Amino Acids (2012) 43:1049–1060
DOI 10.1007/s00726-012-1286-y

INVITED REVIEW

Quantitative proteomics to decipher ubiquitin signaling

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Received: 3 November 2010 / Accepted: 3 April 2012 / Published online: 22 July 2012

Abstract Ubiquitin signaling plays an essential role in controlling cellular processes in eukaryotes, and the impairment of ubiquitin regulation contributes to the pathogenesis of a wide range of human diseases. During the last decade, mass spectrometry-based proteomics has emerged as an indispensable approach for identifying the ubiquitinated proteome (ubiquitinome), ubiquitin modification sites, the linkages of complex ubiquitin chains, as well as the interactome of ubiquitin enzymes. In particular, implementation of quantitative strategies allows the detection of dynamic changes in the ubiquitinome, enhancing the ability to differentiate between function-relevant protein targets and false positives arising from biological and experimental variations. The profiling of total cell lysate and the ubiquitinated proteome in the same sets of samples has become a powerful tool, revealing a subset of substrates that are modulated by specific physiological and pathological conditions, such as gene mutations in ubiquitin signaling. This strategy is equally useful for dissecting the pathways of ubiquitin-like proteins.

Keywords Ubiquitin · Proteasome · E3 · DUB · Mass spectrometry · Proteomics · SILAC

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Introduction

Ubiquitin (Ub), a highly conserved protein of 76 amino acids, regulates almost all cellular events in eukaryotes. Although the purification of Ub was described more than 30 years ago, novel regulatory functions of Ub pathways are continuously being discovered. Ubiquitin, originally known as the ubiquitous immunopoietic polypeptide (UBIP), was first isolated from bovine thymus and then from all tested living cells (Goldstein et al. 1975; Schlesinger and Goldstein 1975; Schlesinger et al. 1975), and it formed an isopeptide bond with a lysine residue in histone 2A (Goldknopf and Busch 1977). The biological function of Ub, however, remained a mystery until 1980, in part because the mono-ubiquitination of histone 2A has no effect on its degradation. Accidentally, ATP-dependent proteolysis factor 1 (APF-1) that induces protein degradation in an ATP-dependent manner in vitro was discovered to be identical to ubiquitin (Ciehanover et al. 1978; Wilkinson et al. 1980); the physiological role of Ub in protein degradation was later demonstrated in cells (Ciechanover et al. 1984; Finley et al. 1984). The role of ubiquitin in degradation is supported by the purification of a protease complex (the proteasome) with the activity of degrading ubiquitinated species (Hough et al. 1987). The 26S proteasome is a 2.5-mDa complex composed of a 19S regulatory particle and a 20S core particle. This 19S particle recognizes the Ub tag on substrates and then de-ubiquitinates, unfolds, and translocates the substrates into the 20S particle for proteolysis, whereas the 20S particle has multiple protease activities to digest protein substrates into small peptides (Finley 2009). These landmark findings established the concept of the ubiquitin proteasome system (UPS) in protein degradation (Hershko and Ciechanover 1998); further studies provide evidence that ubiquitin modification functions not only in the UPS pathway, but also in a

wide range of signaling events, including protein sorting, transcription regulation, DNA repair, and immune responses (Mukhopadhyay and Riezman 2007) (Fig. 1).

Like protein phosphorylation, ubiquitination is a reversible posttranslational process catalyzed by a large number of enzymes. Ub is primarily conjugated to lysine residues of substrates by catalytic action of E1-activating enzymes, E2-conjugating enzymes, and E3 ligases (Ciechanover 2005). In some cases, non-lysine residues (e.g., N-terminal amine group and cysteine residues) are alternative sites for Ub conjugation (Cadwell and Coscoy 2005; Ciechanover and Ben-Saadon 2004). In addition to monoubiquitination, substrates can be modified by additional Ub, resulting in multi-monoubiquitination or polyUb chains. PolyUb chains are assembled through any of the eight amine groups in the Ub sequence: M1, K6, K11, K27, K29, K33, K48, and K63 (Kirisako et al. 2006; Peng et al. 2003; Xu et al. 2009c), enabling the formation of diverse polyUb chain structures (Bremm et al. 2010; Varadan et al. 2004; Virdee et al. 2010). Ub on substrates can be cleaved by five families of deubiquitinating enzymes (DUBs) of metalloproteases or cysteine proteases (Komander et al. 2009; Nijman et al. 2005). These DUBs function to produce Ub monomer from Ub precursors, edit polyUb chains on substrates, recycle Ub from substrates, and rescue substrates from proteasomal degradation (Reyes-Turcu et al. 2009). Specificity in Ub pathways is mediated by E3–substrate interaction, recognition of Ub moieties (monoUb and polyUb with diverse linkages) by Ub receptors (Dikic et al. 2009; Pickart and Fushman 2004), and selective removal of Ub modifications by DUBs. The scope of protein ubiquitination is as vast as that of phosphorylation. The human genome encodes two Ub E1s, approximately 40 E2s, at least 600 E3s, and 95 DUBs (Li et al. 2008; Nijman et al. 2005; Semple 2003), which are proposed to regulate thousands of protein substrates in cells.

Considering the versatile role of Ub in eukaryotic cells, a link between ubiquitination dysregulation and the development of human diseases such as cancer (Voorhees and Orlowski 2006) and neurodegenerative disorders (Ciechanover and Brundin 2003; Goldberg 2007) comes as no surprise. One example is that localization and degradation of the tumor suppressor p53 protein are exquisitely regulated by numerous E3s and at least one DUB (Lee and Gu 2010). Genetic studies reveal that tumorigenesis is directly associated with recurrent mutations in multiple E3s, including BRCA1 (Wang 2007), Fanconi anemia protein complex (Wang 2007), Von Hippel–Lindau disease protein VHL (Kaelin 2008), FBW7 (F-box and WD repeat domain-containing 7) (Welcker and Clurman 2008), and RNF43 (Wu et al. 2011). Deubiquitinating enzymes, such as the cylindromatosis protein CYLD (Bignell et al. 2000) and TNFAIP3/A20 (Musone et al. 2008), are also tumor suppressors. In neurodegenerative disorders, some cases of early-onset Parkinson's disease are associated with mutations in the Parkin E3 ligase (Bandopadhyay and de Belleroche 2010). Missense mutations in a BTB-Kelch protein KLHL7 (functioning in ubiquitination through Cullin E3 ligases) cause retinitis pigmentosa, a progressive degenerative disease of rod and cone photoreceptors in the retina; mutations in another BTB-Kelch protein (gigaxonin) lead to giant axonal neuropathy (Friedman et al. 2009); and UBE3A mutations are linked to Angelman syndrome (Yi and Ehlers 2007). Two DUB genes, UCHL1 and ATXN3, are tied genetically to Parkinson's disease and spinal cerebral ataxia, respectively (Yi and Ehlers 2007). More recently, mutations in UBQLN2, which encodes a Ub receptor regulating proteasomal degradation, have been found to cause familial amyotrophic lateral sclerosis (Deng et al. 2011). Besides these genetic data, Ub-positive inclusion staining is a hallmark of pathology in neurodegenerative diseases, suggesting a key role of Ub in disease development (Ciechanover and Brundin 2003; Yi and Ehlers 2007).

In addition to ubiquitin, a growing family of proteins referred to as ubiquitin-like proteins (Ubls) shares similar protein modification mechanisms and regulates a broad

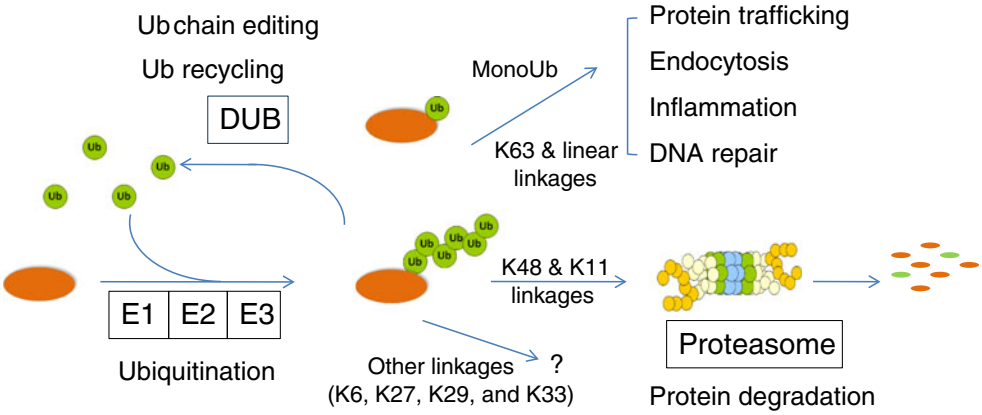

Fig. 1 The chemistry and function of protein ubiquitination

range of cellular functions in eukaryotes and even in prokaryotes (Hochstrasser 2009; Kerscher et al. 2006). Nine out of more than ten Ubls identified can covalently modify other proteins, such as small ubiquitin-like modifier (SUMO), neural precursor cell-expressed developmentally downregulated protein 8 (Nedd8), and interferon-stimulated 15-kDa protein (ISG15). Despite limited sequence similarity, Ubls exhibit a common globular structure of the β-grasp fold or ubiquitin fold (Hochstrasser 2000). Ubl modifications alter the chemical properties and tertiary structure of substrates and are able to compete with modification by Ub and other molecules (Jeram et al. 2009).

In summary, Ub and Ubls are crucial regulators of cellular events, and failure in the Ub and Ubl pathways plays a significant role in the pathogenesis of human diseases. In most cases, however, the molecular mechanisms underlying pathogenesis remain to be illuminated. Current developments in modern mass spectrometry (MS) enable biochemical characterization of proteins in the femtomolar or even sub-femtomolar range (Choudhary and Mann 2010; Cravatt et al. 2007; Gstaiger and Aebersold 2009), providing unprecedented opportunities for dissecting ubiquitin pathways. In this review, we present the strategies for applying MS to unravel the functions of Ub or Ubl modifications, with a focus on protein ubiquitination.

Mass spectrometry (MS) analysis of the proteome modified by ubiquitin

Ub-modified proteins are usually analyzed by capillary liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Choudhary and Mann 2010; Cravatt et al. 2007; Gstaiger and Aebersold 2009). Protein samples are first digested with a protease (usually trypsin) and the resulting peptides are then fractionated by liquid chromatography (e.g., a C_{18} reverse-phase column). These peptides are ionized and analyzed by a mass spectrometer on the basis of mass-to-charge (m/z) ratio. It should be emphasized that tandem mass spectrometry itself is a high-resolution tool to isolate one ion among many coeluting ions. The isolated peptide ion is fragmented to generate a specific MS/MS spectrum containing its sequence information. Next, bioinformatics programs such as SEQUEST (Eng et al. 1994) and Mascot (Perkins et al. 1999) are used to match the experimental MS/MS spectrum with theoretical (computer-generated) peptide spectra in a database to identify the peptide. Since current MS instruments can acquire MS/MS spectra at a rate of up to 10 Hz, this procedure allows the identification of a large number of peptide/protein sequences from complex mixtures. For example, a single run on an optimized LC–MS/MS platform can identify approximately 1,000 proteins from a total yeast lysate (Xu et al. 2009b), and with extensive modifications, may identify more than 4,000 proteins from the yeast proteome (Nagaraj et al. 2011a). To further increase the separation power, it is common to resolve protein samples by one-dimensional SDS gel electrophoresis, or to pre-fractionate peptide mixtures by strong cation exchange chromatography, high pH reverse-phase chromatography, or isoelectric focusing (de Godoy et al. 2008). These deep proteome analysis technologies allow the detection of over 10,000 human proteins (Huttlin et al. 2010; Nagaraj et al. 2011b). Details of these technologies are available in other excellent review papers (Motoyama and Yates 2008; Peng and Gygi 2001; Steen and Mann 2004).

As ubiquitinated proteins are sparse in cells, prior to MS analysis Ub conjugates need to be pre-enriched by affinity approaches (Fig. 2) such as Ub antibodies (Matsumoto et al. 2005; Vasilescu et al. 2005), Ub-binding proteins (Bennett et al. 2007; Layfield et al. 2001; Maor et al. 2007; Weekes et al. 2003), or epitope-tagged Ub (e.g., FLAG, HA, myc, His, and biotin tag) (Kirkpatrick et al. 2005; Wang et al. 2007; Xu and Peng 2006). Most of the analyses have been discussed in previous reviews (Wang et al. 2007; Xu and Peng 2006); here, we focus on a few key experiments and recent developments. The first large-scale study used a His-tagged Ub purification method to isolate the ubiquitinome, followed by LC/LC–MS/MS to identify these ubiquitinated proteins and modification sites from yeast (Peng et al. 2003). In this study, 1,075 proteins were identified as ubiquitinated candidates and 110 ubiquitinated sites were detected. A critical step was the use of a highly denaturing condition (8 M urea) to minimize associated proteins and interrupt Ub protease activities that rapidly disassemble Ub conjugates during purification. When applied to mammalian cell cultures and even animal models (Jeon et al. 2007), the His tag strategy had limited success due to the large number of His-rich native proteins in mammalian cells and the low-level expression of tagged Ub (Peng J, unpublished data). Development of a tandem His-biotin tag strategy alleviated this problem and identified 669 potential ubiquitinated proteins and 44 ubiquitinated sites in HeLa cells (Meierhofer et al. 2008). More recently, a shortened biotin-tagged Ub was expressed under a neuron-specific promoter in fly, which allowed the study of cell type-specific ubiquitination in vivo (Franco et al. 2011). To improve the expression of epitope-tagged ubiquitin in higher organisms, suppression of endogenous Ub by tetracycline-inducible RNAi strategy (Xu et al. 2009a) may be an effective strategy. In contrast to tagged Ub methods that require genetic engineering, other high-affinity reagents have been tested to capture endogenous ubiquitinated species. One approach is the use of tandem-repeated Ub-binding entities to increase Ub binding and to protect Ub conjugates from the action of DUBs (Hjerpe and Rodriguez 2008; Lopitz-Otsoa et al. 2011). Recently, the

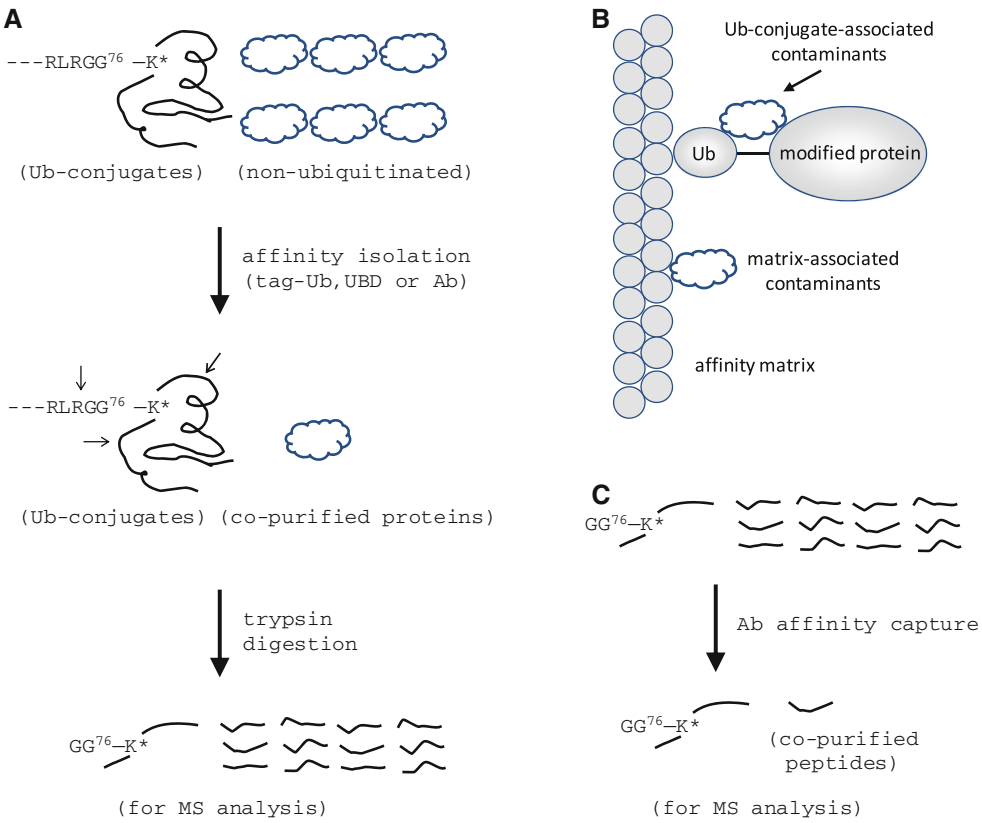

Fig. 2 Strategies for isolating ubiquitinated proteins/peptides from complex mixtures. **a** Schematic representation of the purification strategies based on tagged Ub (e.g., HA, FLAG, His and biotin), ubiquitin-binding domains (UBDs), or Ub antibodies (Ab). *Arrows* indicate tryptic sites surrounding a lysine modification residue in Ub conjugates. Digestion of the Ub moiety generates a small Gly–Gly tag on the lysine residue. **b** Purified Ub conjugate samples contain two classes of contaminated proteins that are not ubiquitinated, one class associated with affinity matrix nonspecifically and the other class interacting more specifically with Ub conjugates. **c** Immunocapture of GG-tagged peptides to enhance the capacity for identifying ubiquitination sites

availability of modern mass spectrometers (Olsen et al. 2007, 2009) prompted the re-analysis of the ubiquitinated proteome in yeast and mammalian cells (Danielsen et al. 2011; Shi et al. 2011a; Starita et al. 2011), increasing the number of identified ubiquitinated proteins and modification sites.

Instead of purifying ubiquitinated proteins from cell lysates, a milestone success is to enrich Ub-modified peptides by antibodies directly from trypsin-digested lysates. As trypsin cleaves proteins at the carboxyl side of lysine or arginine unless followed by proline residues, trypsinization of Ub produces a di-glycine (GG) tag on the modified residues in ubiquitinated substrates, and the GG-modified lysine residues are resistant to trypsin digestion (Goldknopf and Busch 1977). Thus, the GG-tagged, miscleaved lysine residues ensure a monoisotopic mass addition of 114.0429 Da and allow identification by MS (Marotti et al. 2002; Peng and Gygi 2001; Peng et al. 2003). The same principle is applied to determine amino acid residues modified by Ubls (Table 1). Using monoclonal antibodies against GG-tagged peptides, two groups recently identified ~11,000 and ~19,000 modification sites, respectively (Kim et al. 2011; Wagner et al. 2011), representing a marked improvement in analyzing the ubiquitinome. Such a new tool makes it feasible to profile the ubiquitinome in cells under a variety of conditions, although the bias of these antibodies (Xu et al. 2010) needs characterization. Another caveat is that modification by two Ubls (Nedd8 and ISG15) also leads to the generation of the same GG tag. Thus, additional care is required to differentiate these conjugates.

During the isolation of ubiquitinated proteins, non-ubiquitinated species are always copurified, although the level of contamination varies upon experimental conditions and personal expertise (Fig. 2). Therefore, it is essential to confirm whether proteins identified in Ub conjugate-enriched fractions are truly modified by Ub. Three approaches may be used to confirm modification by Ub: (1) identification of ubiquitinated sites in some of these ubiquitinated proteins (Peng et al. 2003); (2) quantitative comparison of samples with different levels of ubiquitination (Meierhofer et al. 2008); and (3) immunoprecipitation and Western blotting to validate large mass shift caused by ubiquitination. As it is impractical to apply this method to every identified candidate, a virtual Western blotting approach was developed on one-dimensional SDS gel and LC–MS/MS, enabling the large-scale examination of mass shift in identified proteins (Seyfried et al. 2008).

During the database search of MS/MS spectra, false-positive identification of ubiquitination sites frequently occurs (Peng 2008; Seyfried et al. 2008; Shi et al. 2011b). For example, GG modification could be falsely assigned to the C-terminal lysine in matched peptides by computer algorithms (Denis et al. 2007; Xu et al. 2010), resulting in a questionable conclusion that trypsin may cleave at the C-terminus of GG-modified Lys residues. However, an

Table 1 Strategy for identifying amino acid residues modified by ubiquitin and Ubls

	Remnant tagged peptides after trypsin digestion	Mass shift	Problem	Problem solving
Ub	GG-K	114.0429 Da	Ubiquitin, Nedd8, and ISG15 share same signature peptide after trypsin digestion	Pre-enrichment of proteins modified by individual Ubl before trypsin digestion
Nedd8	GG-K			
ISG15	GG-K			
SUMO-1	ELGMEEEDVIEVYQEQTGG-K	Multiple large mass shifts	Weak ionization of the modified peptides and complex MS/MS spectra that is difficult to interpret	i. Software such as SUMmOn ii. UBL spectral library iii. Introduction of short tryptic tags at Ubl C-terminus by mutagenesis
SUMO-2	FDGQPINETDTPAQLEMEDEDTIDVFQQQTGG-K			
SUMO-3	FDGQPINETDTPAQLEMEDEDTIDVFQQQTGG-K			
Fat10[a]	GNLLFLACYCIGG-K			

Trypsinization of conjugated proteins produces specific tags (e.g., GG for Ub) on modified residues, resulting in a mass shift

[a] The mass of human Fat10 tag may change due to a sequence variation (Swiss-Prot 015205)

experiment to trypsinize a reported synthetic GG-peptide was not successful (Seyfried et al. 2008). No peptides with the C-terminal ubiquitination survive manual interpretation of MS/MS matches (Seyfried et al. 2008; Shi et al. 2011b). Another question is if a reported longer tag (LRGG due to miscleavage at Arg74 in Ub) is reliable (Warren et al. 2005). The LRGG tag may be generated under certain digestion conditions, but can rarely be identified. The Arg74 residue is usually cleaved at high efficiency during trypsin digestion (Wang et al. 2006; Xu et al. 2006). Hence, acceptance of either C-terminal ubiquitination or LRGG-modified peptides is not recommended. The peptide coverage of identified proteins is another parameter used to evaluate false-positive matches (Shi et al. 2011b). Ubiquitination sites identified on proteins with low peptide coverage need to be accepted with extreme caution when analyzing ubiquitinated proteins.

Iodoacetamide is a Cys-alkylating reagent used during purification to inhibit DUB activities, but it may also modify lysine residues to produce a monoisotopic mass tag of 114.0429 Da, the same mass as the GG tag (Nielsen et al. 2008; Yang and Attygalle 2007). A suggested substitute (chloroacetamide) (Nielsen et al. 2008) might also produce this artifact tag at high temperature, although at a much lower level than iodoacetamide (Xu et al. 2009c). However, at a low temperature (room temperature or below) or low dosage, this side reaction is essentially eliminated (Xu et al. 2009c). As the most abundant ubiquitinated peptide in cells (K48-GG Ub peptide) can be distinguished from its iodoacetamide-modified artifact on the basis of LC retention time and MS/MS patterns, examination of the presence of this artifact peptide in samples can be a quality control measure. Interestingly, the GG-peptide antibodies are able to distinguish the GG moiety from the iodoacetamide-derived artifact (Yang Y. and Peng J., unpublished). Alternatively, by using other alkylation reagents (e.g., *N*-ethylmaleimide or vinylpyridine), this problem may be avoided (Righetti 2006).

Quantitative profiling of ubiquitin-regulated proteome

Quantitative proteomics analysis of wild-type versus mutant cells is an effective strategy to uncover specific substrates of Ub enzymes (e.g., E3 ligases or DUBs) (Kirkpatrick et al. 2005). The quantity of proteins can be evaluated by a number of MS approaches such as label-free quantification based on spectral counting (SC) and extracted ion current (XIC) or stable isotope labeling methods. Spectral counting uses the total number of MS/MS spectra identifying a single protein, which increases almost linearly with protein abundance after normalizing for protein size (Liu et al. 2004). This method works reasonably well for proteins with high spectral counts, but its reliability decreases significantly for proteins of low spectral counts (Zhang et al. 2006; Zhou et al. 2010). The abundance of peptides in samples can also be compared by extracted ion current of corresponding ions (Radulovic et al. 2004; Wang et al. 2003). Because the ionization efficiency of peptides may vary among different LC runs due to fluctuations of the LC system and ion suppression, considerable

variations need to be normalized in this label-free method. The intrinsic LC–MS/MS variations are reduced by introducing stable isotope-labeled peptides as internal standards (Choudhary and Mann 2010). Internal standards and native counterparts are eluted and ionized simultaneously during the LC–MS/MS runs. Relative quantification is achieved by comparing the peptide pairs before fragmentation or by comparing derived product ion peaks after fragmentation. Stable isotopes can be incorporated into samples by in vitro labeling methods such as isobaric tags for relative and absolute quantitation (iTRAQ) (Ross et al. 2004) or tandem mass tags (TMT) (Dayon et al. 2008; Thompson et al. 2003). The iTRAQ/TMT reagents differentially label the amine group at the N-termini and Lys residues of peptides after protein isolation and digestion. In contrast, the strategy of stable isotope labeling with amino acids in cell culture (SILAC) (Ong et al. 2002) allows metabolic labeling of proteins, accounting for the variations during the steps of protein isolation and digestion. SILAC has emerged as a highly accurate in vivo labeling method for large-scale proteomics. Many of these advanced quantitative methods have been used to investigate protein changes in Ub pathways.

Assuming that a single E3 ligase mediates the degradation of substrates, the substrates are expected to accumulate in mutated cells. For example, to identify plasma membrane receptors modified by the transmembrane E3 ligase MARCH9, the SILAC method was used to monitor protein changes in the plasma membrane isolated from wild-type and MARCH9 mutant B cells (Hor et al. 2009). The label-free quantitative method was used to identify the E3 ligase (ASB2) substrates, in which proteins from inducible cell lines expressing wild type or ASB2 mutant were compared (Burande et al. 2009). The same method was also adapted to study the parkin-related pathway underlying the loss of parkin in a *Drosophila* model (Xun et al. 2009). It should be noted that proteins with altered levels in E3 mutants may not be genuine substrates, but instead may represent the adaptation of cells to mutants. Additional confirmation experiments, such as mRNA-level analysis, half-time measurement, protein–protein interaction, and in vitro ubiquitination, are required to validate the putative enzyme–substrate connection.

Direct profiling of isolated ubiquitinated proteome is more informative than the analysis of total cell lysate after perturbation of Ub signaling. For instance, the dynamics of ubiquitinated proteins was studied upon proteasomal inhibition in mammalian cells by the SILAC strategy (Kim et al. 2011; Meierhofer et al. 2008; Wagner et al. 2011). Temporal response of the ubiquitinome indicates different dynamics of individual modification sites and that Ub conjugate accumulation during proteasome blockage relies on ongoing protein translation (Kim et al. 2011). In another scenario, Akimov et al. (2011) analyzed the dynamics of ubiquitination events in cells treated with epidermal growth factor. Ideally, both total cell lysate and ubiquitinated proteome should be profiled from the same set of samples to reveal protein targets. Xu et al. used the SILAC method to compare the two sets of the proteome in wild-type and Ub K11R mutant yeast strains to identify protein substrates modified by K11 polyUb chains (Xu et al. 2009c). As K11 linkage modification directs proteins to proteasomal degradation, two candidate substrates were identified based on their enrichment in the total cell lysate and reduction in the ubiquitinome. The same strategy was also used to probe a subset of Ub conjugates recognized by Rpn10, a Ub receptor of the yeast proteasome (Mayor et al. 2007, 2005). Quantitative proteomics was also utilized to identify substrates of the BRCA1 and HRD1 E3 ligases (Lee et al. 2011; Song et al. 2011). Recently, Kim et al. (2011) used a monoclonal GG-peptide antibody to identify 386 GG-modified lysine sites dependent on cullin-RING Ub ligases. Together, quantitative comparison of the total proteome and ubiquitinome permits background protein subtraction and data set cross-validation to reduce false discoveries in the identification of ubiquitin substrates.

Quantitative analysis of polyubiquitin chains

As the linkages of polyubiquitin chains may determine the consequence of modified substrates (Fig. 1), identifying the type of linkages on protein targets is of importance to dissect the function of ubiquitination events. Classic K48 polyUb linkages direct substrates to the proteasome for degradation (Chau et al. 1989); the functions of newly discovered polyUb linkages (K6, K11, K27, K29, and K33), however, are much less understood and may also contribute to proteasomal targeting (Johnson et al. 1995; Kirkpatrick et al. 2006; Xu et al. 2009c). In contrast, K63 linkages and monoUb modification mainly play roles in protein sorting (Hicke and Dunn 2003), DNA repair (Bergink and Jentsch 2009), and inflammation (Bhoj and Chen 2009). Finally, linear polyUb chains are formed via the Ub N-terminal alpha amino group, but whether the linear chains function in proteolysis remains controversial (Kirisako et al. 2006; Rahighi et al. 2009; Zhao and Ulrich 2010).

To measure all the polyUb linkages, stable isotope labeling peptides have been synthesized for all eight linkages corresponding to ubiquitinated GG peptides (Kirkpatrick et al. 2006; Xu et al. 2006). The labeled peptides used as internal standards are spiked into a protein mixture that is then digested with trypsin to generate native GG peptides from polyUb chains. The pairs of native peptides and internal standards are detected by a mass spectrometer in the setting of selected reaction monitoring [SRM, also termed MRM

(multiple reaction monitoring)] for quantification. By this method, Kirkpatrick et al. (2006) detected mixed chain topologies (K11, K48, and K63) on ubiquitinated cyclin B1 catalyzed by the anaphase-promoting complex in vitro, and the heterogeneous chains were capable of mediating the degradation of cyclin B1 in a reconstituted system in vitro, suggesting a more broad involvement of linkages in substrate degradation. The method has been further improved using different MS platforms (Phu et al. 2010) and by introducing labeled protein standards instead of peptide standards (Kaiser et al. 2011; Xu et al. 2009c). Xu et al. (2009c) performed the measurement in yeast and uncovered a surprisingly high level of unconventional polyUb linkages in His-tag affinity-purified Ub conjugates: K6, 10.9 %; K11, 28 %; K27, 9 %; K29, 3.2 %; K33, 3.5 %; K48, 29 %; K63, 16 %. The measurements in mammalian cells are slightly different, with a lower level of K11 linkage (Dammer et al. 2011; Kaiser et al. 2011). Analyses of Ub linkage under proteasomal inhibition in cell culture and in animals (Bedford et al. 2011) support the concept that all non-K63 linkages (not including linear linkage in M1-tagged Ub chains) contribute to proteasomal degradation. Potentially, the levels of polyUb linkages can be used as an index reflecting the UPS capacity in animal disease models and human patient tissues (Bennett et al. 2007; Dammer et al. 2011). In contrast to the involvement in the UPS, other studies have implicated the non-degradation role of unconventional linkages such as K11 (Boname et al. 2010; Goto et al. 2010) and K33 (Huang et al. 2010).

During fully tryptic digestion of Ub conjugates, some structural information on polyUb chains is lost. For example, the current Ub-SRM method does not account for forked polyUb chains (i.e., one Ub molecule simultaneously modified by two other Ub molecules at Lys29 and Lys33) (Peng et al. 2003). To address this issue, a "middle-down" technology (Garciaa et al. 2007; Wu et al. 2006) has been used to partially digest Ub polymers under native conditions and enable analysis of the forked structure (Xu and Peng 2008). Alternatively, the development of antibodies recognizing specific ubiquitin linkages, including K-11, K-48, and K63, makes it possible to immunoprecipitate specific chains and to perform cell imaging analysis (Matsumoto et al. 2010; Newton et al. 2008). These complementary tools permit a more comprehensive study of Ub chain structure and function.

Interactome studies of ubiquitin enzymes

The function of proteins is regulated by associated partners during cellular signaling in response to physiological conditions. To reveal the regulatory mechanism of Ub signaling, it is important to identify the interactome of core Ub enzymes such as E3 ligases, DUBs, and the proteasome. Interactome studies consist of two major steps: (1) purification of the targeted protein complex and (2) MS analysis to identify all components in the complex. Purification of the protein complex is one of the most critical steps and requires optimization to increase yield and reduce nonspecific binding proteins. A commonly used approach to improve specificity is tandem affinity purification (TAP) (Li 2010; Puig et al. 2001), while a single step of affinity purification is also widely used in laboratories. As signaling proteins may bind to targeted molecules weakly and transiently, quantitative analysis of tandem affinity-purified cross-linked protein complexes (QTAX) was developed to increase the recovery of the interacting proteins (Guerrero et al. 2008). A protein lysate can be made from formaldehyde-fixed cells by the QTAX method, followed by TAP under denaturing conditions. QTAX can be further coupled with SILAC to distinguish specific binding proteins from contaminants. Another strategy is to use the cleavable cross-linker disuccinimidyl sulfoxide to improve the purification of weak interacting proteins (Kao et al. 2011). Alternatively, affinity purification by GST fusion proteins is frequently used as well to pull down interacting proteins.

A few representative examples of interactome studies are discussed here. First, multiple E3 ligases (RNF8, BRCA1, and RNF168) and the E2 enzyme Ubc13 are critical regulating proteins in DNA repair. A strep tag used to identify the RNF8 complex revealed that DNA damage-inducible phosphorylation of HERC2 promotes its interaction with RNF8 (Bekker-Jensen et al. 2010). The TAP tag strategy was used to define the interaction of BRCA1 and NBA1, which is required to maintain the BRCA1 complex and to recruit BRCA1 to chromosomal damage sites (Wang et al. 2009). A recent immunoprecipitation-MS study found that OTUB1, a DUB associated with Ubc13, suppresses RNF168-dependent ubiquitination and inhibits the DNA damage response (Nakada et al. 2010). Second, Sowa et al. (2009) used a global proteomic analysis strategy to identify the proteins associated with DUBs by a single step of antibody affinity purification. They developed an unbiased comparative approach that integrated parallel epitope-tagged purification with LC–MS/MS analysis. With this platform, 774 high-confidence candidate proteins were found to interact with 75 human DUBs. Many of the DUBs were associated with protein complexes, and protein network analysis linked the DUBs to diverse functional processes. Recently, a similar study of DUB interactome and localization was performed in fission yeast (Kouranti et al. 2010). Third, GST affinity purification was performed to isolate interacting proteins of mind bomb 1, a membrane-associated E3 ligase required for Notch signaling. MS analysis revealed that Mib1 primarily interacts with membrane trafficking proteins, cell adhesion

components, several DUBs, and a number of kinases (Choe et al. 2007).

In addition to E3s and DUBs, the interactome of the proteasome has been under intense scrutiny in recent years. Traditional purification of functional proteasome uses high-salt buffer for washing, making the detection of weakly associated protein impossible. In contrast, by using epitope tag and near-physiological washing conditions during purification, a large number of proteasome-interacting proteins have been found (Finley 2009). Guerrero et al. (2008) used the QTAX method to identify transient interacting components of the proteasome, disclosing 42 novel proteins from mammalian cells. To date, proteomic analyses have characterized proteasome complexes from different tissues such as brain, heart, kidney, liver, lung, thymus, spleen, and intestines (Bingol et al. 2010; Ducoux-Petit et al. 2008; Gorbea et al. 2010; Tai et al. 2010). These studies identified a large number of proteasome-associated proteins, many of which play important regulatory roles. For example, the proteasome can regulate Ub chains of substrates dynamically through Hul5 (a proteasome-associated E3) and Ubp6/Usp14 protease. It is of clinical importance that drug targeting proteasome-associated proteins may represent a new way to modulate proteasomal function for disease therapy. Along this line, Lee et al. (2010) identified a small-molecule inhibitor of Usp14 and showed that inhibition of Usp14 catalytic activity increases the turnover of several proteins involved in neurodegenerative diseases, including tau, ataxin-3, and TDP-43. Furthermore, the localization and activity of the proteasome can be mediated through associated proteins in cell signaling pathways. For instance, phosphorylation of CaMKIIα enhances both its association and recruitment of the proteasome to dendritic spines in hippocampal neurons (Bingol et al. 2010). The translocation of proteasome to the spines may play an important role in synapse function and memory.

Conclusions

During the last decade, advances in MS and enrichment methods have enabled the sensitive analysis of the ubiquitinome and interactome of Ub pathway enzymes. It is possible now to profile thousands of ubiquitinated proteins and to map tens of thousands of modification sites from complex cell or tissue lysates. These technologies have played a major role in deepening our understanding of protein ubiquitination, especially in the discovery of complex polyUb chain structures and in the exploration of proteins associated with Ub enzymes. With continuous development of MS and Ub affinity capture reagents, the future holds more extensive applications of MS-based proteomics for biological and medical research on Ub signaling.

Acknowledgments This work was partially supported by National Institutes of Health grant RR025822 and American Cancer Society grant RSG-09-181 and ALSAC (American Lebanese Syrian Associated Charities). We thank Joseph Mertz, and Drs. Zhiping Wu and Vani Shanker for critical reading and editing.

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