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REVIEW

Unraveling the ubiquitin-regulated signaling networks by mass spectrometry-based proteomics

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Ubiquitin (Ub) is a small protein modifier that is covalently attached to the ε-amino group of lysine residues of protein substrates, generally targeting them for degradation. Due to the emergence of specific anti-diglycine (-GG) antibodies and the improvement in MS, it is now possible to identify more than 10 000 ubiquitylated sites in a single proteomics study. Besides cataloging ubiquitylated sites, it is equally important to unravel the biological relationship between ubiquitylated substrates and the ubiquitin conjugation machinery. Relevant to this, we discuss the role of affinity purification-MS (AP-MS), in characterizing E3 ligase-substrate complexes. Recently, such strategies have also been adapted to screen for binding partners of both deubiquitylating enzymes (DUBs) and ubiquitin-binding domains (UBDs). The complexity of the "ubiquitome" is further expanded by the fact that Ub itself can be ubiquitylated at any of its seven lysine residues forming polyubiquitin (polyUb), thus diversifying its lengths and topologies to suit a variety of molecular recognition processes. Therefore, applying MS to study polyUb linkages is also becoming an emerging and important area. Finally, we discuss the future of MS-based proteomics in answering important questions with respect to ubiquitylation.

Keywords:
Cell biology / Deubiquitylating / Diglycine / Post-translational modification / Proteasome / Ubiquitin

1 Introduction

Highly conserved among the eukaryotes, ubiquitin (Ub) is an 8.5 kDa protein that covalently modifies protein substrates so as to regulate their stability, localization, and activity [1]. Importantly, such regulation contributes to protein turnover, endocytosis, immune response, transcription, and DNA repair [2, 3]. Ubiquitin consists of 76 amino acids. During ubiquitylation, an isopeptide bond is formed between the *C*-terminus diglycine (di-Gly) of Ub and the ε-amino group of the targeted Lys residue. Although rare, it is also possible for an *N*-terminal amine or a Cys residue to be ubiquitylated. Analogous to protein phosphorylation, which is regulated by kinases, phosphatases and substrate-binding domains, ubiquitylation is finely orchestrated by a similar writer/eraser/reader paradigm (Fig. 1). First, three classes of enzymes are required for ubiquitylation: Ub activating enzyme (E1), Ub conjugating enzyme (E2), and Ub ligase E3 [4]. They are the "writers". The human genome encodes two E1, approximately 40 E2 and 700 E3 enzymes [5], whereby the

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Abbreviations: AP-MS, affinity purification-mass spectrometry; **CRLs**, cullin RING ubiquitin ligases; **di-Gly**, diglycine; **DUB**, deubiquitylating enzyme; **FBP**, F-box protein; **IAA**, iodoacetamide; **polyUb**, polyubiquitin; **PPI**, protein–protein interaction; **SCF**, SKP1–CUL1–F-box protein; **Ub**, ubiquitin; **UBD**, ubiquitin-binding domain

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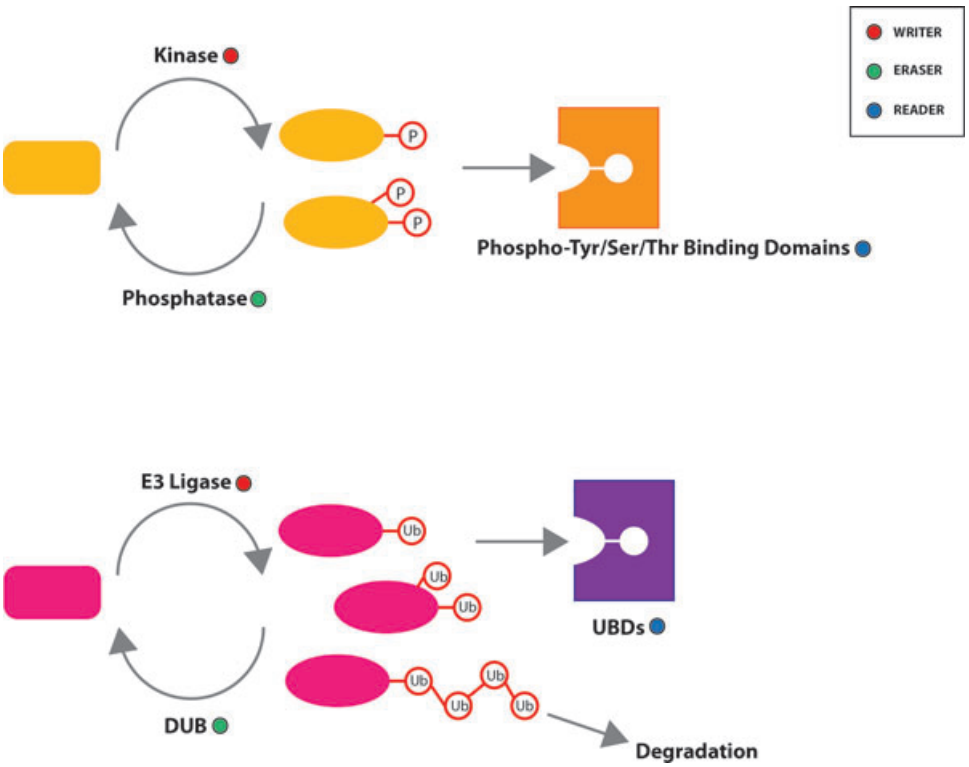

Figure 1. Conceptual similarities between regulatory networks involved in phosphorylation and ubiquitylation. Generally, regulation of PTMs follows a reader/writer/eraser paradigm where the "writers", such as kinases or E3 ligases catalyze the covalent transfer of modifying moieties (phosphates or ubiquitin) onto target proteins while the "erasers" such as phosphatases or deubiquitylating enzymes (DUBs) reverse the modifications. The modifying moieties serve as marks that are then interpreted by "reader" modules such as SH2's, WW's, or FHA's that specifically recognize phosphorylated motifs or ubiquitin-binding domains (UBDs) that recognize ubiquitin tags of different lengths and topologies.

E2 and E3 enzymes confer substrate specificity. Ubiquitin can be attached to one or multiple Lys residues in proximity, forming monoubiquitin (monoUb), or multiple monoubiquitin, respectively. Moreover, all seven internal Lys residues (K48, K63, K6, K11, K27, K29, and K33) of an Ub moiety can be further conjugated to form polyubiquitin (polyUb) [6]. Ubiquitylation can be reversed by approximately 100 deubiquitylating enzymes (DUBs), the "erasers" [7]. Meanwhile, the "readers" consist of approximately 20 ubiquitin-binding domains (UBD) that are used for Ub binding and signal interpretation [8]. Based on the sheer numbers and diversity of writers, erasers, and readers, it can be hypothesized that ubiquitylation is as fine tuned and tightly regulated as phosphorylation.

2 Proteomics of PTMs

One strength of MS-based proteomics is its capability to globally and simultaneously analyze PTMs, which are known to expand the physicochemical and functional diversity of proteins beyond approximately 20 000 encoding genes. Ideally, PTM-oriented proteomics is capable of deciphering the localization of every PTM and precisely quantifying its level and occupancy within a defined cellular space and time. In reality, among the 552 naturally occurring PTM records in the RESID database [9], only phosphorylation [10], *N*-terminal, and Lys acetylation [11] and glycosylation [12] are studied en masse. Due to their generally low abundance and diverse chemistries, each PTM necessitates dedicated enrichment and analysis procedures.

2.1 Protein level enrichment of ubiquitylated proteome

Rational designs exploiting the chemistry of ubiquitin for enriching ubiquitin-modified proteins/peptides have yet to emerge. However, since Ub is a protein, methods tailor made for affinity purification of proteins can also be applied to capture the ubiquitin-modified proteome (ubiquitome). Generally, these methods apply either (i) epitope-tagged ubiquitin, (ii) Ub-specific antibodies, or (iii) UBD domains. A common pitfall of such methods is nonspecific binding, which is diminishable by stringent washing steps or by referring to a contaminant list obtained from control experiments. Still, another more confounding problem arises from a wide variety of undesirable secondary binders that interact specifically with Ub or Ub proteins. Fortuitously, trypsin cleaves after R_{74} of ubiquitin, leaving the *C*-terminal di-Gly intact on the modified Lys [13]. Following MS/MS, this di-Gly remnant can be identified for localizing and validating the ubiquitylation sites.

Peng et al. pioneered the use of His-tagged Ub to target the Ub-proteome in yeast. This resulted in the identification of 1075 Ub protein candidates, and 110 Ub sites [13]. Different epitope tags have been explored to tag Ub, including FLAG, HA, *Myc*, and biotin tags [14]. Currently, the His-tagged Ub

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is the most widely applied technique partly because the coordination of His-rich motifs to immobilized Ni^{2+} ions is unaffected by denaturing conditions [15]. Performing affinity purification under denaturing conditions has benefits as it (i) reduces nonspecific protein–protein interactions and (ii) inhibits DUBs [16]. To achieve even cleaner purification, different epitope tags have been fused to create a tandem-affinity purification tag (TAP-tag) that allows immunoprecipitations to be performed in two consecutive steps [17]. Applying His-biotin-tagged Ub on a yeast sample under fully denaturing conditions, Tagwerker et al. identified 285 Ub protein candidates [18], with a significant reduction in contaminants compared to Peng et al.'s earlier study [13]. Danielsen et al. reported, using Strep-HA-tagged Ub, 753 Ub sites from an U2OS cell line [19]. Recently, FLAG-His-tagged Ub was expressed in a K0-Ub yeast mutant in which all seven internal Lys in Ub were replaced with Arg [20], preventing the formation of polyUb. This allows the steady-state level of Ub substrates to accumulate against proteasomal degradation. In addition, it improves the dynamic range of Ub proteins against Ub, which is the most ubiquitylated substrate in wild-type yeast. This strategy enabled Oshikawa et al. to identify up to 1392 Ub sites. However, the epitope-tagging approach has its caveats, as overexpression of exogenous, tagged Ub may affect cellular physiology. Besides, endogenous Ub can compete with tagged Ub for ubiquitylation sites. In yeast, endogenous Ub can be easily knocked out, but this is more difficult in mammalian cells as they harbor four loci for Ub [21]. In contrast to epitope tagging, Ub-specific antibody-based strategies can be applied to samples not amenable for genetic engineering, such as tissues or primary cells. These antibodies, such as FK2 [22], recognize only conjugated Ub but not the unconjugated forms, the latter being far more abundant [23]. Better still, the binding capacity of the FK2 antibody is unaffected by denaturing conditions as 345 Ub proteins were purified in 8 M urea, as demonstrated by Matsumoto et al. [24]. Recently, K11, K48, and K63 linkage-specific antibodies have emerged [25–27], though not yet thoroughly tested.

Eukaryotic cells evolved more than 20 different UBD families for recognition and binding to ubiquitin modifications. Most of these UBDs have weak affinity for polyUb [8, 28]. To use UBDs for enrichment of Ub proteins, multiple UBDs were fused to create tandem repeated ubiquitin-binding entities to enhance binding avidity [29, 30]. Shi et al. reported the isolation of approximately 300 ubiquitination sites with four tandem ubiquilin-1 fused to a GST tag [31]. Despite the functional heterogeneity of Ub signaling, the diversity and combinatorial capability of UBDs present opportunities to isolate Ub proteins involved in a specific pathway. In recent work, Akimov et al. made ingenious use of different UBDs by first depleting abundant Lys-48 polyUb protein conjugates using GST-UBA domains of human Rad23A (hHR23A). The pre-cleaned lysates were then incubated with recombinant GST-tagged UBDs from the endocytic adaptor proteins Eps15 and Epsin-1, so as to unravel the role of ubiquitin signaling in the EGFR network [32].

2.2 Peptide level enrichment of ubiquitylated proteome

For Ub proteins that are enriched at the protein level, the ensuing proteolytic process often leads to low-abundant di-Gly-modified peptides and highly abundant unmodified peptides. Thus, unmodified peptides tend to suppress di-Gly-modified peptides during MS analysis. To address these shortcomings, Xu et al. developed a monoclonal antibody (i.e. GX41) that specifically recognizes peptides harboring the di-Gly-modified Lys, but not internal Gly-Gly-Lys residues [33]. Using GX41 for peptide-based enrichment, Wagner et al. identified 11 054 endogenous ubiquitylation sites from trypsinized cell lysate [34]. Concurrently, by using a commercial monoclonal antibody, Kim et al. also reported approximately 19 000 Ub sites [35]. These studies demonstrate that enrichment at the peptide level is efficient, requiring only a single step of affinity enrichment, and is amenable to samples from any cells or tissues [36]. More recently, to study the effects of proteasome inhibition by MG-132 and DUB inhibition by PR-619, Udeshi et al. employed limited pre-fractionation of peptides by SCX prior to enrichment with anti-di-Gly remnant antibodies [37]. This combination resulted in the identification of 5533 di-Gly-modified peptides, an increase of threefold to fourfold compared to unfractionated samples.

2.3 Ubiquitomics: Caveats and challenges

Main challenges for ubiquitomics arise from the low steady-state levels of protein ubiquitylation, attributed to its (i) inherently low stoichiometry, (ii) rapid degradation by the proteasomal machinery, and (iii) deubiquitylation by DUBs. By treating cells with proteasome inhibitors such as MG132 during sample preparation, the accumulation of Ub substrates can be enhanced [38]. DUBs can be inhibited by chemical inhibitors such as *N*-ethylmaleimide, choloroacetamide, iodoacetamide (IAA) or 1,10-o-phenanthroline [7, 39] or by performing experiments under fully denaturing conditions. However, since DUBs are associated with Ub molecules, it is difficult to fully inactivate them [16]. Also, there is still some confusion about the specificity of trypsinization of Ub. Previously, it was reported that the longer -LR_{74}GG remnant (+383.228 Da), which originates from the miscleavage of the R_{74} residue, can be detected together with the more common di-Gly remnant [40]. More recently, upon systematic analyses of Ub conjugates, the -LGRR remnant was rarely identified [41]. Instead, R_{74} was shown to be the most accessible residue for trypsin in both native and denaturing conditions [42, 43]. Similarly, it was claimed that trypsin cleaves *C*-terminal to ubiquitylated Lys residues of synthetic Ub [44]. This was, however, contradicted by observation made by Seyfried et al. [41], supported by recent large-scale studies showing that trypsin does not cleave the vast majority of di-Gly-modified lysines [34–36]. Thus, the presence of miscleavage at a di-Gly-modified lysine can, in fact, provide further confidence in site localization.

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It is noteworthy that, Ile (113.084 Da), Leu (113.084 Da), Asp (115.027 Da), and Asn (114.043 Da) have molecular mass very similar to di-Gly (114.043 Da). Peptides containing these amino acids adjacent to an internal Lys can be mis-assigned as Ub peptides if they are analyzed with MS with low mass accuracy and resolution. Except for Asn, which is indistinguishable from di-Gly by mass, this ambiguity can be resolved by acquiring selected precursors in SIM scan, or by using MS with higher resolution and mass accuracy. Assignment of Ub sites can be improved by complementing fragmentation by collision-induced dissociation with electron transfer dissociation [45] to obtain unambiguous product ion series or further confirmed by SRM. Excessive alkylation by IAA has been reported to potentially induce an artifact having the exact chemical composition ($C_4H_6N_2O_2$) as di-Gly [46]. This problem can be solved by replacing IAA with chloroacetamide or by reducing the concentration of IAA. Nonetheless, even with unprecedented mass accuracy, resolution, and complete product ion series, not all Ub peptides can be identified with absolute confidence. For example, NEDD8 and ISG15 modifications also produce di-Gly remnants on modified lysines after trypsinization, and are therefore indistinguishable by MS from Ub. Moreover, identification of the di-Gly remnant itself is insufficient to reveal if the modified lysine is mono- or polyubiquitylated.

Recently, Shi et al. described how different database search algorithms contribute to the discrepancy in sensitivity and specificity in ubiquitome analysis [21], even when followed by peptide validation strategies such as target-decoy search [47] and Percolator that uses semi-supervised machine learning to discriminate between correct and decoy spectrum identifications [48]. Based on our past experiences with PTM analysis, especially phosphorylation, different algorithms written for modification site localization scoring [49] can also be useful for Ub site determination. As the emerging discipline of ubiquitomics presents new challenges, drafting consensus guidelines within the community for data analysis, validation, and reporting would be very welcome.

3 Ubiquitin chain linkages

The identification of ubiquitylation sites by di-Gly signatures on substrates, though useful, does not directly reveal the full picture since ubiquitin moieties can form poly-chains of various lengths and topologies, each giving rise to a wide variety of molecular signals [8]. Ikeda et al. classified Ub chains into four distinct groups [50] (Fig. 2). In homotypic polyUb chains, Ub molecules are sequentially conjugated to another Ub molecule on lysine residues of a fixed position. In mixed-linkage chains, different lysine residues in the Ub monomers are conjugated, thus forming forks or bifurcations. In some cases, polyUb chain can also incorporate other ubiquitin-like (Ubl) modifiers, such as SUMO and NEDD8, forming heterologous Ub chains. Finally, multiple monoubiquitin that are spatially close can be considered as

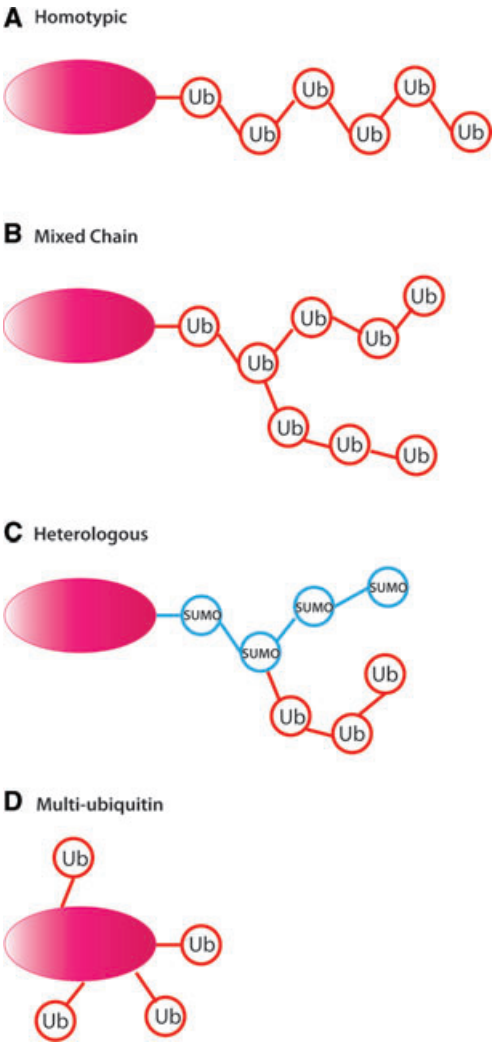

Figure 2. Nature's variety of ubiquitin chain linkages. Illustrated are the ubiquitin chain linkages as proposed by Ikeda et al. [50]. (A) Homotypic linkages consist of typical chains such as Lys 48- and Lys 63-linked Ub chains or atypical chains such as Lys 6-, Lys 11-, Lys 27-, Lys 29-, and Lys 33-linked chains. (B) Mixed-linkage chains are formed by different lysines for consecutive Ub conjugation, resulting in forked chains. (C) Heterologous chains are produced when Ub is conjugated to other ubiquitin-like proteins such as small ubiquitin-like modifier (SUMO) and (D) Multiple monoubiquitination moieties on the same protein substrate.

Ub signals. The precise chain length and topology of polyUb chains are crucial for recognition by different "readers" that subsequently determine the fates of the ubiquitylated substrates. For instance, monoUb has been shown to control diverse cellular processes such as receptor transport, viral budding, and DNA repair [51]. Homotypic K48 polyUb chains with at least four Ub moieties are considered a canonical signal for proteasomal degradation by 26S proteasomes [52]. Meanwhile, K63 polyUb acts in a wide variety of nonproteolytic processes such as protein sorting, DNA repair, kinase

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activation, as well as translational control [50], though it may also target substrates to proteasomal degradation [53]. To further add to the complexity, it has also been reported that substrates that are degraded in the ubiquitin fusion degradation pathway may be tagged with mixed chains that are initiated by K29 but elongated with K48-linked chains [54]. In another atypical Ub chain linkage, ubiquitins are linked linearly by *C*-terminal Gly to amino-terminal Met residue of the substrate-conjugated ubiquitin [55]. This linear Ub linkage was demonstrated to be essential in nuclear factor-kappa B activation and cellular responses to inflammatory cytokines [56, 57].

3.1 Quantifying and deciphering ubiquitin chain linkages with MS

Upon trypsinization, both monoUb and polyUb produce linkage-specific peptides with characteristic mass and sequences. These signature peptides can be used to identify and quantify individual chain linkages. Based on semi-quantitative spectral counts, all seven Lys residues have been found to form Ub–Ub linkages, with the following relative order of abundance: K48 > K11 and K63 ≫ K6, K27, K29, and K33 in *Saccharomyces Cerevisiae* [13]. To achieve precise and accurate quantitation of Ub linkages, another MS-based strategy has been developed that combines absolute quantification of proteins and their modifications (AQUA) [58] with SRM [59]. This so-called Ub-AQUA-SRM method comprises two basic steps. First, isotopic variants of di-Gly-modified Ub peptides corresponding to substrate-, site- and linkage-specific native peptides, are synthesized as internal standards. These peptides are chemically identical to their native counterparts, but are distinguishable by their mass. Second, these internal standards are spiked, at a defined amount, into a sample, which is later trypsinized. This digested sample is then subjected to LC-SRM-MS analysis where co-eluted native di-Gly-containing peptides and internal standards are monitored by SRM to quantify Ub linkages. This approach is very useful, albeit only to identify and quantify known chain linkages.

Ub-AQUA-SRM has stringent pre-requirements. Complete digestion of target proteins is essential, therefore digestion conditions have to be carefully optimized [60]. By systematic optimizations, Xu et al. found that for in-solution digestion, trypsinization in 2 M urea is the most efficient [61], while Phu et al. used 20 ng/μL trypsin diluted in 50 mM ammonium bicarbonate in 5% ACN at pH 8.0 to yield the best results [62]. In addition, peptides representing each Ub-linkage is systematically characterized beforehand for optimal detection and quantitation [61, 62]. It was found that even under optimal conditions, partially digested peptides were observed at K63 and K33 cleavage sites. These miscleavages were attributed to acidic residues flanking K63 and K33. The peptides for K6 linkage, meanwhile, contain a *N*-terminal Met that is susceptible to oxidation to form sulfoxide or sulfone. This complicates quantitation as the distribution of oxidation states cannot be precisely determined. To alleviate this problem, peptides can be treated with 5% formic acid or 5% hydrogen peroxide to fully oxidize Met residues. Finally, the K29-linkage peptide [SK(-GG)IQDK] is very hydrophilic and therefore not well retained by C18 materials. Lowering the organic content of loading buffer improves binding.

Notwithstanding these practical challenges, the Ub-AQUA-SRM method has been successfully applied to measure linkages of total Ub conjugates in yeast, revealing that the ratio of K6:K11:K27:K29:K33:K48:K63 linkages was 11%:35%:7%:4%:1%:30%:11% [63], in agreement with the previously reported data based on spectral counts. This technique was also applied to quantify polyUb topologies attached to the EGF receptor [64] and cyclin B1 [59]. While the EGF receptor is modified with approximately 49% mono-Ub, 40% K63, 6% K48, 3% K11, and 2% K29 linkages, cyclin B1 was found to be conjugated by mono-Ub and short polymers linked by K11, K48, and K63 during in vitro ubiquitylation. Although the stoichiometry of ubiquitin linkages for selected proteins can be determined by Ub-AQUA-SRM, some structural information is missing, especially for forked linkages [16]. To resolve this, a so-called middle-down MS approach was proposed for analyzing Ub-chain linkages [43]. This strategy consists of three steps. In step 1, limited proteolysis of target proteins is performed with trypsin under well-controlled native condition so that only the R_{74} site is cleaved. Therefore after digestion, two types of Ub peptides are produced. The first type is named UbR74, as it comprises the first 74 residues of Ub without any di-Gly remnants. These peptides either originate from monoUb or the distal end-cap of polyUb. The second type is named UbR74-GG because it contains one or more di-Gly remnants on at least one of the seven Lys residues of Ub. UbR74-GGs originate from the internal units of polyUb. In step 2, the tryptic lysate is analyzed using LC-MS/MS. In step 3, the extracted ion chromatograms from different charge states of both UbR74 and UbR74-GG are computed and compared. This is possible because both UbR74 and UbR74-GG co-elute in C8 chromatography and ionize with similar efficiency despite the additional di-Gly tags. This allows both species to be directly quantified and compared in the same MS survey scans without spiking in any isotopic internal standards. In the simplest instance, the ratio between UbR74 and UbR74-GG reflects the actual length of homotypic linkages. Based on the ratio of UbR74 and different variants of UbR74-GG, this analysis can be mathematically extended to other chain linkages to reveal their lengths and topologies.

Independently, Kaiser et al. developed ubiquitin protein standard absolute quantification [65]. Ubiquitin protein standard absolute quantification shows two novel aspects compared to Ub-AQUA-SRM although both methods measure Ub linkages with LC-MS/MS after trypsinzation of Ub proteins. First, specialized UBDs are used to enrich separately different variants of Ub so as to determine the concentration

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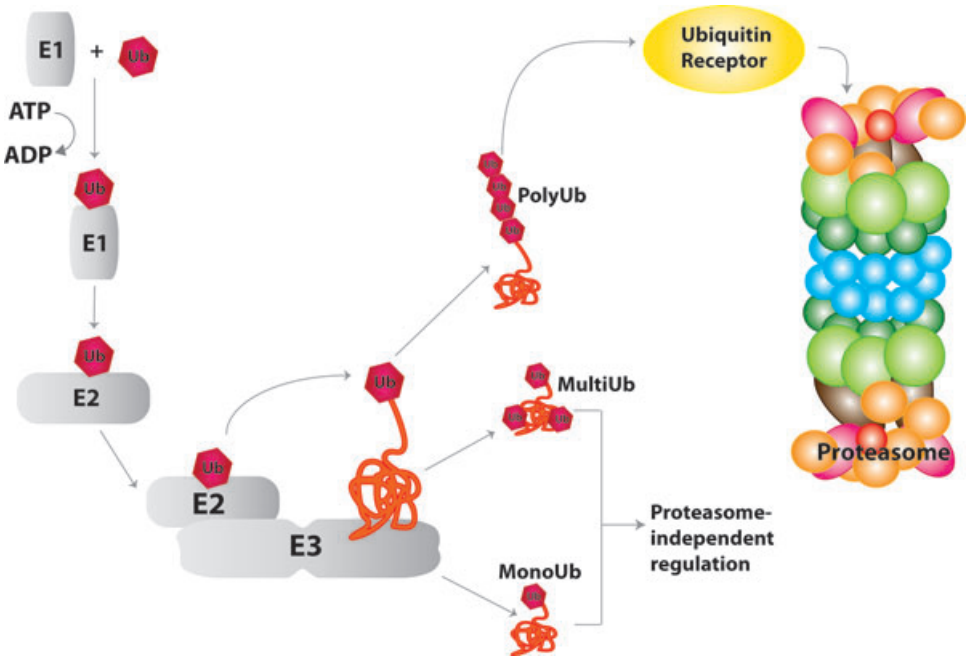

Figure 3. Schematic overview of the ubiquitin proteasome system (UPS). During Ub activation, an E1 enzyme forms a thioester bond between its catalytic cysteine and an Ub molecule in an ATP-dependent manner. Then, E1 enzyme binds to an E2 conjugating enzyme, to which the Ub is transferred. Subsequently, E2 enzymes bind to E3 ligases and pass activated Ub to a protein substrate also bound to E3. While mono- and multi-ubiquitylated substrates are subjected to non-proteasomal regulation, polyubiquitylated substrates are degraded by the 26S proteasome.

of monoUb and free Ub pools, which are not quantified in Ub-AQUA-SRM. Furthermore, heavy isotope-labeled protein that represents each of these three major ubiquitin populations is spiked in as standards instead. By using protein standards [66] rather than peptide standards, one can account for the loss of proteins through sample processing, fractionation, or DUB activity, to achieve more precise determination of absolute abundance.

4 Ubiquitin network: Wiring by writers, readers, and erasers

The afore-mentioned MS-centric strategies enable large-scale identification of ubiquitin sites and their chain linkages, but fail to describe the complex ubiquitin network that is characterized by the writer/eraser/reader paradigm. As described earlier, the "writers" comprise a conjugation machinery of E1-E2-E3 enzymes. An E1 enzyme forms a thioester bond between its catalytic cysteine and an Ub molecule in an ATP-dependent manner. Ub charging induces structural changes in E1 that subsequently promote binding of E1 to an E2 conjugating enzyme, to which Ub is transferred. The specificity of the ubiquitylation is then provided by the E3 ligase enzyme, which physically interacts with both the target substrate and the activated ubiquitin–E2 complex, to complete the transfer of ubiquitin to the target protein (Fig. 3). Several rounds of ubiquitin conjugation can produce long chains of ubiquitin moieties (polyubiquitylation), the first of which is covalently bound to the substrate. At this point, the polyubiquitylated protein must also be able to interact with the proteasome or with shuttling factors that deliver it to the proteasome; whereas monoubiquitylated and multiubiquitylated proteins have nonproteolyic fates. The human genome is estimated to encode for more than 700 E3 ligases, which are classified in four major classes on the basis of their characteristic structural motif and by the presence of unique domains: the RING-finger proteins, HECT-domain proteins or U-box-type and PHD finger-type [67,68]. Many multisubunit, RING-finger type ubiquitin ligases contain a cullin (Cul) protein subunit, a name derived from the ability of cullin RING ubiquitin ligases (CRLs) to "cull" or sort substrates for degradation. In mammals, there are six different CRLs, including the SKP1–CUL1–F-box protein (SCF) complex.

4.1 Identification of E3 ligases and substrate interaction

Matching each ubiquitin E3 ligase to its respective substrates helps unravel mechanism and physiology of the ubiquitin system. It is believed that most modifying enzymes, such as kinases, interact transiently with their substrates in a "hit-and-run" manner [69]. Therefore, it is more feasible to assay the products of ligase activity rather than ligase–substrate interaction itself. An E3 ligase can be perturbed, for instance by RNAi, chemical inhibitors or genetic manipulations, and subsequently the perturbed samples and the controls may be evaluated by quantitative MS for any changes. If the ubiquitylated substrates are degraded by the proteasomal pathway, the differential levels of proteins can be used as readouts for putative substrates. Burande et al. created a myeloid leukemia cell line expressing an ASB2 BC box mutant (ASB2LA) that is unable to interact with the Elongin BC complex,

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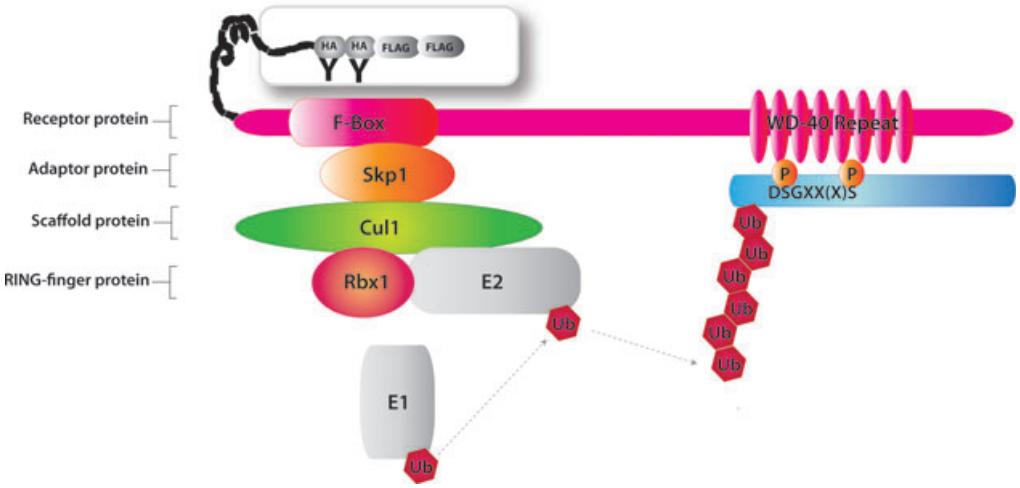

Figure 4. Defining ubiquitin-regulating signaling networks by affinity purification and MS. A schematic of an HA-flag-tagged F-box protein (FBPs) used for affinity-based MS studies featuring β-TrCP as FBP. SCF ligases are composed of Skp1, Cullin 1, a RING-finger protein (Rbx1) and a variable component known as F-box protein. Cul1 is a scaffold protein that interacts via its *N*-terminus with the adaptor protein Skp1, and via its *C*-terminus with the RING-finger protein Rbx1, as well as a specific E2 enzyme. Skp1, in turn, binds to the F-box protein. F-box proteins recognize and recruit specific substrates that are subsequently ubiquitylated by E2 enzymes. The WD40 repeats β-propeller structure of β-TrCP specifically recognize a diphosphorylated motif with the consensus DpSGXX(X)pS, known as phosphodegron.

resulting in the accumulation of ubiquitylated proteins. Label-free proteomics was then used to compare proteomes of wild-type and mutant cell line [70]. Recently, Koo et al. reported on a membrane E3 ligase, RNF43, which is important in regulating the turnover of receptors involved in Wnt signaling. By using cell surface biotinylation to isolate membrane proteins followed by label-free quantitative MS, they demonstrated that LRP5, LRP6, and frizzleds are downregulated upon doxycycline-driven induction of RNF43 [71]. Nevertheless, these protein level-based assays require extensive validation due to the lack of evidence on ligase–substrate interaction and ubiquitylated sites, as proteins can also be indirectly downregulated upon transcriptional repression, or degraded by cellular proteases. One possible way to address this is to enrich for ubiquitylated peptides prior to quantitative MS. In one application, MLN4924, a chemical inhibitor, was used to inhibit the NEDD8 E1 enzyme, Nae1 [72]. The inhibition of Nae1 led to the inactivation of CRLs that are allosterically activated by neddylation [73]. Subsequently, ubiquitylated peptides from untreated controls and MLN4924 treated were separately enriched by anti-di-Gly remnant antibodies followed by quantitative MS using SILAC. A similar approach was used to study HRD1, an E3 ligase implicated in rheumatoid arthritis. In this study, RNAi was used to knock down HRD1, then cell lysates from both treated untreated cells were pooled, followed by enrichment for Ub-proteins and Ub-peptides prior to MS analysis [74].

Contrary to the conventional belief, ligase–substrate interactions can be partially retained upon IP. This feature allows us to employ affinity purification-mass spectrometry (AP-MS) to identify ubiquitin ligases targeting a specific substrate and vice versa. For instance, using AP-MS, Yaron et al. identified β-TrCP as the receptor component of the ubiquitin ligase that targets IκB for proteasomal degradation [75]. After this study, similar AP-MS approaches have led to the identification of additional substrates of $SCF^{\beta\text{-TrCP}}$ [76–78]. $SCF^{\beta\text{-TrCP}}$ belongs to the multi-subunit SCF ligase subfamily [68], whose members are composed of the invariable core subunits Cul1, Rbx1, and Skp1 and the variable subunit F-box protein (FBP) that determines substrate specificity (Fig. 4). Cul1 is a scaffold protein that interacts via its *N*-terminus with the adaptor protein Skp1, and via its *C*-terminus with the RING-finger protein Rbx1, which in turn binds to a specific ubiquitin-conjugating enzyme. Skp1 interacts with the F-box domain of one of approximately 70 FBPs, which recruit substrates via protein–protein interaction (PPI) domains such as WD-40 and leucine-rich repeats. The WD40 repeats of β-TrCP form a β-propeller structure that specifically recognizes a diphosphorylated motif with the consensus DpSGXX(X)pS, known as phosphodegron. Due to the modular structure of these ubiquitin ligases, tagging the actual substrate-binding subunit is expected to give the highest chance of success in identifying their specific substrates. Recently, this approach has led to the identification of novel substrates of $SCF^{\beta\text{-TrCP}}$ [79], $SCF^{FBXO1/cyclinF}$ [80, 81], SCF^{FBXW7} [82], SCF^{FBXO6} [83], SCF^{FBXO25} [84], SCF^{Skp2}, and SCF^{FBXL5} [85]. Two sequential immunoprecipitations are often used in these studies to reduce the co-purification of unspecific contaminants, although this may result in the loss of weakly interacting partners. One possible way to address this problem is to employ quantitative MS and analyze protein abundance in an experimental sample relative to a negative control [86]. Appropriate negative controls are FBPs lacking the F-box domain which, as a result, are unable to bind the other SCF subunits [85], or

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FBPs with specific point mutations in the WD40 repeats that fail to bind their substrates [87].

4.2 Identification and characterization of DUBs

Conjugated Ub moieties can be removed by DUBs, which are cysteine proteases or metalloproteases. DUBs comprise five major classes, based on their catalytic domain structure: ubiquitin *C*-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Machado-Joseph disease proteases, and JAB1/MPN/Mov34 metalloenzymes (JAMMs). DUBs are essential for (i) activating ubiquitin proproteins, (ii) recycling of ubiquitin adventitiously trapped in inactivatable forms, (iii) reversing ubiquitylation from target proteins, and (iv) regenerating of monoUb from free polyUb chains [88]. DUBs are less well characterized compared to ligases, though approximately 100 human DUBs are estimated to exist, by in silico methods [89] and activity-based protein profiling [90]. In activity-based protein profiling, epitope-tagged Ub are used as probes to capture DUBs. These synthetic probes harbor compounds such as vinyl methylsulfone, vinyl methylester, alkyl halides, and vinyl cyanides as substitutes for *C*-terminal Gly residue of Ub. These thiol-reactive groups can act as "suicide" substrates when positioned within the DUB active site. Subsequently, probe-DUB complexes can be purified by their epitope tags followed by identification with LC-MS/MS. This approach has been used to capture 23 DUBs from mouse lymphoma cell lysates, at the same time, discovering new gene products (Otubain) that contain the ovarian tumor domains [91].

Despite their well-defined identities and biological roles, very little is known about the enzymatic targets and modes of regulation for DUBs [92]. Large-scale PPI screens have been performed on 75 of the 95 DUBs encoded by the human genome in order to identify stably associated interacting proteins [93]. Ensuing this, the specific biological functions of the DUBs could be partly inferred from interacting protein partners based on the principle of "guilt-by-association" [94]. Indeed, this study led to the identification of 774 unique high-confidence candidate interacting proteins, one-third of them being involved in protein turnover, transcription, RNA processing, or DNA damage response, providing insights into the possible biological functions and regulatory mechanisms of DUBs. Besides, 26 DUBs were found to associate with one or more proteins that contain domains associated with ubiquitin conjugation, including HECT and Cullin-based E3 ubiquitin ligases, suggesting the existence of larger ubiquitin-regulating signaling scaffolds, enabling cross-regulation within the ubiquitin system.

In a multifaceted study, the entire family of 20 putative DUBs in *Schizosaccharomyces pombe* was studied by determining their endogenous localizations, in vitro activity, and PPIs [94]. This combined study revealed that DUBs are present in nearly every cellular compartment. Besides, stable PPIs for over 55% of the DUBs were identified, including previously uncharacterized protein complexes essential for DUB function, establishing these family members as bona fide deubiquitinating enzymes. In another study, SILAC-based quantification was performed for wild-type *S. cerevisiae* against 20 other strains in which DUBs had been systematically deleted [95]. Major changes in protein expression abundance were observed between individual deletion strains. These effects were attributed to the direct influence of DUBs on their substrates, as well as the indirect effects exerted via altered transcription.

5 Bioinformatic resources designed for the analysis of ubiquitylation

So far, conventional databases such as Swiss-Prot [96] and RESID [9] are two of the most highly curated sources for PTMs. According to the Proteome-wide PTM Statistics Curator [97], there are currently 878 high quality, experimentally observed Ub sites and 1594 putative Ub sites annotated in the Swiss-Prot database. Meanwhile, PhosphoSite Plus has already annotated 19 362 Ub sites [98]. On the other hand, E3Net provides a comprehensive collection of E3-substrate connections and a framework for the analysis of E3-mediated regulatory networks [99]. Currently, E3Net contains 2201 E3s and 4896 substrates in 427 organisms and 1671 E3-substrate specific relations between 493 E3s and 1277 substrates in 42 organisms. Likewise, *S. Cerevisiae* Ubiquitination Database [100] and Database of Plants Ubiquitin Proteasome System (plantsUPS) [101] are specifically designed for storing information for the ubiquitin-proteasome system in yeast and higher plants, respectively. In addition to providing reliable information to assist researchers in data-mining and analysis, collection of highly curated data can also serve as training datasets to help build new bioinformatics tools for predicting Ub sites, motifs, or ligase–substrate relationship. Some of these tools are E3Miner [102], UbiPred [103], and UbPred [104].

6 Conclusion and future perspectives

Here we reviewed how an arsenal of MS-based tools is now enhancing our understanding of different aspects of ubiquitin-regulated biology. Finally, we outline below several pending issues, which may be addressed by high-throughput MS in the near future:

(i) The ability to generate high-density ubiquitomics data [33–36] will allow the examination of positive and negative crosstalk between ubiquitylation and other PTMs such as phosphorylation and Lys-acetylation where dense data have already been acquired [105].
(ii) Despite earlier studies indicated the absence of sequence motifs for ubiquitylation [19,20,33], two independent, large-scale studies demonstrated respectively, the

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enrichment for nonpolar amino acids [34] and acidic amino acids [35] surrounding the di-Gly-modified lysines. A subsequent study implies that these sequence biases can be partially explained by the different sources of anti-di-Gly antibodies used [36]. More upcoming data should help resolve this pending discrepancy.

(iii) Due to its omnipresence in cells, the ubiquitin network is tightly regulated at every level. For instance, some of the E2 and E3 enzymes are expressed in a tissue-specific manner, while others are compartmentalized within specific subcellular structures [106]. Besides, many E3-DUB pairs work closely together to fine-tune the timing of ubiquitin loading and removal. All these different mechanisms of regulation can be studied by spatiotemporal proteomics [107].

Although MS-based proteomics provides valuable insights for Ub-sites, chain linkages and possible network relationship, classical biochemical assays, and molecular biology tools such as in vitro ubiquitylation assays and site-directed mutagenesis remain indispensable for ubiquitin research since they are crucial for validating proteomics data and for probing the diverse influences of ubiquitylation on the biological systems investigated. The pace at which MS-based proteomics is developing is astonishing. As for unraveling the biology of ubiquitin network biology, the work reviewed here represents just a beginning, rather than an end to this challenging and important field.

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7 References

[1] Hershko, A., Ciechanover, A., The ubiquitin system. *Annu. Rev. Biochem.* 1998, *67*, 425–479.

[2] Haglund, K., Dikic, I., Ubiquitylation and cell signaling. *EMBO J.* 2005, *24*, 3353–3359.

[3] Mukhopadhyay, D., Riezman, H., Proteasome-independent functions of ubiquitin in endocytosis and signaling. *Science* 2007, *315*, 201–205.

[4] Pickart, C. M., Eddins, M. J., Ubiquitin: structures, functions, mechanisms. *Biochim. Biophys. Acta* 2004, *1695*, 55–72.

[5] Semple, C. A. M., The comparative proteomics of ubiquitination in mouse. *Genome Res.* 2003, *13*, 1389–1394.

[6] Pickart, C. M., Fushman, D., Polyubiquitin chains: polymeric protein signals. *Curr. Opin. Chem. Biol.* 2004, *8*, 610–616.

[7] Love, K. R., Catic, A., Schlieker, C., Ploegh, H. L., Mechanisms, biology and inhibitors of deubiquitinating enzymes. *Nat. Chem. Biol.* 2007, *3*, 697–705.

[8] Dikic, I., Wakatsuki, S., Walters, K. J., Ubiquitin-binding domains – from structures to functions. *Nat. Rev. Mol. Cell Biol.* 2009, *10*, 659–671.

[9] Garavelli, J. S., The RESID Database of Protein Modifications as a resource and annotation tool. *Proteomics* 2004, *4*, 1527–1533.

[10] Olsen, J. V., Vermeulen, M., Santamaria, A., Kumar, C. et al., Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci. Signal* 2010, *3*, ra3.

[11] Mischerikow, N., Heck, A. J. R., Targeted large-scale analysis of protein acetylation. *Proteomics* 2011, *11*, 571–589.

[12] Zielinska, D. F., Gnad, F., Wiśniewski, J. R., Mann, M., Precision mapping of an in vivo *N*-glycoproteome reveals rigid topological and sequence constraints. *Cell* 2010, *141*, 897–907.

[13] Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C. et al., A proteomics approach to understanding protein ubiquitination. *Nat. Biotechnol.* 2003, *21*, 921–926.

[14] Xu, P., Peng, J., Dissecting the ubiquitin pathway by mass spectrometry. *Biochim. Biophys. Acta* 2006, *1764*, 1940–1947.

[15] Jeon, H. B., Choi, E. S., Yoon, J. H., Hwang, J. H. et al., A proteomics approach to identify the ubiquitinated proteins in mouse heart. *Biochem. Biophys. Res. Commun.* 2007, *357*, 731–736.

[16] Peng, J., Evaluation of proteomic strategies for analyzing ubiquitinated proteins. *BMB Rep.* 2008, *41*, 177–183.

[17] Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M. et al., A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* 1999, *17*, 1030–1032.

[18] Tagwerker, C., Flick, K., Cui, M., Guerrero, C. et al., A tandem affinity tag for two-step purification under fully denaturing conditions: application in ubiquitin profiling and protein complex identification combined with in vivocross-linking. *Mol. Cell Proteomics* 2006, *5*, 737–748.

[19] Danielsen, J. M. R., Sylvestersen, K. B., Bekker-Jensen, S., Szklarczyk, D. et al., Mass spectrometric analysis of lysine ubiquitylation reveals promiscuity at site level. *Mol. Cell Proteomics* 2011, *10*, M110.003590.

[20] Oshikawa, K., Matsumoto, M., Oyamada, K., Nakayama, K. I., Proteome-wide identification of ubiquitylation sites by conjugation of engineered lysine-less ubiquitin. *J. Proteome Res.* 2012, *11*, 796–807.

[21] Shi, Y., Xu, P., Qin, J., Ubiquitinated proteome: ready for global? *Mol. Cell Proteomics* 2011, *10*, R110.006882.

[22] Fujimuro, M., Sawada, H., Yokosawa, H., Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. *FEBS Lett.* 1994, *349*, 173–180.

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[23] Hatakeyama, S., Matsumoto, M., Nakayama, K. I., in: Deshaies, R. J. (Ed.), *Ubiquitin and Protein Degradation, Part B*, vol. *399*, Academic Press, New York 2005, pp. 277–286.

[24] Matsumoto, M., Hatakeyama, S., Oyamada, K., Oda, Y. et al., Large-scale analysis of the human ubiquitin-related proteome. *PROTEOMICS* 2005, *5*, 4145–4151.

[25] Matsumoto, M. L., Wickliffe, K. E., Dong, K. C., Yu, C. et al., K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody. *Mol. Cell* 2010, *39*, 477–484.

[26] Matsumoto, M. L., Dong, K. C., Yu, C., Phu, L. et al., Engineering and structural characterization of a linear polyubiquitin-specific antibody. *J. Mol. Biol.* 2012, *418*, 134–144.

[27] Newton, K., Matsumoto, M. L., Ferrando, R. E., Wickliffe, K. E. et al., Using linkage-specific monoclonal antibodies to analyze cellular ubiquitylation. *Methods Mol. Biol.* 2012, *832*, 185–196.

[28] Hicke, L., Schubert, H. L., Hill, C. P., Ubiquitin-binding domains. *Nat. Rev. Mol. Cell Biol.* 2005, *6*, 610–621.

[29] Hjerpe, R., Aillet, F., Lopitz-Otsoa, F., Lang, V. et al., Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. *EMBO Rep.* 2009, *10*, 1250–1258.

[30] Lopitz-Otsoa, F., Rodriguez-Suarez, E., Aillet, F., Casado-Vela, J. et al., Integrative analysis of the ubiquitin proteome isolated using tandem ubiquitin binding entities (TUBEs). *J. Proteome.* 2011, *75*, 2998–3014.

[31] Shi, Y., Chan, D. W., Jung, S. Y., Malovannaya, A. et al., A data set of human endogenous protein ubiquitination sites. *Mol. Cell Proteomics* 2011, *10*, M110.002089.

[32] Akimov, V., Rigbolt, K. T. G., Nielsen, M. M., Blagoev, B., Characterization of ubiquitination dependent dynamics in growth factor receptor signaling by quantitative proteomics. *Mol. Biosyst.* 2011, *7*, 3223–3233.

[33] Xu, G., Paige, J. S., Jaffrey, S. R., Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling. *Nat. Biotech.* 2010, *28*, 868–873.

[34] Wagner, S. A., Beli, P., Weinert, B. T., Nielsen, M. L. et al., A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. *Mol. Cell Proteomics* 2011, *10*, M111.013284.

[35] Kim, W., Bennett, E. J., Huttlin, E. L., Guo, A. et al., Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol. Cell* 2011, *44*, 325–340.

[36] Wagner, S. A., Beli, P., Weinert, B. T., Scholz, C. et al., Proteomic analyses reveal divergent ubiquitylation site patterns in murine tissues. *Mol. Cell Proteomics* 2012, M112.017905. [Epub ahead of print].

[37] Udeshi, N. D., Mani, D. R., Eisenhaure, T., Mertins, P. et al., Methods for quantification of in vivo changes in protein ubiquitination following proteasome and deubiquitinase inhibition. *Mol. Cell Proteomics* 2012, *11*, M111.016857.

[38] de Bettignies, G., Coux, O., Proteasome inhibitors: dozens of molecules and still counting. *Biochimie* 2010, *92*, 1530–1545.

[39] Altun, M., Kramer, H. B., Willems, L. I., McDermott, J. L. et al., Activity-based chemical proteomics accelerates inhibitor development for deubiquitylating enzymes. *Chem. Biol.* 2011, *18*, 1401–1412.

[40] Warren, M. R. E., Parker, C. E., Mocanu, V., Klapper, D. et al., Electrospray ionization tandem mass spectrometry of model peptides reveals diagnostic fragment ions for protein ubiquitination. *Rapid Commun. Mass Spectrom.* 2005, *19*, 429–437.

[41] Seyfried, N. T., Xu, P., Duong, D. M., Cheng, D. et al., Systematic approach for validating the ubiquitinated proteome. *Anal. Chem.* 2008, *80*, 4161–4169.

[42] Wang, M., Cheng, D., Peng, J., Pickart, C. M., Molecular determinants of polyubiquitin linkage selection by an HECT ubiquitin ligase. *EMBO J.* 2006, *25*, 1710–1719.

[43] Xu, P., Peng, J., Characterization of polyubiquitin chain structure by middle-down mass spectrometry. *Anal. Chem.* 2008, *80*, 3438–3444.

[44] Denis, N. J., Vasilescu, J., Lambert, J.-P., Smith, J. C. et al., Tryptic digestion of ubiquitin standards reveals an improved strategy for identifying ubiquitinated proteins by mass spectrometry. *Proteomics* 2007, *7*, 868–874.

[45] Syka, J. E. P., Coon, J. J., Schroeder, M. J., Shabanowitz, J. et al., Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci. USA* 2004, *101*, 9528–9533.

[46] Nielsen, M. L., Vermeulen, M., Bonaldi, T., Cox, J. et al., Iodoacetamide-induced artifact mimics ubiquitination in mass spectrometry. *Nat. Methods* 2008, *5*, 459–460.

[47] Elias, J. E., Gygi, S. P., Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods* 2007, *4*, 207–214.

[48] Käll, L., Canterbury, J. D., Weston, J., Noble, W. S. et al., Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat. Methods* 2007, *4*, 923–925.

[49] Chalkley, R. J., Clauser, K. R., Modification site localization scoring: strategies and performance. *Mol. Cell Proteomics* 2012, *11*, 3–14.

[50] Ikeda, F., Dikic, I., Atypical ubiquitin chains: new molecular signals. "Protein Modifications: Beyond the Usual Suspects" review series. *EMBO Rep.* 2008, *9*, 536–542.

[51] Hicke, L., Protein regulation by monoubiquitin. *Nat. Rev. Mol. Cell Biol.* 2001, *2*, 195–201.

[52] Thrower, J. S., Hoffman, L., Rechsteiner, M., Pickart, C. M., Recognition of the polyubiquitin proteolytic signal. *EMBO J.* 2000, *19*, 94–102.

[53] Saeki, Y., Kudo, T., Sone, T., Kikuchi, Y. et al., Lysine 63-linked polyubiquitin chain may serve as a targeting signal for the 26S proteasome. *EMBO J.* 2009, *28*, 359–371.

[54] Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D. et al., A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* 1999, *96*, 635–644.

[55] Kirisako, T., Kamei, K., Murata, S., Kato, M. et al., A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J.* 2006, *25*, 4877–4887.

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[56] Tokunaga, F., Sakata, S., Saeki, Y., Satomi, Y. et al., Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. *Nat. Cell Biol.* 2009, *11*, 123–132.

[57] Rahighi, S., Ikeda, F., Kawasaki, M., Akutsu, M. et al., Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation. *Cell* 2009, *136*, 1098–1109.

[58] Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W. et al., Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. USA* 2003, *100*, 6940–6945.

[59] Kirkpatrick, D. S., Hathaway, N. A., Hanna, J., Elsasser, S. et al., Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. *Nat. Cell Biol.* 2006, *8*, 700–710.

[60] Havlis, J., Shevchenko, A., Absolute quantification of proteins in solutions and in polyacrylamide gels by mass spectrometry. *Anal. Chem.* 2004, *76*, 3029–3036.

[61] Xu, P., Cheng, D., Duong, D. M., Rush, J. et al., A proteomic strategy for quantifying polyubiquitin chain topologies. *Israel J. Chem.* 2010, *46*, 171–182.

[62] Phu, L., Izrael-Tomasevic, A., Matsumoto, M. L., Bustos, D. et al., Improved quantitative mass spectrometry methods for characterizing complex ubiquitin signals. *Mol. Cell. Proteomics* 2011, *10*, M110.003756.

[63] Xu, P., Duong, D. M., Seyfried, N. T., Cheng, D. et al., Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* 2009, *137*, 133–145.

[64] Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S. et al., Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. *Mol. Cell* 2006, *21*, 737–748.

[65] Kaiser, S. E., Riley, B. E., Shaler, T. A., Trevino, R. S. et al., Protein standard absolute quantification (PSAQ) method for the measurement of cellular ubiquitin pools. *Nat. Methods* 2011, *8*, 691–696.

[66] Brun, V., Dupuis, A., Adrait, A., Marcellin, M. et al., Isotope-labeled protein standards toward absolute quantitative proteomics. *Mol. Cell. Proteomics* 2007, *6*, 2139–2149.

[67] Nakayama, K. I., Nakayama, K., Ubiquitin ligases: cell-cycle control and cancer. *Nat. Rev. Cancer* 2006, *6*, 369–381.

[68] Cardozo, T., Pagano, M., The SCF ubiquitin ligase: insights into a molecular machine. *Nat. Rev. Mol. Cell Biol.* 2004, *5*, 739–751.

[69] Fukunaga, R., Hunter, T., MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *The EMBO J.* 1997, *16*, 1921–1933.

[70] Burande, C. F., Heuzé, M. L., Lamsoul, I., Monsarrat, B. et al., A label-free quantitative proteomics strategy to identify E3 ubiquitin ligase substrates targeted to proteasome degradation. *Mol. Cell. Proteomics* 2009, *8*, 1719–1727.

[71] Koo, B. K., Spit, M., Jordens, I., Low, T. Y. et al., Tumour suppressor RNF43 is a stem cell E3 ligase that induces endocytosis of Wnt receptors. *Nature* 2012, *488*, 665–669.

[72] Bennett, E. J., Rush, J., Gygi, S. P., Harper, J. W., Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics. *Cell* 2010, *143*, 951–965.

[73] Duda, D. M., Borg, L. A., Scott, D. C., Hunt, H. W. et al., Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation. *Cell* 2008, *134*, 995–1006.

[74] Lee, K. A., Hammerle, L. P., Andrews, P. S., Stokes, M. P. et al., Ubiquitin ligase substrate identification through quantitative proteomics at both the protein and peptide levels. *J. Biol. Chem.* 2011, *286*, 41530–41538.

[75] Yaron, A., Hatzubai, A., Davis, M., Lavon, I. et al., Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. *Nature* 1998, *396*, 590–594.

[76] Dorrello, N. V., Peschiaroli, A., Guardavaccaro, D., Colburn, N. H. et al., S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. *Science* 2006, *314*, 467–471.

[77] Peschiaroli, A., Dorrello, N. V., Guardavaccaro, D., Venere, M. et al., SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response. *Mol. Cell* 2006, *23*, 319–329.

[78] Guardavaccaro, D., Frescas, D., Dorrello, N. V., Peschiaroli, A. et al., Control of chromosome stability by the beta-TrCP-REST-Mad2 axis. *Nature* 2008, *452*, 365–369.

[79] Kruiswijk, F., Yuniati, L., Magliozzi, R., Low, T. Y. et al., Coupled activation and degradation of eEF2K regulates protein synthesis in response to genotoxic stress. *Sci. Signal* 2012, *5*, ra40.

[80] D'Angiolella, V., Donato, V., Vijayakumar, S., Saraf, A. et al., SCF (cyclin F) controls centrosome homeostasis and mitotic fidelity through CP110 degradation. *Nature* 2010, *466*, 138–142.

[81] D'Angiolella, V., Donato, V., Forrester, F. M., Jeong, Y.-T. et al., Cyclin F-mediated degradation of ribonucleotide reductase M2 controls genome integrity and DNA repair. *Cell* 2012, *149*, 1023–1034.

[82] Busino, L., Millman, S. E., Scotto, L., Kyratsous, C. A. et al., Fbxw7α- and GSK3-mediated degradation of p100 is a pro-survival mechanism in multiple myeloma. *Nat. Cell Biol.* 2012, *14*, 375–385.

[83] Liu, B., Zheng, Y., Wang, T.-D., Xu, H.-Z. et al., Proteomic identification of common SCF ubiquitin ligase FBXO6-interacting glycoproteins in three kinds of cells. *J. Proteome Res.* 2012, *11*, 1773–1781.

[84] Teixeira, F. R., Yokoo, S., Gartner, C. A., Manfiolli, A. O. et al., Identification of FBXO25-interacting proteins using an integrated proteomics approach. *Proteomics* 2010, *10*, 2746–2757.

[85] Yumimoto, K., Matsumoto, M., Oyamada, K., Moroishi, T. et al., Comprehensive identification of substrates for F-box proteins by differential proteomics analysis. *J. Proteome Res.* 2012, *11*, 3175–3185.

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[86] Paul, F. E., Hosp, F., Selbach, M., Analyzing protein-protein interactions by quantitative mass spectrometry. *Methods* 2011, *54*, 387–395.

[87] Wu, G., Xu, G., Schulman, B. A., Jeffrey, P. D. et al., Structure of a beta-TrCP1-Skp1-beta-catenin complex: destruction motif binding and lysine specificity of the SCF(beta-TrCP1) ubiquitin ligase. *Mol. Cell* 2003, *11*, 1445–1456.

[88] Reyes-Turcu, F. E., Ventii, K. H., Wilkinson, K. D., Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. *Annu. Rev. Biochem.* 2009, *78*, 363–397.

[89] Nijman, S. M. B., Luna-Vargas, M. P. A., Velds, A., Brummelkamp, T. R. et al., A genomic and functional inventory of deubiquitinating enzymes. *Cell* 2005, *123*, 773–786.

[90] Hemelaar, J., Galardy, P. J., Borodovsky, A., Kessler, B. M. et al., Chemistry-based functional proteomics: mechanism-based activity-profiling tools for ubiquitin and ubiquitin-like specific proteases. *J. Proteome Res.* 2004, *3*, 268–276.

[91] Borodovsky, A., Ovaa, H., Kolli, N., Gan-Erdene, T. et al., Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. *Chem. Biol.* 2002, *9*, 1149–1159.

[92] Ventii, K. H., Wilkinson, K. D., Protein partners of deubiquitinating enzymes. *Biochem. J.* 2008, *414*, 161–175.

[93] Sowa, M. E., Bennett, E. J., Gygi, S. P., Harper, J. W., Defining the human deubiquitinating enzyme interaction landscape. *Cell* 2009, *138*, 389–403.

[94] Kouranti, I., McLean, J. R., Feoktistova, A., Liang, P. et al., A global census of fission yeast deubiquitinating enzyme localization and interaction networks reveals distinct compartmentalization profiles and overlapping functions in endocytosis and polarity. *PLoS Biol.* 2010, *8*, e1000471.

[95] Poulsen, J. W., Madsen, C. T., Young, C., Kelstrup, C. D. et al., Comprehensive profiling of proteome changes upon sequential deletion of deubiquitylating enzymes. *J. Proteomics* 2012, *75*, 3886–3897.

[96] Farriol-Mathis, N., Garavelli, J. S., Boeckmann, B., Duvaud, S. et al., Annotation of post-translational modifications in the Swiss-Prot knowledge base. *Proteomics* 2004, *4*, 1537–1550.

[97] Khoury, G. A., Baliban, R. C., Floudas, C. A., Proteome-wide post-translational modification statistics: frequency analysis and curation of the Swiss-Prot database. *Sci. Rep.* 2011, *1*, 90.

[98] Hornbeck, P. V., Kornhauser, J. M., Tkachev, S., Zhang, B. et al., PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic Acids Res.* 2012, *40*, D261–D270.

[99] Han, Y., Lee, H., Park, J. C., Yi, G.-S., E3Net: a system for exploring E3-mediated regulatory networks of cellular functions. *Mol. Cell Proteomics* 2012, *11*, O111.014076.

[100] Lee, W.-C., Lee, M., Jung, J. W., Kim, K. P. et al., Kim, D., SCUD: Saccharomyces cerevisiae ubiquitination database. *BMC Genomics* 2008, *9*, 440.

[101] Du, Z., Zhou, X., Li, L., Su, Z., plantsUPS: a database of plants' Ubiquitin Proteasome System. *BMC Genomics* 2009, *10*, 227.

[102] Lee, H., Yi, G.-S., Park, J. C., E3Miner: a text mining tool for ubiquitin-protein ligases. *Nucleic Acids Res.* 2008, *36*, W416–W422.

[103] Tung, C.-W., Ho, S.-Y., Computational identification of ubiquitylation sites from protein sequences. *BMC Bioinformatics* 2008, *9*, 310.

[104] Radivojac, P., Vacic, V., Haynes, C., Cocklin, R. R. et al., Identification, analysis, and prediction of protein ubiquitination sites. *Proteins* 2010, *78*, 365–380.

[105] Hunter, T., The age of crosstalk: phosphorylation, ubiquitination, and beyond. *Mol. Cell* 2007, *28*, 730–738.

[106] Grabbe, C., Husnjak, K., Dikic, I., The spatial and temporal organization of ubiquitin networks. *Nat. Rev. Mol. Cell Biol.* 2011, *12*, 295–307.

[107] Lamond, A. I., Uhlen, M., Horning, S., Makarov, A. et al., Advancing cell biology through proteomics in space and time (PROSPECTS). *Mol. Cell Proteomics* 2012, *11*, O112.017731.

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