associated degradation were identified in this manner (34). In the experiments, a yeast strain that lacks two central genes in the pathway was compared to isogenic wild type strain by subtractive proteomics, uncovering a total of 83 potential substrates involved. Recently, the combination of stable-isotope labeling and Ub-affinity isolation provides a better tool for quantitative analysis. The method was used to illustrate specific ubiquitinated substrates that are recognized by a ubiquitin receptor Rpn10 (S5a) in ubiquitin-proteasome system in yeast (35).

Determination of ubiquitination sites by tandem mass spectrometry

Ubiquitin-modified specific residues can be mapped by tandem mass spectrometry after trypsin digestion, as trypsin trims the attached ubiquitin molecule to a di-glycine remnant (GG with monoisotopic mass of 114.043 Da, Fig. 2A) (6, 15, 16). In

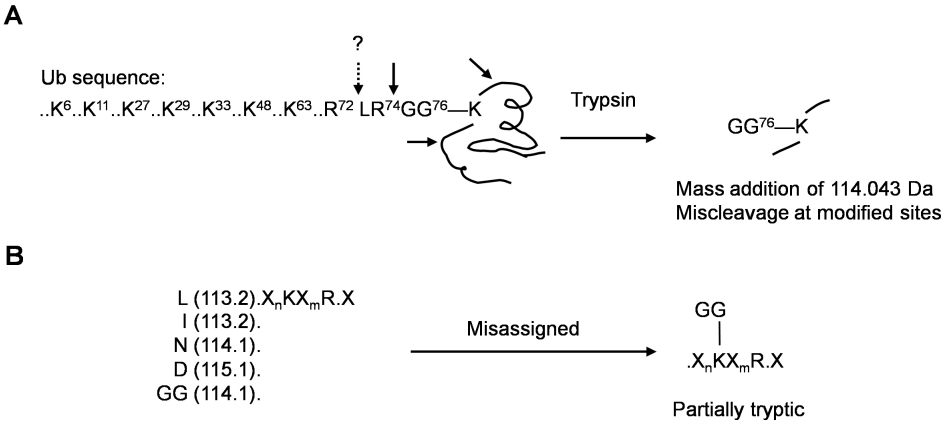

Fig. 2. The scheme for analyzing ubiquitinated residues in proteins. (A) Trypsin specifically cleaves peptide bonds at the C-termini of Arg or Lys unless followed by a Pro residue. During tryptic digestion, ubiquitin is trimmed to a small -GG tag that adds unique mass to modified residues. Moreover, the modified Lys residues are not accessible to trypsin proteolysis, leading to a miscleavage in peptides. It is questionable if the miscleavage at Lys72 in ubiquitin occurs. (B) The list of flanking amino acids that could be misassigned as -GG tag. Whereas "X" represents any amino acids, "n" and "m" indicates certain number of residues in the peptides. The cleavage positions are shown in dots. The number of "n" is usually small, and no major breakage occurs in the region during fragmentation. Thus, the computer software cannot distinguish the listed possibilities.

addition, the GG peptides contain a missed tryptic cleavage at the modified lysine sites. Based on the characteristics, ubiquitin modification sites are identified by unique MS/MS spectra of the GG peptides during database searching. But some ubiquitin-like proteins (e.g. Nedd8 and ISG15) also generate the same GG peptides by trypsin digestion, and therefore it is prerequisite to separate those proteins from ubiquitinated samples by pre-fractionation. Moreover, chemical modification at N-termini may expedite the identification of GG-modified peptides (36), and alternative digestion with Glu C was also tested to map ubiquitination sites (37).

During database matching of MS/MS spectra, the number of ubiquitination sites identified may be inflated by false assignment. For instance, peptides can be mismatched as ubiquitinated if containing internal Lys residue(s) and adjacent N- or C-terminal residues that have similar mass to GG modification (Fig. 2B). The residues consist of GG themselves, Leu, Ile, Asn and Asp residues, provided that the mass of precursor and product ions is still within selected mass tolerance range during database search. This type of mismatches could be caught by determining tryptic state of the peptides, as the mismatched peptides have only one tryptic end (i.e. partially tryptic). Filtering with high mass accuracy is also effective to find the error caused by the flanking Leu, Ile or Asp residues (Fig. 2B). In addition, ubiquitinated peptides could be simply generated by random mismatching, especially when a large number of MS/MS spectra are used to search again a small database.

Although it was reported that miscleavage at Arg74 in the ubiquitin sequence could generate a longer tag (LRGG) (37), we have rarely identified reliable ubiquitinated peptides carrying this large tag in a number of systematic analyses of Ub-conjugates. Indeed, Arg74 residue is readily cleaved at high efficiency under native or denaturing condition (36, 38). Thus, the LRGG modified peptide matches should be validated by manual examination with caution.

In some cases, when a mono-ubiquitinated peptide contains several lysine residues, computer algorithms may assign the GG modification to a wrong Lys residue, such as the C-terminal lysine, because incomplete fragmentation cannot generate sufficient product ions for unambiguous assignment. This led to a questionable conclusion that trypsin is capable of digesting at the C-terminus of GG-modified Lys sites (39). To test this idea, an experiment was performed to cleave the reported synthetic GG-peptide, and the result was negative, suggesting that the C-terminal GG-tagged lysine is likely to be an artifact during database search (33).

Analysis of polyUb topologies by bottom-up and middle-down approaches

The strategy for mapping ubiquitination sites is equally applied to determine Ub lysine residues involved in polyUb assembly. Systematic analysis of yeast Ub-conjugates uncovered startling complexity of polyUb chains: all seven lysine residues can be used for the formation of Ub-Ub linkages (6). Further analysis of ubiquitination proteins in mammalian cells also confirmed the complexity of chain linkages (38, 40). Indeed, signaling outcome of ubiquitination may rely on the linkage of modified ubiquitin chains (10). Conventional K48-polyUb chains are the principle signal for targeting substrates for proteasomal degradation, whereas polyUb chains linked through K63 have non-proteolytic role in a wide range of other processes. The physiological roles of other linkages are poorly characterized. Development of novel MS tools to analyze polyUb structures is crucial to the investigation of their functions.

To quantify polyUb linkages on substrates, stable isotope labeling has been employed by synthesizing all seven internal standards corresponding to ubiquitinated GG peptides (38, 41). The labeled standards for seven linkages are spiked into a protein mixture that is then digested with trypsin to produce native GG peptides from polyUb chains. The native peptides and internal standards are co-eluted during chromatography, but separated in a mass spectrometer. The instrument is set to the mode of selected reaction monitoring (SRM, also termed MRM, multiple reaction monitoring) in which native peptides and internal standards are selected for fragmentation to generate product ions. Pairs of product ions are monitored and their ratios allowed for measurement of linkages in the original sample (Fig. 3A). Using this method, linkages of total Ub-conjugates were measured, revealing that the ratio of K6:K11:K27:K29:K33:K48:K63 linkages was 11%:35%:7%:4%:1%:30%:11% in yeast (38). Surprisingly, unconventional linkages, in particular K11 linkage, are highly abundant in cells in spite of little knowledge of the function. The Gygi's group used the approach to quantify polyUb topologies attached to EGF receptor (40) and cylin B1 (41). EGF receptor is modified with different lengths and linkages including ~49% mono-Ub, 40% K63, 6% K48, 3% K11 and 2% K29 linkages, consistent with the role in protein sorting. Cyclin B1 is conjugated by mono-Ub and short polymers linked by K11, K48 and K63 during *in vitro* ubiquitination, and unexpectedly the modified cyclin B1 is effectively degraded by proteasome *in vitro*. Once established, the bottom-up quantitative approach has been widely applied to many other cases (31, 42-45).

The MS strategy circumvents the requirement of specific antibodies to detect proteins in complex mixtures. However, in the above protocol, the labeled synthetic GG-peptides are added into Ub-conjugate samples after affinity purification, and experimental variation could be generated during the isolation step. If metabolically labeled cells are used as internal standard and added in during the first step of harvest, quantitative errors are greatly reduced (46) (Fig. 3A, Xu and Peng, unpublished data).

Since seven lysine residues and N-terminal amine group in ubiquitin could be used for polyUb chain elongation, the chain structure may be much more diverse than previously anticipated, with the complexity rising exponentially with the length of the chains. For example, Ub dimer would display

eight forms, whereas Ub trimer would display two classes of configurations: branched chains with 64 types (i.e. 8 × 8 possibilities) (Fig. 3B) and forked chains with 28 types (i.e. 8 × 7/2 possibilities) (Fig. 3C). In the bottom-up mass spectrometric analysis described above, some structure information is lost during trypsin digestion. For instance, in a tri-Ub sample with both K29 and K48 linkages, it is difficult to know if two Ub molecules are attached to different Ub molecules, or to the same Ub molecule as in a forked chain. Indeed, forked polyUb chains have been identified in multiple MS analyses (6, 24, 47).

To address this problem, a "middle-down" technology (48, 49) was utilized to partially digest ubiquitin polymers under native condition, in which trypsin cleaves ubiquitin at only one site (R74) in the C-terminal tail, as other tryptic sites in the sequence are generally inaccessible owing to compact ubiquitin folding (36). Therefore, it would be simple to monitor how many GG-tags co-modify a single Ub molecule by mass shift. In the case of a K48-linked Ub dimer (Fig. 3B), it is excised into equal amount of two large peptides, UbR74 (amino acid M1 to R74) and UbR74-1GG-K48 (UbR74 with one -GG tag at the K48 site). A Ub trimer with forked K29 and K48 linkages (Fig. 3C) is digested into two large fragments (UbR74: UbR74-2GG-K29K48) with 2:1 ratio. The molar ratio between UbR74 and UbR74-GG indicates the length of homogeneous polyUb chains (i.e. 1:1 for dimer, 1:2 for trimer, 1:3 for tetramer, and so on). Furthermore, lysine residues in ubiquitin used for chain linkages are detectable by MS/MS and MS/MS/MS of large GG-tagged Ub fragments (50). With the middle-down strategy, it is possible to directly monitor the change of forked polyUb chains in biological samples.

Conclusions

In recent years, analytical approaches to protein ubiquitination

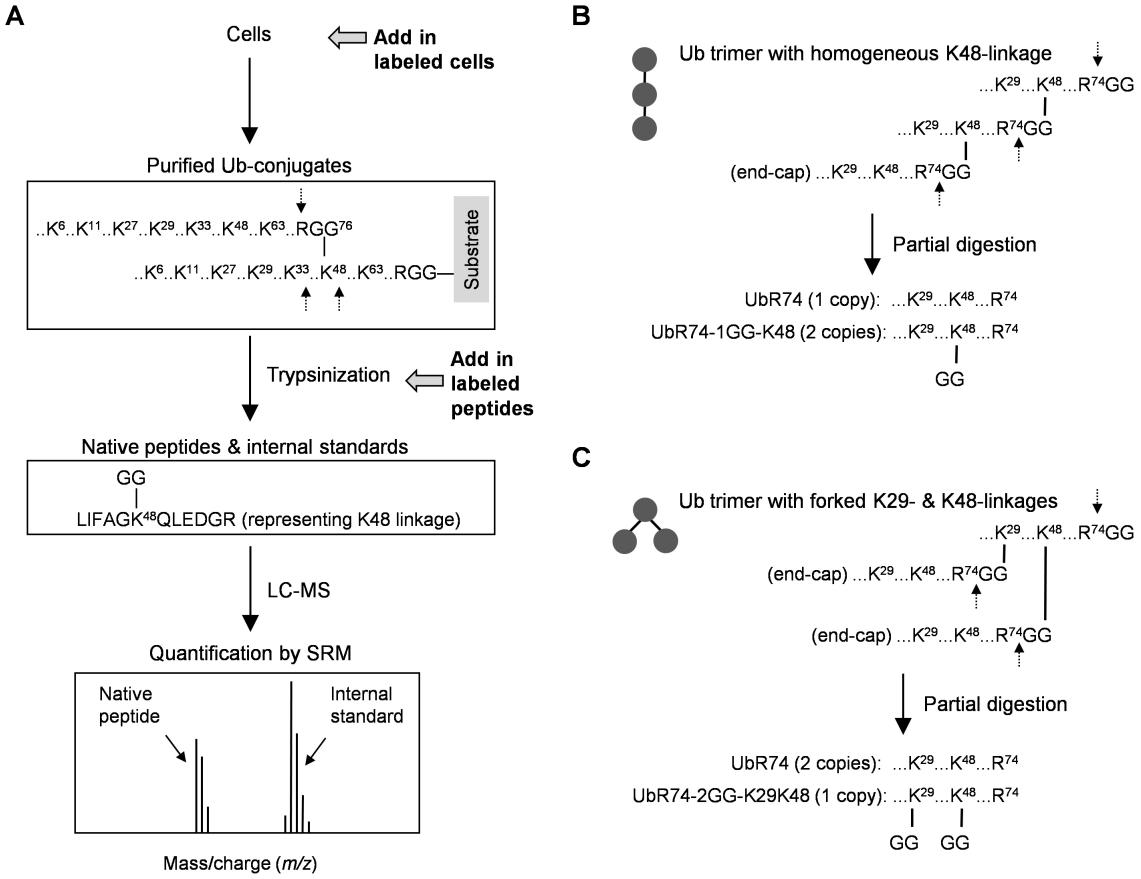

Fig. 3. In-depth analysis of polyUb chains by bottom-up and middle-down mass spectrometry. (A) During the bottom-up strategy, the timing that internal standards are added has impact on experimental variation. If peptide standards are used, they are spiked in during trypsin digestion. Alternatively, labeled cells are mixed in when harvesting cell culture, which greatly minimizes the variation of sample processing. (B) In the middle-down analysis, native ubiquitin is digested only at a single R74 site by trypsin, resulting in the production of an almost full-length ubiquitin (UbR74) and linkage-specific GG-tagged UbR74, such as UbR74-1GG-K48 (one GG tag through K48 residue). The abundance ratio of the two large Ub fragments can be predicted from the structure. (C) Anticipated products during partial digestion of a forked Ub trimer.

have been rapidly developed in parallel with the evolution of modern mass spectrometry. The large-scale proteomics analyses of ubiquitination have expanded the ubiquitin territory, including the scope of substrates and the diversity of polyUb chains. Different polyUb chain linkages may play important regulatory roles in synthesis by E1/E2/E3, recognition by Ub-binding proteins, and disassembly by DUBs. At current stage, the methodology in mammal is not as mature as that in yeast. Further refinement of Ub-affinity strategy is required for routine high-throughput analysis of ubiquitinated proteome. Moreover, seamless combination with quantitative MS approaches will provide powerful tools to profile dynamics of ubiquitinated proteome under different cellular conditions and genetic backgrounds. In addition, many of the strategies discussed will be adapted to analyze protein substrates modified by numerous ubiquitin-like proteins (51).

Acknowledgements
This work was supported in part by NIH grants CA126222 and AG025688 to J. P.

REFERENCES

1. Varshavsky, A. (2005) Regulated protein degradation. *Trends. Biochem. Sci.* **30**, 283-286.
2. Weissman, A. M. (2001) Themes and variations on ubiquitylation. *Nat. Rev. Mol. Cell. Biol.* **2**, 169-178.
3. Pickart, C. M. (2001) Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* **70**, 503-533.
4. Ciechanover, A. and Ben-Saadon, R. (2004) N-terminal ubiquitination: more protein substrates join in. *Trends. Cell. Biol.* **14**, 103-106.
5. Cadwell, K. and Coscoy, L. (2005) Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. *Science* **309**, 127-130.
6. Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D. and Gygi, S. P. (2003) A proteomics approach to understanding protein ubiquitination. *Nat. Biotechnol.* **21**, 921-926.
7. Wilkinson, K. D. (2000) Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. *Seminars in Cell & Developmental Biology* **11**, 141-148.
8. Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K. and Bernards, R. (2005) A genomic and functional inventory of deubiquitinating enzymes. *Cell* **123**, 773-786.
9. Semple, C. A. (2003) The comparative proteomics of ubiquitination in mouse. *Genome. Res.* **13**, 1389-1394.
10. Pickart, C. M. and Fushman, D. (2004) Polyubiquitin chains: polymeric protein signals. *Curr. Opin. Chem. Biol.* **8**, 610-616.
11. Hicke, L., Schubert, H. L. and Hill, C. P. (2005) Ubiquitin-binding domains. *Nat. Rev. Mol. Cell. Biol.* **6**, 610-621.
12. Harper, J. W. and Schulman, B. A. (2006) Structural complexity in ubiquitin recognition. *Cell* **124**, 1133-1136.
13. Aebersold, R. and Mann, M. (2003) Mass spectrometry-based proteomics. *Nature* **422**, 198-207.
14. Yates, J. R., 3rd (2004) Mass spectral analysis in proteomics. *Annu. Rev. Biophys. Biomol. Struct.* **33**, 297-316.
15. Marotti, L. A., Jr., Newitt, R., Wang, Y., Aebersold, R. and Dohlman, H. G. (2002) Direct identification of a G protein ubiquitination site by mass spectrometry. *Biochemistry* **41**, 5067-5074.
16. Peng, J. and Gygi, S. P. (2001) Proteomics: the move to mixtures. *J. Mass. Spectrom.* **36**, 1083-1091.
17. Love, K. R., Catic, A., Schlieker, C. and Ploegh, H. L. (2007) Mechanisms, biology and inhibitors of deubiquitinating enzymes. *Nat. Chem. Biol.* **3**, 697-705.
18. Kessler, B. M. (2006) Putting proteomics on target: activity-based profiling of ubiquitin and ubiquitin-like processing enzymes. *Expert Review of Proteomics* **3**, 213-221.
19. Kirkpatrick, D. S., Denison, C. and Gygi, S. P. (2005) Weighing in on ubiquitin: the expanding role of mass- spectrometry-based proteomics. *Nat. Cell. Biol.* **7**, 750-757.
20. Xu, P. and Peng, J. (2006) Dissecting the ubiquitin pathway by mass spectrometry. *Biochim. Biophys. Acta.* **1764**, 1940-1947.
21. Tomlinson, E., Palaniyappan, N., Tooth, D. and Layfield, R. (2007) Methods for the purification of ubiquitinated proteins. *Proteomics* **7**, 1016-1022.
22. Wang, X., Guerrero, C., Kaiser, P. and Huang, L. (2007) Proteomics of proteasome complexes and ubiquitinated proteins. *Expert Review of Proteomics* **4**, 649-665.
23. Peng, J. and Cheng, D. (2005) Proteomic analysis of ubiquitin conjugates in yeast. *Methods. Enzymol.* **399**, 367-381.
24. Tagwerker, C., Flick, K., Cui, M., Guerrero, C., Dou, Y., Auer, B., Baldi, P., Huang, L. and Kaiser, P. (2006) A tandem affinity tag for two-step purification under fully denaturing conditions: application in ubiquitin profiling and protein complex identification combined with *in vivo* cross-linking. *Mol. Cell. Proteomics.* **5**, 737-748.
25. Jeon, H. B., Choi, E. S., Yoon, J. H., Hwang, J. H., Chang, J. W., Lee, E. K., Choi, H. W., Park, Z. Y. and Yoo, Y. J. (2007) A proteomics approach to identify the ubiquitinated proteins in mouse heart. *Biochemical and Biophysical Research Communications* **357**, 731-736.
26. Matsumoto, M., Hatakeyama, S., Oyamada, K., Oda, Y., Nishimura, T. and Nakayama, K. I. (2005) Large-scale analysis of the human ubiquitin-related proteome. *Proteomics* **5**, 4145-4151.
27. Vasilescu, J., Smith, J. C., Ethier, M. and Figeys, D. (2005) Proteomic analysis of ubiquitinated proteins from human MCF-7 breast cancer cells by immunoaffinity purification and mass spectrometry. *J. Proteome. Res.* **4**, 2192-2200.
28. Layfield, R., Tooth, D., Landon, M., Dawson, S., Mayer, J. and Alban, A. (2001) Purification of poly-ubiquitinated proteins by S5a-affinity chromatography. *Proteomics* **1**, 773-777.
29. Weekes, J., Morrison, K., Mullen, A., Wait, R., Barton, P. and Dunn, M. J. (2003) Hyperubiquitination of proteins in dilated cardiomyopathy. *Proteomics* **3**, 208-216.
30. Maor, R., Jones, A., Nuhse, T. S., Studholme, D. J., Peck, S. C. and Shirasu, K. (2007) Multidimensional protein identification technology (MudPIT) analysis of ubiquitinated proteins in plants. *Mol. Cell. Proteomics.* **6**, 601-610.
31. Bennett, E. J., Shaler, T. A., Woodman, B., Ryu, K. Y., Zaitseva, T. S., Becker, C. H., Bates, G. P., Schulman, H. and Kopito,

R. R. (2007) Global changes to the ubiquitin system in Huntington's disease. *Nature* **448**, 704-708.
32. Mayor, T., Lipford, J. R., Graumann, J., Smith, G. T. and Deshaies, R. J. (2005) Analysis of polyubiquitin conjugates reveals that the Rpn10 substrate receptor contributes to the turnover of multiple proteasome targets. *Mol. Cell. Proteomics.* **4**, 741-751.
33. Seyfried, N. T., Xu, P., Duong, D. M., Cheng, D., Hanfelt, J. and Peng, J. (2008) A systematic approach to validating ubiquitinated proteome. *Anal. Chem. Under. Revision.*
34. Hitchcock, A. L., Auld, K., Gygi, S. P. and Silver, P. A. (2003) A subset of membrane-associated proteins is ubiquitinated in response to mutations in the endoplasmic reticulum degradation machinery. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 12735-12740.
35. Mayor, T., Graumann, J., Bryan, J., MacCoss, M. J. and Deshaies, R. J. (2007) Quantitative profiling of ubiquitylated proteins reveals proteasome substrates and the substrate repertoire influenced by the Rpn10 receptor pathway. *Mol. Cell. Proteomics.* **6**, 1885-1895.
36. Wang, M., Cheng, D., Peng, J. and Pickart, C. M. (2006) Molecular determinants of polyubiquitin linkage selection by an HECT ubiquitin ligase. *The EMBO Journal* **25**, 1710-1719.
37. Warren, M. R., Parker, C. E., Mocanu, V., Klapper, D. and Borchers, C. H. (2005) Electrospray ionization tandem mass spectrometry of model peptides reveals diagnostic fragment ions for protein ubiquitination. *Rapid. Commun. Mass. Spectrom.* **19**, 429-437.
38. Xu, P., Cheng, D., Duong, D. M., Rush, J., Roelofs, J., Finley, D. and Peng, J. (2006) A proteomic strategy for quantifying polyubiquitin chain topologies. *Israel J. Chem.* **46**, 171-182.
39. Denis, N. J., Vasilescu, J., Lambert, J. P., Smith, J. C. and Figeys, D. (2007) Tryptic digestion of ubiquitin standards reveals an improved strategy for identifying ubiquitinated proteins by mass spectrometry. *Proteomics* **7**, 868-874.
40. Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S. and Sorkin, A. (2006) Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. *Mol. Cell.* **21**, 737-748.
41. Kirkpatrick, D. S., Hathaway, N. A., Hanna, J., Elsasser, S., Rush, J., Finley, D., King, R. W. and Gygi, S. P. (2006) Quantitative analysis of *in vitro* ubiquitinated cyclin B1 reveals complex chain topology. *Nature. Cell. Biology.* **8**, 700-710.
42. Crosas, B., Hanna, J., Kirkpatrick, D. S., Zhang, D. P., Tone, Y., Hathaway, N. A., Buecker, C., Leggett, D. S., Schmidt, M., King, R. W., Gygi, S. P. and Finley, D. (2006) Ubiquitin chains are remodeled at the proteasome by opposing ubiquitin ligase and deubiquitinating activities. *Cell* **127**, 1401-1413.
43. Tan, J. M., Wong, E. S., Kirkpatrick, D. S., Pletnikova, O., Ko, H. S., Tay, S. P., Ho, M. W., Troncoso, J., Gygi, S. P., Lee, M. K., Dawson, V. L., Dawson, T. M. and Lim, K. L. (2008) Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. *Human Molecular Genetics* **17**, 431-439.
44. Kumar, K. G., Barriere, H., Carbone, C. J., Liu, J., Swaminathan, G., Xu, P., Li, Y., Baker, D. P., Peng, J., Lukacs, G. L. and Fuchs, S. Y. (2007) Site-specific ubiquitination exposes a linear motif to promote interferon-alpha receptor endocytosis. *The Journal of Cell Biology* **179**, 935-950.
45. Wooten, M. W., Geetha, T., Babu, J. R., Seibenhener, M. L., Peng, J., Cox, N., Diaz-Meco, M. T. and Moscat, J. (2008) Essential Role of Sequestosome 1/p62 in Regulating Accumulation of Lys63-ubiquitinated Proteins. *The Journal of Biological Chemistry* **283**, 6783-6789.
46. Ong, S. E. and Mann, M. (2005) Mass spectrometry-based proteomics turns quantitative. *Nat. Chem. Biol.* **1**, 252-262.
47. Kim, H. T., Kim, K. P., Lledias, F., Kisselev, A. F., Scaglione, K. M., Skowyra, D., Gygi, S. P. and Goldberg, A. L. (2007) Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. *J. Biol. Chem.* **282**, 17375- 17386.
48. Wu, S. L., Kim, J., Bandle, R. W., Liotta, L., Petricoin, E. and Karger, B. L. (2006) Dynamic profiling of the post-translational modifications and interaction partners of epidermal growth factor receptor signaling after stimulation by epidermal growth factor using Extended Range Proteomic Analysis (ERPA). *Mol. Cell. Proteomics.* **5**, 1610-1627.
49. Garciaa, B. A., Siutib, N., Thomasb, C. E., Mizzena, C. A. and Kelleher, N. L. (2007) Characterization of neurohistone variants and post-translational modifications by electron capture dissociation mass spectrometry. *Int. J. Mass Spectr.* **259**, 184-196.
50. Xu, P. and Peng, J. (2008) Characterization of polyubiquitin chain structure by middle-down mass spectrometry. *Anal. Chem. in press.*
51. Kerscher, O., Felberbaum, R. and Hochstrasser, M. (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu. Rev. Cell. Dev. Biol.* **22**, 159-180.