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The Ubiquitin Proteolytic System - A Focus on SUMO: A review

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Abstract

The Small Ubiquitin-like Modifier proteins (Smt3 in yeast and SUMOs 1-4 in vertebrates) are members of the ubiquitin super family. Like ubiquitin, the SUMOs are protein modifiers that are covalently attached to the epsilon amino group of lysine residues in the substrates. The application of proteomics to the SUMO field has greatly expanded both the number of known targets and the number of identified target lysines. As new refinements of proteomic techniques are developed and applied to sumoylation, an explosion of novel data is likely in the next five years. This ability to examine sumoylated proteins globally, rather than individually, will lead to new insights into both the functions of the individual SUMO types and to how dynamic changes in overall sumoylation occur in response to alterations in cellular environment. In addition, there is a growing appreciation for the existence of crosstalk mechanisms between the sumoylation and ubiquitinylation processes. Rather than being strictly parallel, these two systems have many points of intersection, and It is likely that coordination of these two systems is a critical contributor to the regulation of many fundamental cellular events.

Keywords

Mass spectrometry; Proteomics; Tandem Affinity Purification; Cross-talk; Signature Tags; STUbL; Ubl; SUMOome

SUMOs and the ubiquitin super family

The small protein modifier, ubiquitin, is the prototype of the ubiquitin superfamily [1]. The biochemistry of ubiquitinylation is well understood as is the role of polyubiquitinylation in targeting proteins for proteasomal degradation. Over the last decade, a number of ubiquitin-like proteins (Ulp) have been discovered, including the SUMO family (Smt3 in yeast and SUMOs 1-4 in vertebrates), whose biological functions are less well-defined. The three most well-characterized vertebrate SUMOs fall into two subfamilies, SUMO1 and SUMO2/3. SUMO2 and SUMO3 share 95% sequence identity compared to only ~ 50% identity between SUMO2/3 and SUMO1, though the common and distinct functional roles of the various SUMOs has not been clearly delineated. While SUMO1 shares only about 18% with ubiquitin, all the Ulp have very similar tertiary structures with a common ubiquitin fold and a C-terminal diglycine in the mature forms. Like ubiquitin, SUMOs are post-translationally attached to target protein lysine residues using an enzymatic pathway that is biochemically analogous to, but functionally distinct from the classical ubiquitinylation system [2]. The overall process has four steps (Fig. 1): 1) processing of the precursor SUMO by SUMO proteases (SENPS) which remove C-terminal residues to expose the diglycine motif, 2)

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covalent attachment of the processed SUMO to the heterodimeric E1 activating enzyme (SAE1/SAE2 in vertebrates) via a thioester linkage, 3) transfer of SUMO from the cysteine of the E1 enzyme to cysteine 93 of the E2 conjugating enzyme (Ubc9), and 4) covalent attachment of SUMO to a lysine residue in the target protein, which is facilitated by E3 SUMO ligases. While this process is biochemically identical to that for ubiquitin attachment to substrates, the SUMO proteases, E1, and E2 are specific for SUMO and do not function with ubiquitin or other Ulp3 [3,4]. Interestingly, the number of components in the ubiquitinylation pathway is much greater than for the sumoylation pathway. Most dramatically, there are roughly 30 ubiquitin E2s and hundreds of ubiquitin E3s, while there is only one SUMO E2 and only a few known SUMO E3s. Why there is so much greater diversity in the number of ubiquitin pathway components is unknown, but may reflect a greater number of overall substrates requiring more combinations of components to provide appropriate regulatory control. For more comprehensive general information on sumoylation, there are excellent recent reviews by Kerscher [5], Hay [6], and Seeler et al. [7].

It should also be noted that the sumoylation state of target proteins is not static, but instead reflects a dynamic equilibrium between the forward process of SUMO addition and its removal by cellular desumoylating enzymes called SUSPs or SENPs [8,9]. The mammalian SUMO proteases differ greatly in their sequences and are related primarily in the conserved region critical for cysteine protease catalytic activity. Furthermore, individual proteases have been shown to differ in intracellular localization with both nuclear [8,10] and cytoplasmic [9,11,12] species observed. The existence of multiple mammalian desumoylating enzymes, along with the demonstrated differences in intracellular distribution, suggests that desumoylation is likely to be a complex process that contributes to the regulation of activity of SUMO substrates. In addition, the abundance of the SUMO proteases plus their intrinsic stability has hindered isolation and identification of SUMO substrates since desumoylation can readily occur in cell extracts where the SUMO proteases have not been sufficiently inactivated.

Even though the enzymology and biochemical mechanism of sumoylation are now well understood, the biologic consequences remain less clear. The general importance of the sumoylation system is highlighted by several observations. First, proteins sharing identity with SUMO-1 and its enzymatic partners are conserved throughout eukaryotes including yeast, protozoa, metazoa, and plants [2]. In many cases, these conserved proteins can be exchanged across species and will functionally substitute confirming their homology. Second, mutational analysis in *S. cerevisiae* demonstrated that the sumoylation components are all essential for viability, indicating that this modification system plays a critical role in one or more basic cellular [13,14]. Third, embryonic knockouts of SUMO components are devastating to *C. elegans* development [15] and are lethal for mice [16], pointing to critical roles for sumoylation in fundamental cellular processes. While ubiquitinylation is largely associated with targeting proteins for degradation, sumoylation does not generally appear to be a degradative signal. Instead, sumoylation is associated with a wide variety of effects including intracellular targeting, antagonizing degradation by competing for the same lysines as used for ubiquitinylation, modulating protein-protein interactions, and altering protein activity [17]. Most of this information has been gleaned from studies of individual SUMO substrates, but the recent application of proteomics to this field has the potential to reveal new insights into more global contributions of sumoylation to cellular function.

SUMO proteomics: methods

Prior to 2004, identification of sumoylated proteins was on an individual substrate basis and only a limited number of substrates had been identified. This changed dramatically with the

application of proteomics methods by several groups to generate unbiased surveys of total sumoylated proteins. The most common method involves identification with mass spectrometry (MS), though other approaches such as yeast two-hybrid screens and *in vitro* expression cloning (IVEC) have also been applied successfully to define sumoylated targets. However, no matter which method is used to study sumoylation proteomics it should be kept in mind that sumoylation is a dynamic process with sumoylation and desumoylation taking place concurrently, so the sumoylated form of any particular substrate may be present in very low quantities. Coupled with the possibility that the amount of the target protein itself may be in low abundance, detection of the sumoylated form may be very difficult, though a recently described Ubc9 fusion-directed sumoylation method has promise for enhancing sumoylation of specifically targeted substrates [18]. Furthermore, sumoylation of a protein may depend on certain cellular conditions not met by the study. For example, many studies use cloned SUMOs ending in the diglycine motif that do not require intracellular processing, yet the potential contribution of processing to regulation of sumoylation is largely unknown. Finally, some sumoylated proteins may not be isolated very well due to limited solubility in the choice of lysis buffers or their tight interaction with any of the numerous organelles and structures in the cell. These issues make it impossible for any single study to be comprehensive, and each will only be capturing a fraction of total sumoylated proteins. When developing a proteomics study involving sumoylation, the aforementioned consideration must be evaluated to develop the most comprehensive approach. The aim of this section is to provide information on the techniques used in the proteomic study of the sumoylation system, and to provide insight into the advantages, limitations, and possible problems faced with each technique.

MS Approaches

There are several different approaches to conducting proteomic studies on SUMO targets, though they can generally be divided into MS and non-MS techniques. The exquisite sensitivity and robustness of MS have made it a powerful tool for large-scale analysis of complex protein mixtures. (While MS plays an important role in identifying proteins modified by SUMO, a discussion of even the more common MS techniques is beyond the scope of this review; for a more complete summary of the application and issues involved with mass spectrometry in proteomics see the following reviews by Lane [19] or Lubec et al. [20].) Even with the sensitivity of MS, the low abundance of most sumoylated proteins requires enrichment for these proteins as a prerequisite for MS analysis. Most studies to date involved over expression of tagged SUMOs (Table 1) followed either by single [21-26] or double (tandem) affinity purification [27-29], or by immunoprecipitation [24,30]. A variety of possible affinity tags are available, so the choice of tag or tags should be conducive to the ultimate analysis scheme. One of the primary considerations is to reduce nonspecific binding to the affinity matrix. Additionally, many non-sumoylated proteins contain SUMO binding motifs (SIMs) that promote association with sumoylated proteins [5], and these non-covalently bound proteins must be eliminated during purification. In particular, use of the His tag has been widespread as proteins fused with this tag can be isolated under denaturing conditions to reduce non-covalent associations. Lastly, the development of stable cell lines expressing these tagged SUMOs has also been commonly applied to these studies. The advantage of cell lines is the ability to manipulate SUMO expression to provide more thorough and consistent substrate coverage than with transient expression systems. However, one caveat with the use of any tagged SUMOs is that the tagged version should retain the properties and substrate specificity of the native SUMO. While impossible to prove this later point globally, several studies have demonstrated that N-terminally tagged SUMOs appear to retain normal properties and are effectively recognized by the sumoylation system for conjugation to substrates [31-33]. Consequently, the use of tagged

SUMOs has become the standard approach for isolation of large cadres of sumoylated substrates.

Single affinity purification has been reported in several studies, but the background in control samples purified in parallel is high. The high background may be due to “sticky” proteins, proteins specifically interacting with the bound substrate, or SIM-containing proteins that are associating with the SUMO moiety, and careful controls must be used to discriminate between truly sumoylated proteins and false positives. In addition to false positives, the high background also may cause false negatives. Since typical MS approaches are not quantitative, the relative amounts of protein identified in the control samples versus the experimental sample are not determined. Consequently, even though a protein may be highly enriched in the affinity purified sample due to its sumoylation, if that same protein is present at low levels in the control sample then it will be excluded as background. This inability to discriminate between truly sumoylated proteins and low-level contaminants is a major impediment to the single affinity purification method. Even with these limitations, successful studies have been reported. Using a Protein-A tagged Smt3 (the yeast homolog to SUMO-1 in humans), Panse et al. used SDS-PAGE fractionation of the affinity purified sample, coupled with LC-MS/MS, to identify 138 potentially sumoylated targets in yeast [26]. However, no Smt3-modified peptides were found for any of the identified proteins, nor were some proteins previously known to be sumoylated in yeast, such as topoisomerase II and sister chromatid cohesion protein (Pds5), detected in the study. To attempt to verify the authenticity of these proteins as SUMO substrates, the authors developed a SUMO fingerprint assay. While this assay confirmed that a number of the identified proteins were sumoylated *in vivo*, several tested proteins did not appear to be sumoylated, illustrating the potential for false positives with the single affinity purification process. In a similar study, His₆-Smt3 was used to allow purification of sumoylated substrates under denaturing conditions, though even this more stringent process still resulted in significant contamination with background proteins in the control sample [34]. Nonetheless, using two-dimensional LC-MS/MS (multidimensional protein identification technology, MudPIT), 271 proteins unique to the His₆-Smt3 sample were identified, and further analysis of a selected subgroup confirmed sumoylation. In addition to the yeast studies, single affinity purification approaches have also been applied to human 293 [35] and HeLa [33] cells with many of the same issues of high background. The human cell studies also identified fewer total SUMO substrates, likely reflecting the increased cellular complexity and low abundance of the sumoylated forms.

A variation of single affinity purification is immunoprecipitation (IP), which typically involves a single tag that is used to enrich proteins from a cellular extract using antibody coated beads. IP faces many of the same challenges as other types of affinity purification due to nonspecific interactions with the antibodies or the beads, however, the use of a very specific antibody may allow for better detection of bona fide targets than affinity purification. Immunoprecipitation has been used in conjunction with MS to identify SUMO substrate proteins in human HEK-293 cells [22]. This study used HA-tagged SUMO1 and anti-HA antibodies coupled to Sepharose beads. The use of denaturing conditions to prepare the cell extract reduced background binding, and elution of SUMO1-conjugated proteins from the beads with 1X SDS sample buffer ensured efficient recovery of bound protein. In addition, the use of a SUMO conjugation-deficient cell line as a negative control aided in eliminating those proteins that were bound nonspecifically. After SDS-PAGE fractionation and HPLC-MS/MS, 21 candidate SUMO targets were identified, 4 of them known sumoylated proteins and 18 novel substrates, validating this as a useful SUMO proteomics strategy. Overall, the combination of single affinity purification with MS has been successful in greatly expanding the array of known sumoylated substrates. Not only have many additional nuclear proteins been identified in the pools of SUMO targets, these studies

have extended the range of targets to other cellular compartments including the cytoplasm and the endoplasmic reticulum, emphasizing the impact of sumoylation on numerous cellular processes.

In contrast to single affinity purification methods, tandem affinity purification (TAP) makes use of a SUMO moiety containing two sequential tags. The option of using two tags with very different properties allows increased specificity and reduced background due to nonspecific binding in the purification process, thus providing a greatly decreased recovery of non-sumoylated proteins. However, the potential for at least some nonspecific binding still exists, and appropriate controls and verifications still need to be performed. In the first application of TAP-tagged Smt3 in *S. cerevisiae*, Zhou et al. purified 13 proteins that possessed the tag as confirmed by Western blotting [32], and each band was in-gel digested with trypsin prior to HPLC-ESI-MS/MS. In addition to the band-specific peptides, each band produced Smt3-derived peptides as well, consistent with their sumoylation. Also using the TAP-tag strategy, two subsequent large-scale studies identified 146 [29] and 159 [27] sumoylated *S. cerevisiae* proteins, respectively. Both groups used cells that didn't express the tag protein as a negative purification control, and the total complement of proteins in the control purifications was greatly reduced compared to the single affinity purification studies. The TAP strategy has also been used successfully in mammalian cells [24,31]. In the first application of TAP-tagging to SUMO in human cells, our group developed two stable, inducible cell lines expressing His-S-tagged SUMO-1 and SUMO-3 [25]. Tandem affinity purification and LC-MALDI-MS/MS identified 122 likely sumoylated proteins, with 27 modified by both SUMO-1 and SUMO-3. As with the *S. cerevisiae* studies, very few proteins were detected in the control samples, increasing the confidence that the experimental sample proteins were truly sumoylated. Interestingly, while nuclear proteins were still the single largest group of sumoylated substrates, a significant number of targets were cytoplasmic proteins, including cytoskeletal components. Once again, MS-based proteomics offers the ability for broad definition of novel targets in a relatively unbiased fashion. However, it must be kept in mind that false-positives are possible with any of these affinity approaches, and independent verification of sumoylation should be employed for specific substrates chosen for further study.

Non-MS Approaches

As discussed above, the MS-based methods are not without issues. In addition to issues with nonspecific contaminants yielding false positives, only relatively few of the known sumoylated proteins have been identified in MS studies. The failure to detect these known SUMO substrates is likely due to the difficulty of detecting minor components in complex mixtures dominated by highly abundant proteins, even when the minor components are demonstrably present in the purified sample [31]. While various fractionation techniques have been widely applied prior to MS, there are still issues with resolution, sample loss, and peptide masking. Consequently, development and application of creative non-MS strategies have also been pursued to identify potential SUMO targets. Other methods such as chip-based analysis [36], *in vitro* expression cloning [37], and yeast two-hybrid analysis [29,38] allow for the identification of sumoylation targets without the need for enrichment of sumoylated targets or MS analysis.

Chip based analysis of SUMO substrates is conceptually similar to microarray experiments [36]. In this procedure, potential candidate proteins are spotted on a glass slide and *in vitro* sumoylation reactions are conducted on the arrayed proteins. This is followed by the use of fluorescent antibodies against SUMO to detect those proteins that have been sumoylated. The report by Oh et al. was a proof-of-principle study, and the only protein actually tested was RanGap1, which is a known SUMO target. The purpose was essentially to develop the procedure, optimize conditions, and confirm its feasibility as a sumoylation identification

system. However, protein chips are inherently more difficult to construct and analyze than nucleic acid chips due to the extreme biochemical heterogeneity of proteins. Consequently, it may prove difficult to obtain sufficient quantities of a large array of different proteins to ensure broad proteome coverage for unbiased screening. Also, the conditions for effective sumoylation may vary for different proteins spotted on the slides, and poor or absent sumoylation would yield false negatives. Lastly, this method does not allow mapping of specific modified lysine residue(s), which now appears to be possible with MS (see below). Nonetheless, with rapid improvements being made in protein arrays, this approach may prove less labor intensive than some expression/purification systems and could provide quantities of target information that rival the MS techniques.

Another promising approach is *in vitro* expression cloning (IVEC) [37]. This approach makes use of cDNA libraries that are divided into groups of 100 clones for *in vitro* transcription and translation reactions, followed by *in vitro* sumoylation reactions. Parallel reactions are set up without the *in vitro* sumoylation reactions to provide the band migration pattern for the unmodified translated proteins on SDS-PAGE. Comparison between sumoylated and unsumoylated pools reveals new species that represent sumoylated forms of one or more proteins in the pool. Positive pools are subdivided and rescreened to identify single substrates. This method was applied to a library predicted to cover 10,000 genes, but only 40 SUMO-1 substrates were found, suggesting that identification coverage was limited. This may be due to the fact that a very specific tissue (brain) was examined, that there may be highly variable transcription/translation efficiencies for different clones in each pool, and that only SUMO-1 was tested. Another potential problem with this approach is inefficient sumoylation of some proteins in the initial pools, similar to that discussed for the chip-based assay. The authors did show that *in vitro* sumoylation of some individual substrates was stimulated by inclusion of SUMO ligases, suggesting that inclusion of ligases during the initial screening might enhance detection, though this was not reported. Nonetheless, this strategy is an attractive complement to MS, and this technique warrants further optimization.

Lastly, yeast two-hybrid analysis is another approach that can be used to identify SUMO targets. Unlike most of the other approaches discussed above, the yeast two-hybrid allows for detection of proteins that are either covalently or non-covalently associated with SUMO [29]. Using a GAL-SUMO fusion with the normal SUMO diglycine C-terminus as the bait will identify proteins that are either conjugated to SUMO or simply bound to it. In contrast, using a bait SUMO lacking the diglycine prevents conjugation and only detects proteins non-covalently interacting with SUMO. Comparison of the two screens allows identification of the truly conjugated SUMO substrates. While this approach successfully identified 7 substrates, this was a relatively small number considering that approximately 500,000 clones were screened. The inefficiency may result in part from *in vivo* desumoylation, and better detection may be possible in yeast strains with conditional defects in Ulp1, the yeast SUMO protease. Again, optimization and clever application of yeast genetics may further refine and improve the coverage of this technique.

In conclusion, the methods employed in the proteomic study of SUMO are becoming more and more diverse. Clearly, these unbiased approaches to global identification of sumoylated proteins have great potential for expanding our understanding of how sumoylation impacts diverse cellular processes across the intracellular environment. While each method has their inherent advantages and disadvantages, it must be noted that multiple approaches should be used to ensure that the full spectrum of the SUMO proteome is studied. For simple identification of substrates, any of the discussed methods may be sufficient. However, when conditional sumoylation or a quantitative approach is needed (see below), enrichment, purification, and MS are likely to be the methods of choice. In either case, the field of

proteomics holds enormous promise for gaining insight into the nature of the sumoylation system, and for unraveling its various roles in the cell.

SUMO proteomics: applications

Due to the sensitivity, accuracy, and proteome coverage of the various MS techniques, this has rapidly become a standard approach for addressing global questions about cellular sumoylation. The last two years have seen numerous studies, in both yeast and metazoans, examining the SUMO proteome under various conditions. In the sections below, three major applications of MS to the sumoylation system are discussed.

SUMO type-specific substrate differences

Consistent with their sequence divergence, it is apparent from numerous studies that there are distinct differences between SUMO1 and SUMO2/3 families. These differences include unique susceptibilities to various SENPs [39,40], distinct responses to environmental conditions [35,41,42], dissimilar cellular abundance and localization [35,41,43], and in a few examined cases different preferences for individual substrates [25,31,41]. Furthermore, one of the first comparative studies demonstrated in COS-7 monkey cells that the overall patterns of SUMO1-modified proteins differed from that of SUMO2/3-modified proteins by 1-dimensional SDS-PAGE, suggesting that their target substrate populations were not identical [41]. Differences in total sumoylated protein patterns for different SUMO types have also been observed in human cells [25,35,43] and other cell types [44,45], consistent with a general distinction in substrates for the different SUMOs. Nonetheless, the demarcation in the biological functions of SUMO1, SUMO2, and SUMO3 remains uncertain, and substrate by substrate examination of SUMO type preference seems unlikely to provide significant insight into the roles of each SUMO type. In contrast, several proteomic studies have begun more global identifications of the common and distinct substrates for individual SUMOs [25,31,33,35], and this unbiased approach may generate a broader perspective of what is unique about the substrate population for each SUMO type.

In the first direct comparative study, Manza et al. identified 40 SUMO1-modified proteins (51%), 24 SUMO3-modified proteins (31%), and 14 proteins (18%) that could be modified with either SUMO1 or SUMO3. Both SUMO1 and SUMO3 modified proteins were predominantly nuclear and involved in regulation of nucleic acid structure and function; similarly, a study of just SUMO2 substrates also found that they were mostly nuclear [33]. While there are some protein functional categories that are not represented for both SUMO1 and SUMO3 in the Manza et al. study, this likely reflects the relatively small sample size rather than a true difference in functional substrate preference. A subsequent study by Rosas-Acosta et al. [31] identified a larger total cadre of sumoylated proteins, but found a very similar distribution: 50% exclusively SUMO1-modified, 28% exclusively SUMO3 modified, and 22% modified by both SUMO1 or SUMO3. Once again, there was no obvious difference in protein functional types that were modified by SUMO1 versus SUMO3. More recently, Vertegaal et al. have performed an elegant quantitative proteomics comparison of SUMO1 and SUMO2 substrates using the SILAC isotopic labeling method [25]. Like the previous studies, this report found that SUMO1 and SUMO2 have both distinct and common substrates with a preponderance of nuclear proteins. Unfortunately, the overall sample size is still too small to detect any specific themes concerning which substrate types are modified by which SUMO type. Ultimately, a large catalog of unique and common targets for all three SUMOs needs to be developed to allow careful analysis of trends that may reflect differences in biological function. This will be further complicated by the fact that the pool of sumoylated proteins will undoubtedly change with cell type and environmental conditions (see below).

Conditional sumoylation

One of the most powerful applications of proteomics to the SUMO field is the identification of substrates that are differentially sumoylated in response to various cellular growth conditions. A variety of stress conditions, including elevated temperature, ethanol or H₂O₂ exposure, and hypertonic conditions have been shown to cause changes in the sumoylation pattern for both vertebrates [41,46], yeast [32], and plant [45] cells. In addition to changes in response to stressors, significant changes in overall sumoylation patterns have also been observed for normal differentiating cells [42,47], during induction of apoptosis [48], and for cancerous cells [28]. These combined results suggest the sumoylation contributes to multiple cellular processes that are activated by changes in cellular environment. However, the multitude of observed changes in the overall sumoylated protein profiles make it a significant challenge to distinguish the critical targets from the collateral substrates. The sensitivity and specificity of proteomic mass spectrometry allow identification of individual sumoylated proteins, and this information should be useful for prioritizing important candidates for individual characterization.

To date, the most thoroughly evaluated conditional effector of sumoylation has been the stress response, and several groups have used proteomics to determine individual proteins that are preferentially sumoylated under stress conditions. From yeast [32] to plants [45] to human cells [35], overall sumoylation is generally increased by reagents that produce cellular stress. In both yeast [32] and human HEK293 [35] cells, stress induced a dramatic redistribution of the sumoylated substrates resulting in de novo sumoylation of many stress-specific target proteins. Among the newly sumoylated proteins identified by proteomics are several chaperones, heat shock proteins, and enzymes known to be important in the stress response, including DNA damage and repair proteins. The implication of these results is that sumoylation of these pertinent target proteins enhances their function, though this has not been directly established in most cases. If functions are enhanced, it might be through direct activation, protection from proteolytic degradation, and/or modulation of intracellular location, all known effects of sumoylation. Interestingly, the enzymes of the sumoylation system are themselves sumoylated under stress conditions, and this may increase their activities as well leading to the increased sumoylation of downstream targets. Much additional work will be needed to sort out the important targets from the secondary events, and to define specific targets that are relevant to different types of stress.

Several reports have suggested a link between the sumoylation system and cancer [49-55], though the mechanistic role of sumoylation in prevention or promotion of cancer is unclear. As for the stress studies, identification of proteins whose sumoylation states differ between normal and cancerous cells should provide clues as to relevant substrates whose modification may have functional implications for the disease. Additionally, even sumoylated substrates that are not directly related to the disease process may still be useful biomarkers for the disease state. Recently, a proteomics approach was used to identify sumoylated proteins in a melanoma cell line [28]. Forty-three SUMO substrates were identified, including proteins with known functions in signal transduction, splicing, and transcription. Interestingly, several proteins specifically expressed in cells of the neural lineage were identified as SUMO1 targets in this study, though the significance of this observation is unclear. A major limitation of this study was the lack of a corresponding normal cell line for comparison, so whether or not any of the sumoylated targets were specific to or necessary for the malignant state could not be assessed. Nonetheless, more extensive studies of this type should be informative about malignancy-specific SUMO targets. Given the multitude of cancers and affected cell types, there may be an enormous amount of useful data that can be mined from well-implemented comparative studies.

One general and key problem with all these comparative studies by MS approaches is that important substrates may exist in sumoylated forms under both tested conditions, but may be sumoylated to a quantitatively different degree under one condition. Such changes in the amount of sumoylation could have dramatic effects on protein function, but would not be detected by typical MS screens which would simply identify the substrate as sumoylated under both conditions. Currently published studies have primarily identified substrates with such absolute differences where the substrates are exclusively sumoylated only under one condition, not both. Clearly, application of quantitative MS approaches, such as described by Vertegaal et al. [25], will be needed to develop more robust evaluation of sumoylation changes during conditional growth.

Identification of target lysine in SUMO substrates

Mutational analysis of several of the first identified individual SUMO1 substrates revealed a motif common to many target lysines: $\Psi KxE/D$, where Ψ is a hydrophobic residue (typically Val, Ile, Leu, Met, or Phe), K is the target lysine, x is any amino acid, and the fourth position is an acidic residue [6]. While this motif has proven valuable for predicting potential sumoylated lysines to characterize by site-directed mutagenesis, the mutagenic approach is more problematic when substrates have multiple possible sumoylation sites. Furthermore, it may be difficult in mutational studies to distinguish between lysines that are actual sites of sumoylation and lysines whose mutation simply changes the substrate and results in loss of sumoylation at a distal lysine. Consequently, development of techniques that can directly map sumoylated lysines would greatly facilitate definitive assignment of the target residues. In addition, some early examples of sumoylated proteins were found to have sites that did not match the consensus motif, suggesting that alternative sequence features could also specify a particular lysine for SUMO modification [56-58]. Thus, predictions and analyses based solely on the simple $\Psi KxE/D$ motif would undoubtedly miss pertinent sites, so there is great need for more unbiased approaches that can identify target lysines regardless of sequence context.

Several recent reports have confirmed the potential of mass spectrometry to provide a more specific and global technique for mapping sumoylation sites. In the first reported use of this approach, Zhou et al. identified ten SUMO sites in seven yeast proteins by searching for substrate peptides with masses consistent with both a missed tryptic cleavage (SUMO modified lysines are not cleaved by trypsin) and the addition of a SUMO signature peptide tag (derived from the C-terminus of SUMO after tryptic cleavage; see Table 2) [32]. Interestingly, even from this limited analysis, five of the ten sumoylation sites determined were in non-canonical sequences. In a similar approach, Denison et al. identified sumoylation sites in six yeast proteins, of which two were in classical $\Psi KxE/D$ motifs [27]. This approach is more problematic in humans, as all three human SUMOs lack lysines or arginines near the C-terminus (Table 2), so their signature tag peptides are quite large. Nonetheless, Chung et al. examined SUMO2 conjugation sites for *in vitro* sumoylated proteins by first screening SDS-PAGE bands for ones that produced tryptic digests containing both substrate and SUMO2 peptides [59]. Subsequently, peptide samples from these bands that were in the correct high mass range were subjected to MALDI MS/MS for sequencing and localization of the conjugated lysine. Once again, half the identified sumoylation sites (three of six) were in sequences which did not conform to the $\Psi KxE/D$ motif. In a more limited study that looked only at polysumoylation of RanBP2, a SUMO E3 ligase, Fourier transform ion cyclotron resonance (FT-ICR) was shown to be highly effective for identifying SUMO attachment sites [60]. The resolution and mass accuracy of this technique is ideal for analysis of large isopeptides containing the human SUMO signature tags, though it is still likely limited to samples of low complexity. What should further help these analyses are the development of improved software packages, such as

SUMnOn [61]. This program can be tailored for identification of fragment ion patterns generated by complex post-translational modifications, such as sumoylation. In validation studies using LC-ESI-MS/MS, SUMnOn was significantly more effective than the commonly used SEQUEST and Mascot programs for identifying sumoylated peptides, though this low mass accuracy approach still restricted the analysis to fairly simple protein mixtures. As an alternative to developing approaches for analysis of the native large signature tags of the SUMO family proteins, two groups have developed strategies to alter the SUMO sequence to create proteins whose tryptic cleavage generates smaller signature tags that are more amenable to MS analysis [62,63]. In both cases, amino acids upstream of the terminal diglycine motif were mutated to lysine or arginines to generate a trypsin site adjacent or close to the diglycine. For SUMO-1, the T95R mutant was effectively conjugated to RanGAP1 *in vitro* and yielded a ubiquitin-like diglycine signature tag after tryptic digestion which could be detected with appropriate setting of the data inclusion list [62]. Similar mutation (I96R) of the yeast SUMO, Smt3, also provided greatly enhanced detection of the diglycine-tagged peptides compared to the wild-type EQIGG tag [63]. The diglycine tagged peptides showed a 5-10 fold increased precursor ion intensity, which was the likely explanation for the enhanced identification of these peptides. However, as effective as the use of mutant SUMOs appears to be for identifying SUMO sites in proteins modified *in vitro*, there are still issues for *in vivo* application. First, steady-state level of the sumoylated forms of most substrates is quite low, so even the use of mutant SUMO forms will still require methods to enrich the sumoylated protein population prior to MS analysis. Second, the efficiency and specificity of incorporation of the mutant SUMOs into substrates *in vivo* has not been extensively examined, and differences between wild-type and mutant SUMOs could result in both false negative and false positive identifications. Lastly, while the diglycine tag produced by tryptic digestion of these SUMO mutants facilitates MS analysis, this is the same tag would also be produced if the proteins were naturally modified *in vivo* by ubiquitin, NEDD8, or ISG15. Consequently, even with affinity enrichment for sumoylated proteins, there is some chance that diglycine-tagged peptides could have arisen from these other modifications. Fortunately, none of these issues are insurmountable, and alternative mutations coupled with other non-tryptic digestion protocols should provide a variety of SUMO tags that can be adapted to the needs to the particular analysis. Future combinations of improved software, high mass accuracy MS techniques, and clever modifications of SUMO will undoubtedly facilitate SUMO target site identification from more complex samples.

Cross-talk between the ubiquitin and SUMO systems

Initially, the ubiquitin and SUMO systems were considered completely separate and parallel processes since their enzymes did not cross function with the other modifier. However, it has become apparent that these systems do in fact communicate, both at the level of individual substrates and through direct modification of each other=s enzymatic machinery (reviewed in [64]). The interplay between these two major modification systems will undoubtedly provide levels of co-regulation that have previously not been appreciated. Studies on how these two systems interact will likely become an increasingly exciting area in the near future, and while a comprehensive discussion is outside the scope of this review, a few seminal studies are discussed here (Fig. 2).

Modification of common lysine residues on substrates

Since all the ubiquitin-like modifiers attach to the epsilon amino group of lysine side chains, it is possible for multiple modifiers to target the same lysine residue. In several cases, such as for I κ B α , sumoylation stabilizes the protein by competing with ubiquitin for a common lysine target, lysine 21 [65]. The inability of polyubiquitinylation to occur due to blockage of the target lysine with SUMO prevents proteasomal degradation. Similar competition has

been reported for Smad4 [66] and huntingtin [67] though this does not appear to be a uniformly common mechanism as sumoylation of most substrates does not affect their stability. Alternatively, modification of substrates at common lysines may have drastically different effects on protein function or localization depending on whether SUMO or ubiquitin is attached. This is related to the observations that, in addition to the common role for K48 linked polyubiquitin in targeting proteins for proteasomal degradation, monoubiquitin and alternatively linked (K63) polyubiquitin also have regulatory roles as protein modifiers [68]. Two well-characterized examples of this type of modification-dependent functional switching are NEMO and PCNA. NEMO is the regulatory subunit of the IKK complex and can be both sumoylated or K63 polyubiquitinated at lysine 309 [69]. These modifications appear to be sequential and both required for control of IKK activity (reviewed in [64]). In response to genotoxic stress, NEMO is sumoylated at both K277 and K309, leading to release of NEMO from the IKK complex and nuclear import of NEMO. Within the nucleus, NEMO is desumoylated, phosphorylated at K277, and ubiquitinated with K63 linked ubiquitin. This modified form of NEMO exits the nucleus and rejoins the IKK complex to form active IKK. Thus, it appears that sumoylation and ubiquitination cooperate on NEMO to create a regulated sequence of events controlling activation of IKK. Similarly, alternative modifications at lysine 164 of PCNA subtly regulate the contributions of PCNA to DNA replication and repair [64]. During DNA damage, ubiquitination of PCNA at K164 facilitates bypass of DNA lesions by two mechanisms: monoubiquitin addition recruits error-prone DNA pol η [70] while polyubiquitination triggers an error-free, RAD6-dependent pathway that uses sister chromatid information to bypass the damaged region [58]. Recent work in yeast has now shown that PCNA can also be sumoylated at K164, and that this modification recruits the helicase Srs2p [71]. During normal DNA replication, this recruitment of Srs2p prevents unscheduled homologous recombination and would facilitate subsequent ubiquitin-dependent damage repair at stalled replication forks. Thus, these two modifiers appear to work in concert to control the precise type and degree of PCNA-mediated DNA damage repair. Intriguingly, whether these modifications are consecutive events or can occur simultaneously on different subunits of the homotrimeric PCNA has not yet been determined. In either case, these studies with NEMO and PCNA exemplify how the complex and coordinated interplay between the ubiquitin and SUMO modification systems can provide exquisite and subtle regulation of substrates through targeting of a common lysine residue.

Co-modification of substrates

As opposed to the mutually exclusive modification of a common target lysine discussed above, the SUMO and ubiquitin systems also can be co-regulate through mutual substrate modification at independent lysines. One of the most exciting and potentially far-reaching examples of this is histone sumoylation. Histones have been shown to be sumoylated both in *S. cerevisiae* [72] and in mammalian cells [73], adding SUMO modification to histone code along with acetylation, phosphorylation, methylation, glycosylation, ADP ribosylation, and ubiquitination. Sumoylation has generally been found to have a negative effect on the activity of specific transcription factors, and this transcriptional repression is also being extended to effects on chromatin structure (reviewed in [74]). The recent studies on histone sumoylation support a direct effect on nucleosomes that represses transcription, in contrast to histone ubiquitination, which enhances transcription. However, these opposing effects of sumoylation and ubiquitination are not known to be mediated through alternative modification of the same lysine residues. For *S. cerevisiae*, ubiquitination of H2B at K123 is the only well-established ubiquitin modification. Nathan et al. found that sumoylation sites were detected for H2B K6 or K7 by mass spectroscopy (MS); additionally, the K6/7 region sequence is repeated downstream in H2B suggesting that there would also be sumoylation at K16/17, though MS did not detect these sites [72]. While the existence of

other lysines that could be targeted by both SUMO and ubiquitin is possible, these current results support separate lysine modifications of H2B by these two modifying systems. Whether or not the identified SUMO and ubiquitin sites could be simultaneously modified in H2B has not been determined, but raises combinatorial possibilities. Interestingly, mutation of the K123 ubiquitin site resulted in increased H2B sumoylation, suggesting that even though K123 is not a sumoylation site, that ubiquitinylation of this residue negatively regulates sumoylation at other sites on H2B. In summary, this H2B work adds a new component to histone regulation, though comparable studies have not been performed for the mammalian homologs, so the extent to which ubiquitin and SUMO co-modify and coordinate the activity of metazoan histones remains to be determined. Nonetheless, it has been shown that a human H4-SUMO fusion can recruit HDAC1, and this may be the mechanism by which histone sumoylation antagonizes ubiquitin-stimulated transcription [73].

Sumoylation of ubiquitin system components

There are now a few examples of sumoylation of ubiquitin enzymes, suggesting that SUMO modification is a direct regulator of the ubiquitin system. One of the earliest examples is the ubiquitin ligase, MDM2 [75,76]. MDM2 is the E3 ligase for p53 and is a critical regulator of p53 stability and activity [77]. This regulation is complex, and it has been proposed that monoubiquitinylation of p53 by MDM2 leads to subsequent sumoylation and nuclear export, while polyubiquitinylation leads to typical proteasomal degradation [78]. However, MDM2 itself appears to be multiply sumoylated, first by RanPB2 during nuclear import and then by PIAS proteins within the nucleus [79], and its sumoylation is enhanced by the p14ARF tumor suppressor [75]. The precise role of MDM2 sumoylation is still poorly understood, but a new study by Lee et al. presents evidence that sumoylation of MDM2 prevents self-ubiquitinylation and degradation [80]. This group identified a novel murine homolog of SENP2, designated SUSP4, which both displaced p53 from MDM2 and specifically cleaved SUMO1 conjugates from MDM2. The net effect of these actions was to stabilize p53 as it was no longer targeted for ubiquitinylation upon overexpression of SUSP4. These results are consistent with sumoylation being a positive regulator of the MDM2 ubiquitin ligase function by protecting MDM2 from degradation. In contrast, sumoylation may be a negative regulator of another ubiquitin ligase, Parkin [81]. It is not yet established that Parkin is actually modified by SUMO1, but Parkin does interact with both SUMO1 and the SUMO E3 ligase, RanBP2 [82], and the interaction with SUMO1 stimulates the nuclear localization and self-ubiquitinylation of Parkin. These results are again suggestive of an interplay between the sumoylation system and the ubiquitin system that has regulatory implications for the function of ubiquitin system.

In addition to sumoylation of ubiquitin ligases, SUMO modification of a ubiquitin conjugating enzyme has also been reported [83]. E2-25K is implicated in ubiquitinylation of several neuronal proteins such as huntingtin and polyglutamine proteins [84] and may contribute to pathological damage in those diseases. Work by Pichler et al. showed that E2-25K was modified at lysine 14 in a noncanonical sumoylation motif. *In vitro*, sumoylation of E2-25K inhibited its activity, though no corresponding studies were performed *in vivo* so the actual biological effect of sumoylation on E2-25K function remains unknown. Nonetheless, this study strongly suggests a regulatory role for sumoylation on E2-25K activity. Thus, sumoylation of both the E2 and E3 components of the ubiquitin system has been observed and has functional consequences. Given the multitude of ubiquitin E2 and E3 enzymes, it is highly likely that additional examples of important SUMO regulation of these components await discovery. As with other types of modifications, whether sumoylation has positive or negative effects on ubiquitin E2s and E3s will probably be substrate specific.

Dual Ligases

There is currently only one example of a ligase with functionality for both ubiquitin and SUMO, TOPORS [85-87]. While a large, multi-protein complex with both SUMO ligase activity and potential ubiquitin ligase activity has been reported [88], TOPORS is the sole known protein capable of performing ligation with either modifier. Using p53 as the substrate, TOPORS enhances its sumoylation *in vivo* and *in vitro*, thus fulfilling the criteria for an authentic SUMO ligase. In a subsequent study, Pungalaya et al. used MS to identify twenty additional substrates for the TOPOR SUMO ligase activity, most of which are involved in chromatin modification or mRNA processing [85]. However, unlike p53 which can be both ubiquitinated or sumoylated by TOPORS, whether or not these new substrates were also targets for TOPORS-mediated ubiquitinylation was not addressed. Consequently, while these studies confirm the existence of a dual modifier ligase, little is known about the biological significance of having both ligase activities in a single protein. For example, what are the advantages of a dual modifier ligase, and is TOPORS unique or do other dual ligases exist? Much further study is required before the scope and significance of this type of ligase can be determined.

SUMO-targeted ubiquitin ligases (STUbLs)

An emerging theme in recent months is the existence of ubiquitin E3 ligases that are specifically targeted to sumoylated substrates through SUMO interaction motifs (SIMs) present in the E3 ligases [89-92]. Studies in *S. pombe* identified the Slx8-Rfp complex as a ubiquitin E3 ligase [89,90], and subsequent work in *S. cerevisiae* showed that the related Hex3-Slx8 complex is also a ubiquitin ligase [91,92]. In both yeast types, mutants of these ligases have numerous defects in morphology and genome stability, and exhibit enhanced levels of sumoylated proteins. Interestingly, these mutants can be complemented with human RNF4, indicating that this particular ligase function is well conserved; inspection of the RNF4 sequence suggests that it may perform the function of both Slx8 and Rfp [89]. Also, the effect of these ubiquitin ligase mutants can be suppressed by a SUMO E3 ligase mutant, which confirms that there is important cross-talk between these two systems to maintain SUMO pathway homeostasis. Using Rad52 as a substrate *in vitro*, Xie et al. demonstrated that ubiquitinylation of a Rad52-SUMO fusion is markedly enhanced compared to Rad52 alone, suggesting that the Hex3-Slx8 ligase will preferentially interact with sumoylated proteins [91]. Consistent with this observation, using RNF4 on an artificial substrate, Sun et al. found that mutation of either the RNF4 SIM or the SIM binding region of SUMO itself reduced sumoylation of the substrate [90]. The overall conclusion from this series of studies is that Hex3-Slx8/Slx8-Rfp/RNF4 represent a new class of ubiquitin ligases (STUbLs) with a preferential targeting to sumoylated proteins.

The existence of one or more ubiquitin ligases with a specificity for sumoylated proteins raises the question of biological function. It seems unlikely that these STUbLs would target any sumoylated protein indiscriminately, so additional specificity determinants must exist, and it has been proposed that the Hex3-Slx8/Slx8-Rfp/RNF4 ligase may target DNA repair proteins [90]. If there is substrate specificity, then why do mutants of these ligases have global effects on overall cellular sumoylation levels? There is no evidence that these complexes have SUMO protease or SUMO ligase activity, so it is their function in ubiquitinylation that is critical, but it seems unlikely that their role is to globally target sumoylated proteins for proteasomal degradation. One possible explanation is that selective targeting of certain sumoylated proteins may have regulatory consequences for overall sumoylation. For example, the enzymes of the sumoylation pathway are themselves sumoylated, so targeting one or more of these enzymes for ubiquitin-mediated degradation via a STUbL would help regulate the levels of sumoylation activity. Further insight into STUbL functions and mechanisms will require identification and characterization of

additional STUbLs and their substrates, and both of these tasks are well suited for MS-based approaches.

Expert opinion

In the present report, we review the properties of the ubiquitin super family, with a focus on the SUMO system. Soon after its discovery in the 1990s, it became clear the sumoylation was distinct from ubiquitinylation, both in terms of the enzymatic components and the function of the modifications. Early studies identified several prominent nuclear proteins as SUMO substrates, including known transcription factors, leading to a bias in the literature where reports on sumoylation were heavily weighted towards transcription factors. The eventual application of more global and less biased proteomics approaches revealed that sumoylation was a more wide-spread modification with both nuclear and non-nuclear substrates. While these studies indicated that transcription factors still comprised a large fraction of the total sumoylated population, it became apparent that a much wider array of protein functional categories was targeted for sumoylation. Furthermore, given the limitations of current proteomics for detection of sumoylated substrates, including issues of low abundance, conditional modification, and poor extraction due to subcellular location, it is likely that only a small fraction of the total ASUMOome[®] has been identified. Development of improved methods for isolation and identification of sumoylated proteins will be key to further defining the global SUMO substrate pool and to understanding how changes in substrate sumoylation reflect response to cellular growth conditions. Intrinsic in all of this will be an increased appreciation for and discrimination between the individual roles of SUMO 1, 2, and 3 in vertebrates

A second benefit of SUMO proteomics has been the realization that the initially defined SUMO conjugation site motif, ΨKxE/D, again for historical reasons, reflects only a portion of the authentically targeted lysine residues. The ΨKxE/D motif was developed from the early list of mapped SUMO addition sites, and even though occasional exceptions were noted, most of the limited number of mapped target lysines conformed to this sequence. It wasn't until more extensive SUMO target lysine mapping was performed with MS techniques that the diversity of SUMO attachment became apparent. While lysine remains the only target residue, the sequence context shows considerable variation, and there appear to be structural contexts that influence usage. Similarly, not all ΨKxE/D motifs are actually utilized for sumoylation, and again structural context may be important. Certainly the identification of large numbers of SUMO targeted lysines will provide more sequence information for discovery of additional factors that regulate lysine usage by the SUMO system.

Lastly, the initial belief that sumoylation was a completely parallel and distinct pathway from ubiquitinylation has faltered. As discussed above, there is increasing evidence of complex and varied mechanisms for cross-talk between these systems. This should perhaps not be surprising as the ubiquitin superfamily is an ancient system that seems intimately involved with fundamental and essential nucleic acid metabolic processes such as DNA replication, repair, and transcription. Having connections between the branches of this family would allow signaling, feedback regulation, and coordination between pathways that have evolved to have distinct yet cooperative functions. One of the most exciting and important advances for this field will be to delineate these connections, identify the mechanisms for cross-talk, and define the scope of the overall network as it controls specific cellular events. The combination of proteomics with standard biochemistry, molecular biology, and cellular biology is poised to make spectacular advances in understanding sumoylation and its more global physical and functional context in the ubiquitin superfamily.

Five-year view

Sumoylation is a complex and extensive process that impacts numerous cellular proteins. In the previous 10 years, studies have focused primarily on individual SUMO substrates and have attempted to understand the role of sumoylation on a protein-by-protein basis. The next 5 years will likely see an increased appreciation of the more global role of sumoylation in coordinating multiple processes and pathways. There should be an explosion of new sumoylation data coming from continued application of improving proteomics techniques, which should expand the pool of known SUMO substrates from the current hundreds to thousands. Coupled with the increased number of identified targets will be the careful evaluation of sumoylation dynamics during cell growth, differentiation, and response to environmental stimuli. Lastly, the interplay and cross-talk between sumoylation and other ubiquitin family pathways should become more clearly defined, leading to improved ability to manipulate these systems to facilitate cell growth and repair.

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☐ of interest

☐ of considerable interest

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Key issues

! Novel techniques for SUMO proteomics have been reported and need further refinement.

! MS technologies are now widely applied to sumoylated proteins, but improvements for detection of low abundance proteins would enhance coverage.

! Natural human SUMOs have poor signature tags, and mutated SUMOs with improved signatures have been developed. Additional improvements giving unique and easily detected signature peptides will facilitate mapping of conjugation sites on substrates.

! Current studies have begun to identify broad arrays of sumoylated proteins and have greatly expanded the repertoire of known SUMO substrates. More complete identification of total sumoylated proteins and evaluation of global sumoylation changes in response to environment and growth conditions are needed.

! The proteomic studies have revealed differences in substrate preference between SUMOs 1, 2, and 3, but the distinct and common functional roles of the different SUMOs remain unclear.

! There are clear examples now of cross-talk between the SUMO and ubiquitin systems. The extent and significance of this cross-talk requires further study.

Fig. 1. Schematic representation of the enzymatic pathway for attachment of Ulp to substrate proteins. Below the diagram are comments about the number and features of the particular enzymes involved in attachment of ubiquitin or SUMO as indicated.

Fig. 2. Cross-talk between the sumoylation and ubiquitinylation systems. The diagram illustrates five mechanisms for cross-talk between the SUMO and ubiquitin modifier systems. SE1, SE2, and SE3 refer to the E1-E3 enzymes for the sumoylation system, and UE1, UE2, and UE3 are for the ubiquitin system; S/UE3 represents a dual function ligase. SUMO moieties are represented by the gray ovals and ubiquitin by the black ovals. The rounded rectangles are the protein substrates with target lysines. (1) Modification of a common lysine by either SUMO or ubiquitin, (2) co-modification of a substrate with SUMO and ubiquitin addition at different lysines, (3) specific targeting of sumoylated proteins by STUbLs (**S**UMO-**T**argeted **U**biquitin **L**igases), (4) dual function ligase that can stimulate both SUMO and ubiquitin addition to the substrate, and (5) modification of ubiquitin E2 and E3 enzymes by sumoylation. Details of these five mechanisms are discussed in the text.