Deciphering the ubiquitin code by mass spectrometry

Trulsson, C.F.B.

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Chapter 2

Site-specific proteomic strategies to identify ubiquitin and SUMO modifications: Challenges and opportunities

Fredrik Trulsson[1], Alfred C.O. Vertegaal[1]

[1]Department of Cell and Chemical Biology, Leiden Univerisity Medical Center, Albinusdreef 2, 2333 ZA, Leiden, the Netherlands

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Abstract

Ubiquitin and SUMO modify thousands of substrates to regulate most cellular processes. System-wide identification of ubiquitin and SUMO substrates provides global understanding of their cellular functions. In this review, we discuss the biological importance of site-specific modifications by ubiquitin and SUMO regulating the DNA damage response, protein quality control and cell cycle progression. Furthermore we discuss the machinery responsible for these modifications and methods to purify and identify ubiquitin and SUMO modified sites by mass spectrometry. We provide a framework to aid in the selection of appropriate purification, digestion and acquisition strategies suited to answer different biological questions. We highlight opportunities in the field for employing innovative technologies, as well as discuss challenges and long-standing questions in the field that are difficult to address with the currently available tools, emphasizing the need for further innovation.

1. Introduction

1.1. Ubiquitin and ubiquitin-like modifiers

The post-translational modifications (PTMs) ubiquitin (Ub) and Small Ubiquitin-like Modifiers (SUMOs) are conjugated through unique and dedicated cascades of enzymes termed E1 (activating enzyme), E2 (conjugating enzyme), and E3 (ligase enzyme). Before being conjugated to substrates, precursor ubiquitin-like modifiers (UbLs) are processed by UbL specific isopeptidases, which cleave these precursor proteins to expose their C-terminal GlyGly moiety. UbLs are loaded on their E1 by a thioester bond in an ATP-dependent manner, transferred to E2s and conjugated to acceptor lysines through an isopeptide bond in coordination with E3s. The majority of identified SUMO acceptor sites are also known as Ub acceptor sites (Fig. 1a), and almost all SUMO substrates can also be ubiquitinated (Fig. 1b).

1.2. Ubiquitination in brief

Ub modification governs the protein turnover of many substrates. The large number of cellular proteins with distinct half-lives, that range from minutes to days, requires considerable specificity of the Ub machinery. This substrate specific degradation pathway is a highly complex and tightly regulated process that is implicated in every major cellular process [1]. However, evidence suggests that half of all Ub signaling is non-proteolytic, indicating that Ub signaling serves other biological purposes in addition to protein degradation [2]. Assigning function to specific Ub modification events is one of the great challenges of the field. In addition to targeting substrates for proteasomal degradation, monoUb and polyUb chains may change the activity or location of the substrate by enabling or preventing protein-protein interactions [3].

1.3. SUMOylation at a glance

The two major isoform groups of mammalian SUMO are SUMO1 and SUMO2/3. SUMO2 and SUMO3 share 96% sequence identity whereas they differ about 50% with SUMO1. SUMO1 and SUMO2/3 have overlapping and distinct substrates and associated E3s [4], [5], [6]. SUMO is required for progression through mitosis, chromatin organization and stress response [7], [8], [9]. SUMO interacting motifs (SIMs) are common and enable protein-protein interactions [10], [11], [12]. SUMO has also been implicated in protein degradation through SUMO Targeted Ubiquitin Ligases (STUbLs) and Ub modified SUMO2/3 consequently accumulates upon proteasome inhibition [13], [14]. These, and other mixed Ub and UbL chains come

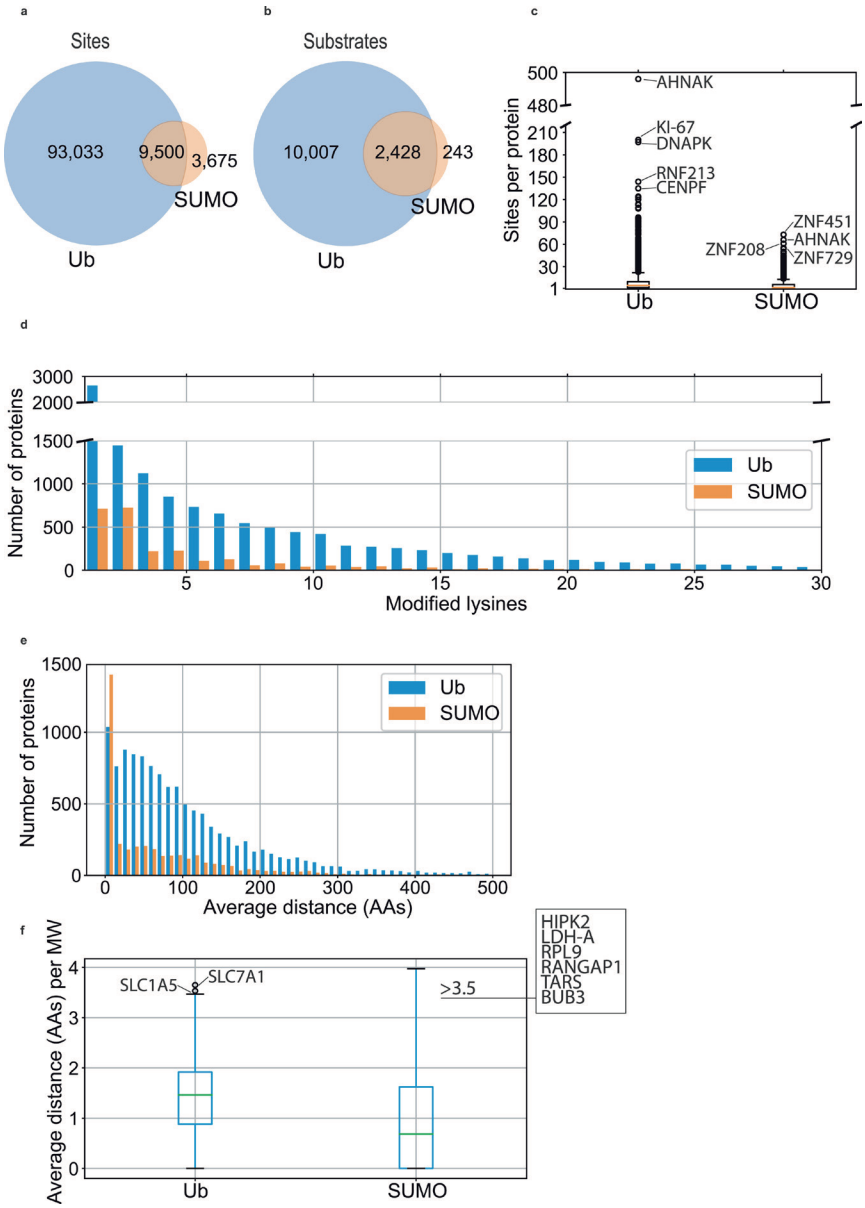


Fig. 1. Ub and SUMO by numbers. a, b Overlap of Ub and SUMO modification sites (a) and protein substrates (b). c, d Number of lysine modification sites per protein for Ub and SUMO of all proteins (c) or for proteins with less than 30 modified sites (d). e, f The average sequence distance (Amino Acids) between modification sites of Ub and SUMO (e) and relative to protein substrate Molecular Weight (AA distance / MW) (f). Data for this figure were obtained from the PhosphositePlus PTM database.

in many flavors and increase the complexity of the UbL code. They constitute largely unexplored signaling pathways that have recently been reviewed [15].

2. Substrate specificity

2.1. Substrate specificity of ubiquitin conjugation

The Ub machinery achieves substrate specificity through interactions of E1, E2 and E3 enzymes. The vast numbers of enzymes involved in Ub conjugation (E1s: 2, E2s: ~40 and E3s: ~600) employ selective interactions of E2 and E3 enzymes that have been reviewed previously [16], [17], [18].

Ub modifications are often targeted through recognition of the substrate, without specificity for unique lysine residues. The purpose of such Ub modifications is usually proteolytic and includes predominantly poly-Ub signals that direct the substrate to the proteasome for degradation through recognition of the Ub molecules rather than the substrate. Examples of such Ub substrates are proteins with rapid turnover such as the Cyclins or Cyclin Dependent Kinase (CDK) inhibitors p16, p21 and p27 [19], [20], [21]. The Cullin Ring Ub ligases (CRLs) are a class of ligases that often targets its substrates for degradation through ubiquitination after recognition of a degron motif on the substrate. Once phosphorylated, the degron motif facilitates interaction with the E3 ligase for ubiquitination and subsequent degradation [19], [22], [23]. After substrate recognition, the CRLs ubiquitinate the substrate without preference for unique lysine positions [24], [25]. Consequently, mutating specific or multiple lysines on Cyclins, DDB1, p21 or p27 does not abolish ubiquitination by CRLs [24], [26]. Interestingly, p16 does not have any internal lysines, instead it is ubiquitinated and degraded through conjugation of Ub at its N-terminal residue [27]. A subset of CRLs, referred to as Skp1–Cdc53/Cullin–F box (SCF) complexes, achieves substrate specificity through more than 50 adapter proteins with an F-box motif (which binds Skp1) and leucine-rich- or WD40-repeats, which bind and recruit the substrates, often through interactions with a phosphorylated degron motif [28], [29], [30].

In yeast, the CDK inhibitor SIC1 is targeted for degradation by the E2 enzyme Cdc34 in conjunction with CRLs. Charged residues at position −1 and −2 from K50, a majority Ub site on SIC1, negatively impact its ubiquitination [31]. Thus, proximal residues have importance, yet no clear Ub motif for lysine selection has been found, in contrast to SUMO or phosphorylation (discussed in section 3.4). However, SIC1 has 14 Ub sites and remains ubiquitinated with 13 of its lysines mutated [32], [33].

Any single lysine residue present in SIC1 sustains its ubiquitination and degradation, thereby releasing CDK inhibition and enabling progression through the cell cycle [32].

2.2. Substrate specificity of SUMO conjugation

In contrast to the abundance of Ub ligases, the SUMO conjugation machinery includes about ten E3 ligases and a single E2 conjugating enzyme [34]. SUMO modifications are often targeted to SUMO consensus motifs (discussed in 3.4) but how SUMO obtains substrate specificity is still poorly understood. Proteins with a SUMO consensus motif are often targeted for SUMOylation by interaction with the SUMO-loaded E2 conjugating enzyme UBC9 [35], [36], [37]. UBC9 auto-SUMOylates, thereby further promoting interactions and proximity with proteins containing SIMs, which are subsequently SUMOylated by UBC9 [38].

The SUMO E3 ligase RanBP2 facilitates SUMOylation independently of substrate recognition, by aligning the SUMO thioester of UBC9 towards a proximal lysine residue [39]. In general, E3 ligases are not required for in vitro SUMOylation of many substrates. Furthermore many substrates can be SUMOylated by several E3s [40].

At sites of DNA damage, SUMOylation occurs on several DNA damage repair proteins without clear specificity for substrates, but SUMOylation is still required for efficient repair [41], [42]. In this instance, SUMOylation is mediated through proximity of the SUMOylation machinery to a lysine residue and may act as a binding platform that supports protein-protein interactions and recruitment of additional DNA damage repair factors.

3. Site specificity

3.1. Ubiquitin site specificity

Experimental evidence suggests that the number of Ub modification sites on a protein is not dependent on molecular size or lysine density of the substrate [43]. Most Ub substrates have less than five Ub modified sites according to the data available on Ub and SUMO modified sites in the database PhosphositePlus (Fig. 1c and d) [44]. In general, Ub sites are spaced further apart than SUMO sites (Fig. 1e), although far more Ub sites have been identified (Fig. 1e). The standard deviation of SUMO site distances is large with many outliers with long sequence distance between sites relative to their molecular weight (Fig. 1f). For the vast majority of Ub substrates it is unknown whether the sites are employed simultaneously, act in a redundant manner,

have synergistic or antagonistic functions or if they combine to constitute a substrate specific Ub code.

Some Ub substrates are modified at a preferred lysine position, usually with a non-proteolytic outcome. The Proliferating Cell Nuclear Antigen (PCNA), histones H2A and H2B and the Fanconi-group proteins FANCD2 and FANCI are examples of such substrates [45], [46], [47], [48], [49], [50]. Histones are targeted by Ub modifications in a site-specific manner, where the modified lysine position is important for downstream signaling. For instance, the E3 ligase HUWE1 ubiquitinates several lysines on histone H1 in response to UV-induced DNA damage, which are then elongated into K63-linked Ub chains by the E2 enzyme UBC13 and the E3 ligase RNF8 [51], [52]. The K63-linked Ub chains on H1 act as a docking platform for the E3 ligase RNF168, which ubiquitinates H2A at K13 and K15, leading to recruitment of downstream DNA damage repair proteins [45], [52]. 53BP1 and BRCA1/BARD1 determine the induced double-stranded break (DSB) repair pathway, stimulating non-homologous end joining or homologous recombination respectively [53]. Non-homologous end joining is induced by 53BP1 binding to ubiquitinated H2A K15 [54], [55]. Similarly, the E3 ligase complex BRCA1/BARD1 recognizes the same ubiquitinated H2A K13/15, while also having affinity for newly synthesized histones that are integrated into chromatin during DNA replication, characterized by unmethylated histone H4 at K20 [56], [57], [58]. 53BP1 has affinity towards mono- and di-methylated K20 on H4, and so these two histone PTMs work in tandem to govern the equilibrium of these competing DSB repair pathways [59].

PCNA is a ring-shaped trimeric protein that slides along the DNA during DNA synthesis and is required for DNA replication [60]. In response to replication stress, PCNA is monoubiquitinated at K164 by the E3 ligase RAD18 and mutating this single lysine is sufficient to abolish ubiquitination of PCNA and inhibit translesion synthesis signaling [49], [50], [60], [61]. Once ubiquitinated, PCNA binds the translesion synthesis DNA polymerase eta, to bypass replication obstacles and ensure efficient DNA replication [60], [61]. This process is also dependent on FANCD2 and FANCI (ID2 complex), two DNA binding proteins essential for DNA damage repair. PCNA may function as a platform to facilitate mono-ubiquitination of FANCD2 via its PCNA interaction motif (PIP-box) [62]. A monoUb modification on K561 of FANCD2 promotes a conformational change of the ID2 complex, enabling binding surrounding DNA [48]. FANCI is monoubiquitinated on K523, resulting in a stabilization of the ID2 complex and protection from deubiquitination by USP1 and WDR48 [48]. The

responsible E3 ligase FANCL mediates K561 site specific mono-ubiquitination of FANCD2 through interaction with the E2 enzyme UBE2T [47]. Interestingly, FANCL interaction with UBE2T mediates a reconfiguration of the E2 active site that directs it towards K561 on FANCD2, thereby achieving site specificity [47].

Misfolded proteins in the endoplasmic reticulum (ER) are exported through retrotranslocation, from the ER to the cytosol, where they are ubiquitinated and degraded by the proteasome through Endoplasmic Reticulum Associated protein Degradation (ERAD) [63]. The transmembrane E3 ligase Hrd1 forms a retrotranslocation pore that binds misfolded proteins and transports them to the cytosol [64]. Hrd1 must be ubiquitinated to assume an open state, and auto-ubiquitinates itself in the RING domain at several possible lysine residues [64]. Interestingly, K48-linked polyUb chains are necessary for steady high channel flow of Hrd1, as demonstrated by experiments with K48R mutants of Ub [64]. The transmembrane DUB Ubp1 deubiquitinates Hrd1, effectively closing the pore [65]. Thus, Hrd1 achieves regulation of its pore through ubiquitination and deubiquitination within a protein domain rather than a single lysine residue.

Site-specific ubiquitination may also aid in protein folding of ER proteins. The transmembrane protein LRP6 is ubiquitinated at its cytosol exposed lysine residue, K1403, which promotes efficient folding of the protein [66]. Before ER exit, LRP6 is deubiquitinated by USP19 and palmitoylated, then transported through the Golgi to the plasma membrane where it acts as a Wnt signaling coreceptor [66], [67]. If folding of LRP6 is unsuccessful, it is polyubiquitinated and degraded through ERAD [66].

3.2. Ubiquitin machinery responsible for ubiquitin chain architecture

Additionally, E3 ligases and DUBs have specificity for single lysine residues on Ub, or polyUb chain linkages [68], [69], [70], [71]. Combinations of specific E2 and E3 enzymes cooperate to generate Ub chains linked through specific lysine residues on Ub. The different polyUb chains seem to have distinct biological roles that have been reviewed elsewhere [72]. While some E2/E3 pairs make only one kind of Ub chain, other enzyme pairs can make multiple Ub chain types. Cdc34 preferentially makes K48-linked Ub chains but can make K11- and K63-linked chains as well [33]. The incorporation of a glycine (G47Q) close to K48 of Ub, abolished K48-linked Ub chains formed by Cdc34, while retaining monoUb activity [33]. This indicates that amino acids neighboring K48 are important for chain formation. However, mimicking K48 sites close to K11 or K63 of Ub, did not alter Cdc34 favored polyUb chain

formation [33]. Preferential chain formation of Cdc34 must therefore be determined by additional factors, other than site proximal amino acids. However, the linkage specificity of E2/E3 pairs has conventionally been assessed using K to R mutants of Ub, to restrict in vitro reactions to a single Ub chain linkage type. This approach, and the detection of Ub chain linkages by immunoblotting, may create biases towards some linkage types and may exaggerate the ability of some E2/E3 pairs to make additional chain linkages [73].

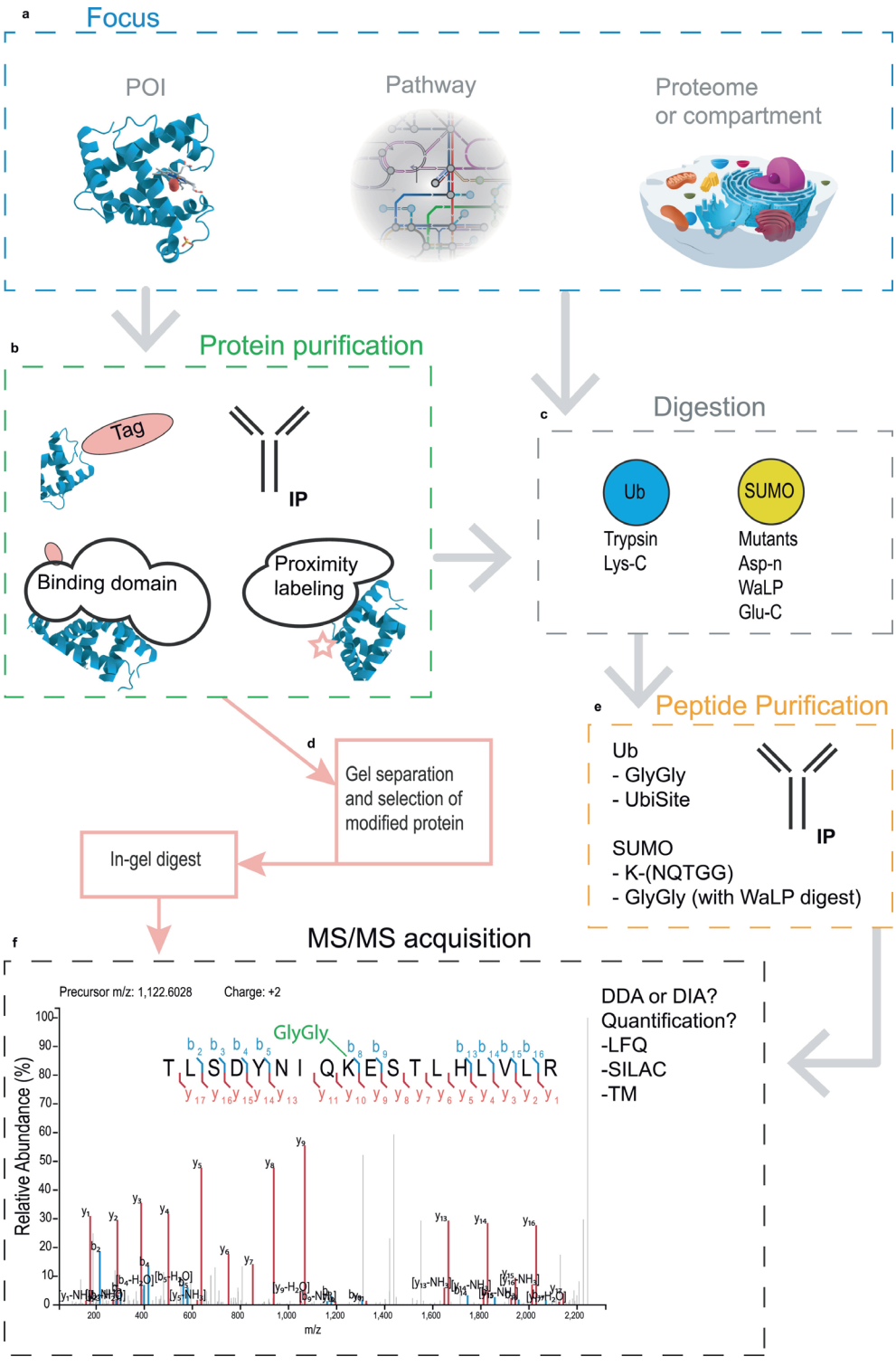
3.3. SUMO site specificity and motifs

SUMOylation is often targeted to the consensus motif ψKxE, where ψ represents a hydrophobic amino acid (I, V, L, A, P, or M) and x is any amino acid [36], [74]. The SUMO consensus motif also exists as a minimal KxE motif and inverted [D/E]xK motif and these motifs constitute at least half of all SUMOylation sites under non-stress conditions [75], [76]. The SUMO consensus motif is bound by UBC9 (the single E2 conjugating enzyme of the SUMO machinery) to facilitate SUMOylation of the target lysine, with the aid of an E3 ligase or additional interactions with UBC9 [35], [77]. In addition to the conventional SUMO consensus motifs, a Phosphorylation-Dependent SUMOylation Motif (PDSM) has been identified (ψKxExxSP), and a Negatively charged amino acid-Dependent SUMOylation Motif (NDSM) with acidic residues downstream of the target lysine [78], [79]. Several tools have been developed to predict SUMOylation sites based on consensus motifs, such as GPS-SUMO, SUMOsp2.0, JASSA, and SUMOgo [80], [81], [82], [83].

However, there are numerous SUMOylation sites identified that do not adhere to the conventional consensus motifs. SUMOylation of PCNA is not dependent on the SUMO consensus motif. PCNA SUMOylation prevents recombination during replication and is predominantly located to specific lysine residues K127 and K164 [50], [84]. Whereas K127 contains a UBC9 consensus motif and can be SUMOylated independently of an E3 ligase, SUMOylation of K164 is mediated by E3 ligase Siz1 in yeast and E3 ligases PIAS1 and PIAS4 in humans, indicating an E3-dependent mechanism for SUMOylation of residues that are not situated in consensus motifs [84], [85]. Furthermore, during stress such as DNA damage or heat shock, SUMOylation is massively enhanced, in a SUMOylation consensus motif-independent manner [86].

These examples of site-specific modifications by SUMO or Ub, involved in virtually all cellular processes, highlight their importance. A multitude of strategies

and tools are available to identify the precise modification sites, and we aim to give an overview of them in this paper. For many biological questions the modification sites of a given protein-of-interest is central and we hope that this paper will aid in the resolution of these questions by highlighting key aspects of sample preparation and data acquisition. In addition, we provide some outlooks on challenges currently present in the field, where tools and technologies have shortcomings, and opportunities where technologies are available but have yet to be applied.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Charge: +2
GlyGly
T L S D Y N I Q K E S T L H L V L R
Relative Abundance (%)
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DDA or DIA?
Quantification?
-LFQ
-SILAC
-TM

Fig. 2. Overview Ub and SUMO site mapping strategies. a The biological focus of each project determines the optimal method that should be employed. When the focus is on a small number of Proteins Of Interest (POI), they can easily be purified individually, but if the study encompasses a large signaling pathway or analysis of full proteomes, a peptide purification step is required, independently or consecutively after protein purification. b Proteins or a subset of the proteome can be purified using tagged versions of a POI, by ImmunoPrecipitation (IP) of tagged or endogenous proteins, using tagged protein binding domains or using proximity labeling. c Proteins are subsequently digested into peptides. Extra care should be taken when investigating SUMO modified peptides since the WT tryptic fragment of SUMO is too large for efficient analysis by MS. C-terminal SUMO mutants or alternative proteases should be used instead. d Purified POIs can be separated by gel electrophoresis; the modified version of the POI can then by excised, digested and identified by Mass Spectrometry (MS). This shortcut generally yields less identified modified peptides. e SUMO and Ub modified peptides can be purified by IP which circumvents the problem of low abundant protein modification states compared to their unmodified counterparts. f Preferred acquisition and quantification method should be tailored for each study. Once a modified peptide has been identified, a high quality spectra with overlapping b- and y-ions around the modified residue is needed to gain confidence in the identification.

4. Strategies to purify ubiquitin and SUMO sites for identification by mass spectrometry

4.1. General workflow

Identification of modifications by shotgun proteomics usually includes specific purification methods tailored to each modification, digestion of proteins into peptides (sometimes done prior to purification), separation of peptides by liquid chromatography and identification of precursor ions by a first round of mass spectrometry (MS1). For Data Dependent Acquisition (DDA) methods, the most abundant peptides are then fragmented into smaller ions and sent for a second round of mass spectrometry (MS2), the sequence of the original peptide and the location of modified residues can then be deduced based on fragment ions identified by MS2. MS identification of PTM sites thus offer precise location of the modified site and quantitative differences in abundance among variable conditions such as inhibitory or stimulatory treatments and genetic alterations of cell lines or organisms. An overview of the workflow is provided in Fig. 2. The optimal method is always dependent on the biological question that is addressed (Fig. 1a). The key to identification of site-specific modifications is sequence coverage of the substrate, which preferably includes all the proteolytically generated peptides. It is therefore typically necessary to purify the protein(s) of interest and the UbL modified peptides consecutively, prior to MS identification, since the modified substrate often represents a small fraction of the total population of any given protein (Fig. 2b, c, e) [87], [88]. Alternatively, the modified version of the purified protein of interest can be separated from the unmodified version by gel electrophoresis and processed for MS identification (Fig. 2d) [89]. However, this method generally has a lower yield of modified peptides.

4.2. Proteases for protein digestion

In shotgun proteomics, trypsin is by far the most widely used protease for digesting proteins into peptides, but using alternative proteases can be advantageous for several reasons. Using several proteases in parallel increases the depth and coverage of analysis as some peptides generated by digestion will be too small to be uniquely mapped to a single protein, or too large to ionize well, because of many closely spaced cleavage sites or too few. Using an alternative protease may enable identification of peptides that were missed in the first analysis, because the cleavage sites are now located after other amino acids.

4.3. Ubiquitin site purification strategies

Since the discovery of 72 modified peptides with a mass difference of 114.04 Da corresponding to the tryptic remnant of Ub (GlyGly), significant improvements to purification methods have been made [90]. Today, tens of thousands of Ub modified peptides can be purified from cells. The biggest limiting factors for purifying Ub conjugates are the low abundance of modified proteins relative to the absolute protein levels and the active DUBs present during purification [90], [91], [92]. An enrichment step is usually required in order to achieve optimal coverage of modified proteins. Affinity purification using tandem UBDs or antibodies requires conditions that are not denaturing, thereby allowing deubiquitination to happen during the purification [93]. Such methods typically require the addition of DUB-inhibitors to sustain Ub conjugates, which routinely include alkylating agents targeting the active cysteine of cysteine-based DUBs (USPs, OTUs, MJDs and UCHs). N-ethylmaleimide (NEM), iodoacetamide (IAA) and chloroacetamide (CAA) are examples of such compounds. Although this still leaves zinc metalloprotease (JAMMs) active, they can be inactivated by EDTA [94]. Each of the alkylating agents has drawbacks that should be considered, for instance modification of amino acids other than cysteines can be problematic if several variable modifications are already included in the analysis. Acetylation of an unexpected amino acid that was not included as a variable modification during data analysis could prevent the identification of a given peptide. In addition, NEM can be hydrolyzed, splitting the spectral peak in two, potentially weakening the signal. Both NEM and CAA are less reactive towards cysteines than IAA, but IAA can produce dialkylation modification of lysine residues that is indistinguishable from the GlyGly modification, although this happens only at higher temperatures [95].

By utilizing peptide purification rather than protein purification, the DUBs are rendered inactive by digestion prior to purification by site-specific antibodies. The most conventional strategy to purify Ub sites employs an antibody recognizing the tryptic fragment of Ub modified lysine residues (GlyGly antibody). This method in combination with data-independent acquisition has recently been used to obtain reproducible identification of > 35,000 Ub sites per MS run [96]. However, this antibody will also purify NEDD8 and ISG15 sites as they share the same tryptic fragment with Ub. Akimov et al. developed the UbiSite antibody that recognizes the Lys-C fragment of Ub modified sites, enabling purification of Ub sites in a specific manner [43]. In addition, the UbiSite antibody is not limited to lysine-modified residues. It enables

identification of N-terminal ubiquitinated peptides and could potentially identify any Ub modified residue, although this remains to be shown [17]. Furthermore, the recent development of N-terminal ubiquitination specific antibodies by Davis et al. enables selective purification of these low abundant ubiquitination events [97].

In addition to immunoprecipitation strategies, there are other ways of purifying Ub sites without the need for overexpressed tagged Ub or any other protein of interest. COmbined FRActional DIagonal Chromatography (COFRADIC) utilizes chemical acetylation agents to block free lysines. Ub modifications are subsequently removed using the catalytic domain of USP2 and free lysine residues are then tagged with a chemical handle to enable purification [98].

Lastly, a machine learning algorithm for identifying Ub sites is now available termed DeepUbi, which can predict Ub sites based on sequence information [99]. Such methods could be useful to predict Ub modifications on MS-incompatible peptides.

4.4. SUMO site purification strategies

Identifying SUMOylated sites is more complicated because the tryptic remnant of SUMO is relatively long (SUMO1: K ELGMEEEDVIEVYQEQTGG, SUMO2: R FDGQPINETDTPAQLEMEDEDTIDVFQQQTGG, SUMO3: R FDGQPINETDTPAQLEMEDEDTIDVFQQQTGG) and generally makes the modified peptide ionize poorly, which makes identification by MS difficult. SUMOylation sites have previously been identified using exogenous SUMO with an introduced tryptic cleavage site close to the C-terminus (SUMO1: Q92R or T95R, SUMO2: Q88R, T91R, SUMO3: Q87R and Q87R/Q88N [100], [101] or T90K [75]) (Table 1). Following purification of SUMOylated proteins and digestion, the tryptic remnant of the Q87R/Q88N SUMO3 mutant can be used for sequential peptide immunoprecipitation using the anti-K-(NQTGG) antibody [102] and for the T90K SUMO3 mutant using the conventional GlyGly antibody [75], thereby increasing the number of identified SUMOylation sites considerably by removing non-modified peptides. Additionally, K0 SUMO mutants with all lysines mutated to arginines have been used to increase the number of SUMO sites identified, by enabling purification of SUMOylated proteins followed by Lys-C digestion and a second round of purification since SUMO K0 is unaffected by Lys-C digestion [103].

UbL	Common Protease (s)	Proteolytic fragment	Proteolytic fragment mass (Da)
Ubiquitin	Trypsin/Lys-C	-GG	114.043
SUMO1	Glu-C	-EQTGG	472.192
SUMO2	Asp-N	-DVFQQQTGG	960.430
SUMO2	WaLP	-GG	114.043
SUMO3	Asp-N	-DVFQQQTGG	960.430
SUMO3	WaLP	-GG	114.043
SUMO1 – Q92R	Trypsin	-EQTGG	472.192
SUMO1 – T95R	Trypsin	-GG	114.043
SUMO2 – Q88R	Trypsin	-QQTGG	471.208
SUMO2 – T91K	Lys-C	-GG	114.043
SUMO2 – T91R	Trypsin	-GG	114.043
SUMO3 – Q87R	Trypsin	-QQTGG	471.208
SUMO3 – T90K	Lys-C	-GG	114.043
SUMO3 – Q87R+Q88N	Trypsin	-NQTGG	457.192

Table 1. Common proteases for site mapping of Ub and SUMO. The different UbLs have different C-terminal sequences and therefore leave distinct proteolytic fragments after digestion. To enable efficient identification by MS, an appropriate protease should be selected. Common proteases for each UbL, with common digestion mutants, are detailed with respective proteolytic fragment and mass of proteolytic fragment. A modified peptide mass is equal to the proteolytic fragment mass of the UbL, in addition to its own mass.

The PRISM method to identify SUMO sites employs His-tag purification of SUMOylated substrates, acetylation of all non-occupied lysines, followed by cleavage of SUMO by SENP1 or SENP2 and subsequent identification of lysines lacking acetylation [104]. This way, the SUMOylation site can be identified while bypassing the issue of the large tryptic fragments of SUMO. In theory, this method of identifying a modified lysine could be applied to any lysine modification with a known de-modifying enzyme. This could be particularly useful when trying to identify modifications that produce poorly ionizing peptides.

Using the protease WaLP, which cleaves after threonine residues, purification of endogenous SUMO sites is possible using the conventional GlyGly antibody [105]. This approach cannot distinguish between a SUMOylation site of SUMO1, 2 or 3 because they would all result in a GlyGly remnant after WaLP digestion. Similarly, the 8A2 anti-SUMO2/3 antibody has been used to purify Lys-C digested SUMOylated peptides followed by additional digestion with Asp-N, Glu-C or WaLP to obtain SUMO conjugate fragments small enough to be compatible with MS identification [106].

The large tryptic fragment of SUMO will fragment into b- and y- ions together with the modified substrate peptide. The resulting fragment ion pattern, specific for SUMO (1 or 2/3) can be identified in the spectra using the SUMmOn tool [107], thereby identifying modified peptides without the need for SUMO mutants, although this tool has seen limited use in practice.

5. Mass spectrometry strategies

In addition to purification strategies, consideration should be given to MS acquisition and fractionation methods employed. Both can significantly enhance the depth and sequence coverage of samples. However, fractionation is not an alternative to purification methods as it does not solve the problem of low abundant protein modification states compared to their unmodified counterparts. Once a modified peptide has been identified, it is key to inspect the MS2 spectra for b- and y- ions overlapping the modified residue to gain confidence in the identification (Fig. 2f).

5.1. Fractionation

Fractionation of samples simply means splitting one sample into multiple MS runs. Several fractionation methods exist, but the most frequently employed method separates proteins by molecular weight. Proteins are first separated by gel electrophoresis and subsequently any molecular weight area or any specific band

corresponding to a protein of interest, or modified version of the protein of interest, can be selected (Fig. 2d). Alternatively, the whole lane of the sample can be cut in several slices to be analyzed as separate MS runs.

Samples of digested peptides can be fractionated through high pH fractionation over stage-tips containing C18 or similar material. The high pH adds another dimension of peptide separation as the hydrophobic properties of peptides are different compared to conventional acidic chromatographic conditions during LC-MS/MS. In brief, peptides are separated by elution with increasing concentration of acetonitrile, while collecting multiple fractions of a single sample. The LC-MS/MS run of early fractions (fractions that eluted first) will still contain peptides with a high retention time, since their hydrophobicity is dependent on low pH conditions.

5.2. MS/MS acquisition methods

The MS acquisition method determines which peptides are sent for sequencing by MS2, which can be key when trying to obtain high sequence coverage of a specific protein of interest or low abundant modified peptides (Fig. 2f).

5.2.1. Data independent acquisition or data dependent acquisition

DDA selects the most abundant peptides from MS1 scans for identification by MS2. Generally, the top 8–13 peptides are sent for fragmentation and subsequent identification by MS2. DDA suffers from the inherent problem of only selecting the most abundant peptides and may not identify low abundant peptides. An alternative is Data-Independent Acquisition (DIA), which uses a preexisting library of MS1 spectra and retention times to select peptides of interest for MS2 identification. Recent efforts employing the GlyGly antibody in conjunction with DIA identified ~90,000 Ub sites [96]. However, DIA is dependent on the preexisting library, which usually consists of a deeply fractionated set of DDA runs. As a result, if a peptide was not identified in the library, it cannot be identified during the DIA acquisition. MaxDIA builds on the classical DIA library based approach but includes the capability of using a discovery library generated by the prediction tool DeepMass:Prism [108], [109]. This enables DIA acquisition without the need to build a deep library through DDA beforehand. Discovery libraries for an abundance of organisms are already available through MaxQuant [109]. However, these libraries do not yet include modified peptides.

5.3. Quantification strategies

5.3.1. Label-free quantification (LFQ)

Uses a dominant population of proteins that remains unchanged between samples to normalize variation introduced during sample preparation or MS performance [110]. The MaxLFQ algorithm achieved similar quantification accuracies as SILAC [110]. The ease of use and unlimited multiplex nature of LFQ makes it an excellent go-to strategy of quantification for most MS experiments.

5.3.2. Stable Isotope Labeling by Amino acids in Cell culture (SILAC)

Can reduce MS run time by combining several isotope-labeled samples into one and eliminates variation from purification protocols since samples are combined prior to purification [111]. However, SILAC increases the complexity of the sample and comparison of isotope intensities takes place at the MS1 level, the peptide intensities are split in multiple peaks and are therefore effectively diluted. Similar methods of chemical labeling, such as dimethylation via reductive methylation with formaldehyde, is significantly cheaper and can be used for any type of sample, not just from cultured cells [112], [113], [114].

5.3.3. Isobaric tag for relative and absolute quantitation (TMT or iTRAQ)

Offers a more sophisticated labeling method, with a greater number of channels and without any impact on sample complexity or splitting of MS1 peaks since the differential labels all have the same mass [115], [116]. Instead, the comparative quantification takes place at MS2, where the differential labels each release a unique reporter ion after fragmentation. TMT and iTRAQ minimize missing values, which can be a problem in quantification of PTMs and normally requires a significant amount of imputation. However, the method is relatively expensive and requires an MS with an ion trap with a low mass range. The DIA method Sequential Window Acquisition of all THeoretical mass spectra (SWATH-MS) has been used in combination with TMT labeling to simultaneously quantify Ub and SUMO peptides [114].

5.3.4. Absolute QUAntification-Ub (AQUA-Ub)

Or other spike in peptides can be used for absolute abundance quantification. Isotope labeled Ub modified peptides spiked into the sample provide a reference used to obtain the absolute quantification of Ub chains or Ub modified peptides in a complex

sample [117], [118]. The method can also be used to identify and quantify the exact constituents of a Ub modified protein, effectively pinpointing the composition of polyUb modifications from an in vitro reaction [118].

6. Challenges

Some biological questions are especially challenging to answer with technologies available today and will require innovative solutions. We discuss some of these challenges below.

6.1. Identifying polymer architecture and total modification status of a protein copy

Information on the modification status of intact protein is lost when digesting proteins into peptides, which makes it impossible to deduce the total modification status of a single protein copy. Even though several peptides of a protein are identified with Ub modifications, these modifications may never co-exist on the same protein molecule. Similarly, assigning polymers to substrates by shotgun proteomics is challenging since only the digested fragment of the first Ub or SUMO molecule is identified on the substrate. In vitro experiments using AQUA-Ub have identified complex Ub polymers formed by the Anaphase-Promoting Complex (APC) on cyclin-B1, demonstrating that vital information of Ub modifications may be lost after digestion [118]. Furthermore, using an engineered viral protease, Lbpro, branched Ub chains were identified on 10–20% of all Ub polymers, indicating further complexity of the Ub code [119].

6.2. Co-modifications

A considerable amount of Ub and SUMO co-modification has been identified on Ub itself and on Ub or SUMO substrates. These co-modifications include, but are not limited to phosphorylation, acetylation, ribosylation and other UbLs [15], [86], [120]. Studying how these modifications work together requires overcoming several hurdles. For instance, if a modification at a specific position of a protein leads to a co-modification with another PTM somewhere else on the protein, this information would be lost when purifying one kind of modified peptides by IP. PTMScan Direct offers an interesting solution to this problem by utilizing multiple antibodies to enrich several PTMs from the same sample [121]. A wide range of antibodies for probing phosphorylation is already available as well as the previously discussed Ub or SUMO targeted antibodies. Essentially any strategy to purify modifications could be combined in a similar manner to gain insights in PTM crosstalk. However, the problem of assigning a total modification status of an intact protein copy remains.

6.3. Redundancy of modification sites and enzymatic substrates

Assigning function to a SUMO or Ub modification of a substrate is often challenging. Producing lysine deficient mutants to investigate any phenotypes associated with a lack of modification is a common strategy but it has its shortcomings. For instance, the modification could simply move to a redundant lysine position on the substrate, and with enough lysines mutated any apparent phenotype could be explained by the numerous mutations themselves rather than a lack of modification. Similarly, there are numerous examples of Ub substrates with many associated E3 ligases, seemingly fulfilling the same function with redundancy. One explanation for this could be to ensure the degradation of a particularly important substrate when one ubiquitination pathway is dysfunctional. An extreme example of this is p53, with ~20 E3 ligases potentially regulating its activity [122]. Alternatively, the cellular localization provides regulation, this way different E3 ligases could regulate subpopulations of the same protein based on their location [123]. It has been shown that expression of E3 ligases differs between cell types and organs, which could account for some of the redundancy of substrate associated E3s and perhaps substrate modification sites [124]. Mapping E3s to substrates and specific sites in this context is still a daunting task.

6.4. Differentiating between ubiquitin, Nedd8 and ISG15 sites

Tryptic digestion will result in C-terminal GlyGly fragments for ubiquitin, Nedd8 and ISG15 that cannot be distinguished by mass spectrometry. Since ISG15 is encoded by an interferon-stimulated gene, ISG15 conjugates will only be present in response to interferon signaling [125]. The Cullins are the canonical substrates for Nedd8, but other substrates have been reported as well [126]. Since Neddylation of Cullins is required for their activity, inhibiting Neddylation will strongly alter ubiquitination and is therefore an inappropriate approach to avoid misidentifying Nedd8 sites as Ub sites. UbiSite technology employs Lys-C digestion, resulting in larger C-terminal fragments of ubiquitin, Nedd8 and ISG15 [43]. The UbiSite antibody uniquely recognizes the C-terminal fragment of ubiquitin, enabling specific enrichment of ubiquitinated peptides that are subsequently cleaved with trypsin to enable mapping of bona fide ubiquitination sites by mass spectrometry.

7. Opportunities

Several aspects of the Ub and UbL signaling pathways are largely unexplored and present promising research topics that are being perused by investigators around the world. These aspects include novel insight in the roles of Ub and UbLs in fundamental cell processes while newly identified Ub an UbL signaling inhibitors can potentially be applied as therapeutics. We discuss some of these opportunities below.

7.1. Chain linkages of ubiquitin-like proteins

Although polyUb chains are well studied and differential biological roles have been assigned to most chain linkages, the roles of other UbL polymers remain largely unknown. SUMO1 has 11 internal lysines but behaves more like a chain-capper rather than a polymer extension, although SUMO1 can be modified by SUMO2/3 under stress conditions [106], [127]. SUMO2 and −3 on the other hand, readily make chains and contain 8 internal lysines with K7, K11, K21 and K33 identified as endogenous SUMO chain linkages, although SUMO2/3 K11 chains are by far the most prominent chain type [106]. The linkage specific biological functions of these SUMO chains, other UbL chains and mixed chains remains elusive.

7.2. Non-lysine modifications of ubiquitin-like proteins

Emerging evidence indicates that Ub can be conjugated to residues other than lysine [128], [129]. The presence of these unconventional ubiquitination events raises the question whether this is exclusive for Ub or if it is true for other UbLs as well. Furthermore, the biological functions of these unconventional ubiquitinations, as well as the required conjugation machinery, are still poorly understood.

7.3. Specific inhibitors for E1, E2 and E3s or DUBs

The development of selective inhibitors of specific enzymes of the Ub and SUMO machinery enables investigation of their substrates, biological function and provides promising therapeutic potential. Inhibitors of the E1 activating enzyme of Ub have been used with success in a leukemia mouse model and preclinical trials of acute myeloid leukemia [130]. Ub involvement in tumorigenesis and drug development targeting E1, E2s, E3s and DUBs have been recently reviewed [131]. The development of a specific USP7 inhibitor highlights the efficacy of targeting the Ub system [132], [133]. Similarly, inhibitors of the SUMO machinery have been employed as cancer treatments and have been reviewed elsewhere [134]. Lastly, Nedd8 E1 and E2 inhibitors have been used in mouse models demonstrating anti-

tumor effect [135], [136].

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