Chapter 1

Concepts and Methodologies to Study Protein SUMOylation: An Overview

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Abstract

Protein modification by the small ubiquitin-related modifier (SUMO) was simultaneously discovered by several groups at the middle of the 1990s. Although distinct names were proposed including Sentrin, GMP1, PIC1, or SMT3, SUMO became the most popular. Early studies on the functions of SUMOylation focused on activities in the nucleus, including transcription activation, chromatin structure, and DNA repair. However, it is now recognized that SUMOylation affects a large diversity of cellular processes both in the nucleus and the cytoplasm and functions of SUMOylation appear to have undefined limits. SUMO-conjugating enzymes and specific proteases actively regulate the modification status of target proteins. The recent discoveries of ubiquitin-SUMO hybrid chains, multiple SUMO-interacting motifs, and macromolecular complexes regulated by SUMOylation underscore the high complexity of this dynamic reversible system. New conceptual frameworks suggested by these findings have motivated the development of new methodologies to study pre- and post-SUMOylation events in vitro and in vivo, using distinct model organisms. Here we summarize some of the new developments and methodologies in the field, particularly those that will be further elaborated on in the chapters integrating this book.

Key words SUMOylation, History, Methodologies

1 A Brief History of the Discovery of Small Ubiquitin-Related Modifiers

In 1978, Avram Hershko and his graduate student, Aaron Ciechanover, discovered that proteins added to reticulocyte extracts become covalently conjugated to a protein called ubiquitin [1]. They subsequently demonstrated that these ubiquitylated proteins are degraded in an ATP-dependent process, thus establishing entirely new fields of cellular, molecular, and biochemical research [2]. These pioneering studies paved the way for subsequent work by a large number of laboratories that revealed regulatory roles for ubiquitylation in virtually every aspect of cell function [3], and ultimately earned Hershko, Ciechanover, and their collaborator Irwin Rose the 2004 Nobel Prize in Chemistry [4].

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Despite the clear utility of the ubiquitylation pathway for regulating cellular processes through exquisite spatial and temporal control of posttranslational protein modification, the existence of related pathways remained uncertain for nearly 20 years following the initial discoveries of Hershko and Ciechanover. This abruptly changed in the mid-1990s with the near-concomitant discoveries by several laboratories of the family of proteins now known as small ubiquitin-related modifiers, or SUMOs. The newly emergent SUMO field benefited enormously from the groundwork and insights obtained through more than 20 years of ubiquitin research. Thus, it was established very quickly that SUMOylation proceeds through an enzyme cascade paralleling ubiquitylation and functions as a posttranslational modification with impact and consequences as broad and profound as ubiquitylation [5]. This review highlights the earliest studies and experimental evidence that identified either the genes coding for SUMOs or the SUMO proteins themselves, and thus pointed to the existence of parallel, ubiquitin-like posttranslational protein modification pathways. Notably, the discovery of SUMOs represented an important milestone, paving the way for the subsequent discovery and characterization of more than ten functionally distinct ubiquitin-like proteins and pathways (UBLs) [6].

1.1 First Discoveries of Genes and cDNAs Coding for SUMO Proteins

The earliest reported studies hinting at the existence of SUMO proteins came in 1995 through the work of Pamela Meluh and Douglas Koshland. Their discovery of SUMO resulted from work focused on analysis of Mif2, a protein linked to accurate chromosome transmission in the budding yeast, *Saccharomyces cerevisiae* [7]. Through genetic and molecular analysis, they verified the importance of Mif2 in chromosome segregation and provided evidence that it is a centromere-associated protein with homology to human CENP-C. Importantly, they also identified several high-copy suppressors of a temperature-sensitive Mif2 mutant allele, including the Smt3 (suppressor of mif two, clone 3) gene that codes for what we now know to be the yeast ortholog of human SUMO1. Although evidence that Smt3 is conjugated to Mif2 or other centromere-associated proteins was not presented in this early study, it is now recognized that multiple centromere and kinetochore-associated proteins are regulated through SUMOylation and that SUMOylation is essential for accurate chromosome segregation in organisms ranging from yeast to human [8]. In this regard, it is also interesting to note that the SUMO E2-conjugating enzyme, Ubc9, was also first characterized in 1995 as an essential protein required for chromosome segregation and progression through mitosis in *S. cerevisiae* [9]. In this study, however, Ubc9 was misidentified as a ubiquitin E2-conjugating enzyme.

Following the identification of the yeast SUMO gene, three studies reporting the identification of cDNAs coding for human SUMO1 appeared in 1996. All three studies involved yeast two-hybrid screens using varying proteins as bait. In the first of these

studies, Michael Boddy and colleagues found that SUMO1 interacts with the promyelocytic leukemia (PML) protein and it was therefore referred to as PIC1 (for PML-interacting clone 1) [10]. Again, this study did not present evidence to suggest that SUMO1 is covalently conjugated to PML or any other proteins. However, immunofluorescence microscopy was used to document for the first time what later emerged to be a very important association of SUMO1 with PML nuclear bodies. A large body of subsequent work from many groups has shown that PML is directly SUMOylated and moreover that SUMOylation is vital to the assembly and functions of PML nuclear bodies [11].

The second protein found to interact with human SUMO1 in yeast two-hybrid screens was the Fas/APO-1 receptor [12]. In this study by Edward Yeh and his colleagues, transient overexpression of SUMO1 was shown to protect cells from anti-Fas/APO1-mediated cell death, and thus the protein was named Sentrin (after sentry, because of its guardian effect against cell death). It is now clear that SUMOylation can act at multiple points to affect signal transduction pathways, and particularly through effects on gene expression [13]. The molecular basis for how SUMOylation affects anti-Fas/APO1-mediated cell death, however, remains unknown.

The third protein involved in the early identification of SUMO1 using yeast two-hybrid screens was the DNA recombinase, RAD51 [14]. This study is notable as being the first to suggest a functional link between SUMOylation and DNA damage repair. A large body of work has subsequently established that SUMOylation is intimately involved in nearly all facets of DNA damage repair and that cross talk between SUMOylation and ubiquitylation pathways is crucial for efficient and accurate maintenance of genome integrity [15]. Notably, several studies have specifically reported that non-covalent interactions between RAD51 and SUMO are important for RAD51 recruitment to DNA double-strand breaks, validating the functional significance of this early discovery [16, 17]. In Chapter 2, Wilson and Hochstrasser review in more detail the broad roles of SUMOylation in regulating chromatin structure and function [18].

1.2 Early Discoveries of SUMO Proteins as Posttranslational Protein Modifications

Although an important part of the early discovery of SUMO and the functions of SUMOylation, the above studies all fell short of providing evidence that SUMO proteins are in fact covalently conjugated to other proteins and thus function as posttranslational protein modifications. It did not take long however, for two independent studies, one published in late 1996 by Michael Matunis and colleagues [19] and the other in early 1997 by Frauke Melchior and colleagues [20], to establish for the first time that SUMO1 is covalently and reversibly conjugated to the Ran GTPase-activating protein, RanGAP1. In both studies, antibodies specific to RanGAP1 were found to detect two proteins differing in molecular mass by

~15 kDa. Peptide sequence analysis confirmed the identity of both proteins as forms of RanGAP1, but also revealed the presence of unique peptides specific to the larger protein. Both groups identified expressed sequence tagged (EST) clones in available cDNA databases that encoded the unique peptides and also a predicted 11.5 kDa protein with 18 % sequence identity to ubiquitin. Matunis and colleagues originally referred to the predicted protein as GAP-modifying protein 1 (GMP1) whereas Melchior and colleagues called the protein small ubiquitin-related modifier 1 (SUMO1).

Due to the absence of stop codons 5′ to the predicted methionine in available EST clones, both groups initially considered the possibility that alternative mRNA splicing could explain the two forms of RanGAP1. However, Melchior and colleagues were ultimately able to demonstrate an ATP-dependent interconversion of the higher and lower molecular mass forms of RanGAP1 using isolated cell-free cell extracts. Matunis and colleagues demonstrated that extraction of rat liver nuclear envelopes in the presence of DTT led to the conversion of the higher molecular mass form of RanGAP1 to the lower form, with the concomitant release of the 11.5 kDa SUMO1. This conversion could be inhibited by extraction in the presence of NEM, providing the first evidence for the association of cysteine-dependent SUMO isopeptidases with nuclear pore complexes. Both groups subsequently went on to demonstrate that SUMOylation functions to promote the association of RanGAP1 with Nup358/RanBP2 at the nuclear pore complex, a finding that was also supported by work from Hisato Saitoh, Mary Dasso, and their colleagues [21–23].

These early studies of RanGAP1 SUMOylation established a number of important paradigms that have proven useful for thinking about the functions and regulation of SUMOylation: (1) that SUMO is reversibly conjugated to proteins and affects protein fate through molecular mechanisms similar to ubiquitylation, and (2) that SUMOylation functions to affect protein-protein interactions and assembly of multi-protein complexes, without necessarily affecting protein degradation. At the same time, RanGAP1 has also proven to be a highly unusual SUMO substrate, and in some cases an exception to more universal paradigms. In this regard, RanGAP1 is unusual in that it is stably SUMO1 modified as a consequence of its tight association with Nup358/RanBP2 and consequent protection from deconjugation by isopeptidases [24]. It is now recognized that the majority of sumoylated proteins are modified only transiently, and at relatively low steady-state levels. In addition, RanGAP1 is modified at a single site by a single SUMO1 protein, whereas it is now recognized that proteins can also be modified at multiple sites and by polymeric SUMO chains. It has also become increasingly clear during the past several years that cross talk between the SUMOylation and ubiquitylation pathways includes roles for SUMO as a signal for protein degradation [25].

1.3 Initial Characterization of SUMO Paralogs

Although yeasts and invertebrate organisms express a single SUMO protein, vertebrates possess multiple genes encoding for unique SUMO paralogs. The presence of SUMO paralogs in mammalian cells was first suggested by sequence analysis of human cDNAs, whereby a family of up to three SUMO-related proteins (SUMO1, SUMO2, and SUMO3) was originally identified [14, 26]. A gene coding for SUMO4 was subsequently identified through analysis of single-nucleotide polymorphisms associated with type 1 diabetes [27]. Experimental evidence that SUMO2 and SUMO3 function as posttranslational protein modifications similar to SUMO1 was first provided through transient transfection and overexpression studies that demonstrated modification of the PML protein [28]. Studies by Hisato Saitoh and Joseph Hinchey, however, were the first to report on the analysis of SUMOylation by endogenous SUMO2 and SUMO3 [29]. Notably, this study was also the first to suggest functionally distinct properties for SUMO1 in comparison with SUMO2 and SUMO3, based on their differential activations in response to environmental stresses.

SUMO2 and SUMO3 share ~95 % sequence identity (and are therefore often referred to as SUMO2/3) but are only ~45 % identical to SUMO1, further suggestive of possibly distinct signaling properties and functions. Among the sequence differences between SUMO1 and SUMO2/3, perhaps the most significant is the presence of a SUMOylation consensus sequence surrounding lysine 11 that is specific to SUMO2/3. This consensus sequence is efficiently recognized as a SUMO conjugation site, and SUMO2/3 therefore readily form polymeric chains both in vitro and in vivo, as first documented by Tatham and colleagues [30]. Polymeric chains can also form through other lysines in SUMO2/3, and whether chain linkages affect downstream signaling is an important question that remains to be fully evaluated. Polymeric chains have, however, been shown to be functionally distinct from monomeric SUMO due to enhanced affinity for proteins containing tandem SUMO-interacting motifs. Thus, proteins modified by polymeric SUMO2/3 chains are preferentially recognized and ubiquitylated by SUMO-targeted ubiquitin ligases (STUbLs) which contain tandem SUMO-interacting motifs (SIMs) and RING E3 ligase domains [25, 31–33]. Whereas monomeric SUMO1 modification may antagonize ubiquitylation and protein degradation [34], polymeric SUMO2/3 chains have the ability to direct ubiquitin-mediated protein degradation. Consistent with unique signaling properties and functions, multiple studies have also provided evidence for selective modification of proteins by SUMO1 and SUMO2/3 [24, 35–37]. Thus, it was somewhat surprising that gene knockout studies in mice revealed nonessential roles for SUMO1 and SUMO3 expression [38, 39]. Whether these mice have subtle growth defects or conditional phenotypes and whether SUMO paralogs have essential and unique functions in other organisms including humans are important questions for future studies.

2 The Complexity of Protein SUMOylation

As other members of the ubiquitin family, SUMOs can be attached to target proteins as monomers but also as polymers. PolySUMO-2/3 chains have been identified by mass spectrometry under different stress conditions [40–42]. Furthermore, hybrid SUMO-ubiquitin chains were also reported by several groups [32, 33]. The status of these homologous or heterologous SUMO chains is actively regulated by modifying and de-modifying enzymes in a cellular compartment or time/stimuli-dependent response (Fig. 1). The way these heterologous chains are recognized and connected with distinct functions is still an open domain of investigation. Tandem SUMO-interacting motifs (SIMs) and ubiquitin-interacting motifs (UIMs) appear to play an important role in hybrid chain recognition [43–45]; however, it is not clear if other motifs are also relevant to recognize hybrid chain architectures.

2.1 SUMO Conjugation Machinery

SUMOs are conjugated to target proteins by an enzymatic cascade involving an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3) (Fig. 1). The E1 is a heterodimer containing SAE1 and SAE2 subunits (known as Aos1 and Uba2 in yeast) [46–49]. The E1 catalyzes the formation of SUMO-AMP and the subsequent transfer of SUMO to the E1 active-site cysteine sulfhydryl group. In the second step of the enzyme cascade, SUMO is transferred from the E1 to the active-site cysteine of the E2-conjugating enzyme, Ubc9 [50, 51]. Ubc9 has the ability to directly recognize substrate proteins and catalyze formation of an isopeptide bond between SUMO and the ε-amino group of a lysine in the substrate protein. Alternatively, SUMO E3 ligases may also bind Ubc9 and increase the rate of SUMOylation. Most SUMO E3s appear to target multiple proteins with recognizably similar features, although exact mechanisms of specificity in many cases are not well understood [52]. SUMOylation is highly dynamic, with the removal of SUMO from proteins (deSUMOylation) being mediated by SUMO-specific proteases/SENtrin proteases (SUSPs/SENPs) that also contribute to the processing of the SUMO precursors [53]. The six members of the SENP family of proteases localize to unique subcellular compartments and the distribution of SUSPs/SENPs is therefore thought to play an important role in the spatial regulation of SUMO turnover and function [53–56].

2.2 SUMO Consensus and Interacting Sequences

Ubc9 recognizes a SUMO consensus motif, ψKxE (where ψ is a large hydrophobic residue and x is any residue, K a lysine, and E/D a glutamic or aspartic amino acid) [57, 58]. SUMOylation of a majority of substrates occurs within this consensus motif; nevertheless ~30 % of proteins are modified on lysine residues not conforming to this consensus sequence [59, 60]. Furthermore, not all proteins

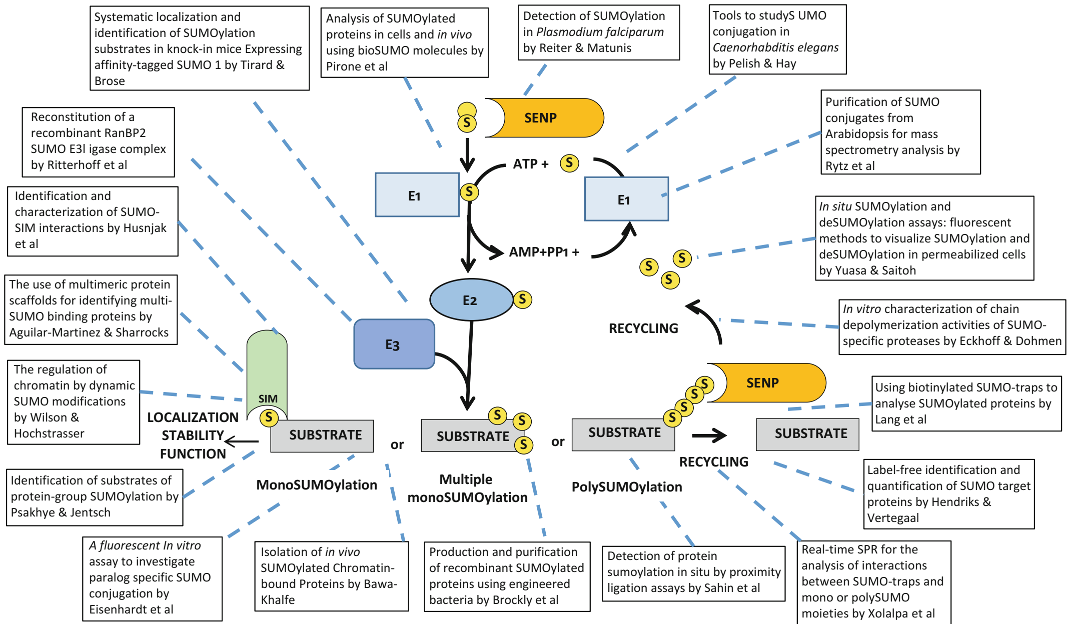

Fig. 1 Regulation of protein SUMOylation and chapters contributing to explore these molecular and cellular events. Multiple steps control the status of SUMOylated proteins and its connection with effector functions. The chapters included in this book contribute to explore some of these events using different biological models and systems. This conceptual and methodological framework should contribute to progress in our knowledge of protein SUMOylation and the development of translational research

containing the ψKxE sequence are SUMOylated, indicating that other factors such as protein structure or localization may influence modification [61, 62]. Interestingly, SUMO-2/3 have functional SUMO consensus motifs used to form polymeric SUMO chains [63]. In addition, polymeric chains are also formed using other non-consensus lysine residues in both SUMO1 and SUMO-2/3 [60, 64]. To avoid the time-consuming approach of systematic mutation of lysine residues on target proteins, bioinformatic tools were developed to identify SUMO consensus sites, including SUMOplot (http://www.abgent.com/tools/sumoplot/) and SUMOsp (http://sumosp.biocuckoo.org/). Unfortunately, since these tools do not consider atypical sequences, structural, temporal, or cellular distribution requirements, predicted SUMOylation sites have not always been confirmed. The contribution of mass spectrometry (MS) approaches has been crucial to identify and/or confirm SUMOylation sites [60] and reviewed in [65]. The identification of conjugation sites has been particularly advanced by recently developed MS-based approaches that allow for enrichment and identification of peptides containing modified lysine residues [66, 67].

Proteins can also interact with SUMO in a non-covalent manner due to the presence of SUMO-interacting motifs (SIMs). The first evidence of SIMs was published by Minty and collaborators in

2000 [68]. Using a two-hybrid approach, the authors observed that some proteins interacted specifically with the SUMOylated version of p73, a member of the p53 family. Further analysis revealed that these interacting proteins contained a common SxS sequence, in which x is any amino acid surrounded by two serine residues, flanked by a hydrophobic core on one side and acidic amino acids on the other. Subsequent studies further confirmed the presence of a Val/Ile-x-Val/Ile-Val/Ile (V/I-x-V/I-V/I) consensus SIM in proteins of varying functions that could facilitate interactions with SUMO [69]. Several enzymes within the SUMO pathway, including the SUMO ligases PIASX and Ran-binding protein 2 (RanBP2/Nup358) and multiple SENPs [55], contain consensus SIMs, suggesting that non-covalent interactions with SUMO facilitate modification and demodification of substrates. In support of this, the SIM in RanBP2/Nup358 is directly adjacent to the minimal IR1-IR2 domain that has E3 activity. However, although this SIM has been shown to bind SUMO, it does not appear to be essential for ligase activity in vitro [70]. The hydrophobic core of a SIM can bind to an interaction surface on SUMO in parallel or antiparallel orientations. Acidic residues, either upstream or downstream of the core, determine binding orientation and may also affect affinity and paralogue specificity [71]. From these initial SIM studies, a more complex type of SUMO recognition domain, named the SUMO-binding domain (SBDs), containing several hydrophobic cores of 3–4 residues often surrounded by a cluster of acidic amino acids was realized [72, 73]. Recent analysis performed by Hoffman revealed three different types of SIMs with the following PROSITE format: (SIMa) (PILVM)- (ILVM)-x-(ILVM)-(DES>) (3), (SIMb) (PILVM)-(ILVM)-D-L-T, and (SIMr) (DSE) (3)-(ILVM)-x-(ILVMF) (2) [74]. The identification and validation of these SIMs using site-directed mutagenesis has been an important approach to investigate the role of SUMO in the regulation of particular processes or pathways. The identification of new SIMs will be crucial for the integration of the many functions regulated by SUMOylation.

2.3 Diversity of SUMO E3s

The Siz proteins in *S. cerevisiae* were the first SUMO E3s identified. These ligases have domains that are homologous to the RING domains of ubiquitin E3 ligases [75]. Co-deletion of Siz1 and Siz2 genes in *S. cerevisiae* eliminates most SUMOylation and affects growth under a variety of conditions, underlining the importance of these SUMO E3 ligases [76, 77]. The protein inhibitors of activated STAT (PIAS) proteins are homologs of the Siz proteins in higher eukaryotes. However, PIAS proteins appear to play additional roles apart from being SUMO E3 ligases [78]. In humans the five PIAS proteins (PIAS1, PIASxα, PIASxβ, PIASγ, and PIAS3) encoded by the genome contain the RING domain. Individual deletion of these genes results in distinct phenotypes.

Using PIAS1−/− mice it was demonstrated that PIAS1 regulates interferon-inducible gene expression and is important in innate immunity [79]. Nevertheless, there was no detectable impact of PIAS1 deletion on total SUMOylation patterns as compared to WT mice. A similar situation was observed with the PIASγ−/− mice, where mild defects in transcriptional responses induced by interferon γ and Wnt agonists were also observed [80, 81]. Although PIAS E3 ligases show some redundancy in vitro and in transient overexpression studies, differences in substrate preferences and regulation likely exist under normal in vivo conditions.

The SUMO-specific ligases RanBP2 and polycomb group protein 2 (Pc2) are unrelated to known ubiquitin E3s. RanBP2/NUP358 is located at nuclear pore complexes and enhances Ubc9-mediated conjugation of SUMO-1 and SUMO-2/3 to a variety of protein substrates. For instance, it enhances SUMOylation of Sp100 and histone deacetylase 4 (HDAC4) by SUMO-1 and preferentially modifies PML with SUMO-2 [30, 82, 83]. The domain of RanBP2 that contains SUMO E3 ligase activity includes the IR1, M1, and IR2 regions involved in binding Ubc9 [30, 84, 85]. The lack of interaction between the RanBP2 ligase domain and substrates indicates that RanBP2 alters the structure of the SUMO-Ubc9 thioester, thereby increasing the capacity to transfer SUMO to protein substrates. However, the mechanism used by RanBP2 to enhance SUMO1 or SUMO2/3 modification of substrates is distinct since M-IR2 binds SUMO1 but not SUMO2.

The Pc2 component of the polycomb chromatin-modifying complex also possesses SUMO E3 ligase activity. One Pc2 substrate is the C-terminal-binding protein (CtBP) transcriptional co-repressor [86]. The N-terminal region of Pc2 alone binds Ubc9 and exhibits E3 ligase activity in vitro. However, the C-terminal region that binds CtBP is also required for activity in vivo. Although the mechanism of action of this SUMO E3 is not completely clear, it is likely that the C-terminal domain of Pc2 functions to recruit CtBP to PcG subnuclear domains where the active N-terminal domain recruits Ubc9 and drives SUMOylation of CtBP [86].

2.4 Regulation of SUMOylation by Specific Proteases

Protein SUMOylation is reversible and is removed from targets by specific cysteine proteases known as SUMO-specific proteases or SUMO isopeptidases. These enzymes remove SUMO from protein conjugates and depolymerize poly-SUMO chains. Some of these enzymes also function to process SUMO precursors by cleaving and releasing carboxy-terminal residues, thereby exposing the signature double glycine required for SUMO conjugation. SUMO-specific isopeptidases/proteases are classified into three families: the Ulp/SENP (ubiquitin-like protease/sentrin-specific protease) family, the Desi (deSUMOylating isopeptidase) family, and USPL1 (ubiquitin-specific peptidase-like protein 1) [53].

Ulp1/Ulp2, discovered in *Saccharomyces cerevisiae*, belong to the C48 family of thiol proteases [87, 88]. In higher eukaryotes, the family includes six enzymes called SENPs 1–3 and 5–7 [89]. SENP8 acts on the ubiquitin family member NEDD8, but not on SUMO paralogs [90, 91]. The catalytic domain of the Ulp/SENP family spans ~200 amino acids in the carboxy-terminal part of the enzyme. The catalytic domains of the human Ulp/SENP family members share 20–60% sequence identity. The amino-terminal regions of all SENPs contain amino acids susceptible to phosphorylation or ubiquitylation, modifications that may affect their stability or interactions with substrates or adaptor proteins that determine their subcellular distribution [92–98]. The N-terminal domains of most SENPs also contain one or more SIMs which are believed to contribute to the recognition of SUMOylated substrates.

The deSUMOylating isopeptidases Desi-1 and Desi-2 belong to the C97 family of cysteine proteases [99]. No orthologs of Desi-1 and Desi-2 have been described in yeast. USPL1 is the only mammalian SUMO-specific protease of the C98 family [100]. Desi-1 and Desi-2 are small proteins characterized by PPPDE (permutated papain fold peptidases of the double-stranded RNA viruses and eukaryotes) domains of around 140 amino acids. Desi-1 and Desi-2 share about 20% sequence identity within this region. The active site contains two conserved cysteine and histidine residues that form a catalytic dyad [99, 101]. The catalytic domain of USPL1 shows homology to the C19 family of ubiquitin-specific proteases. The catalytic domain of USPL1 contains a catalytic triad composed of Cys-His-Asp residues [100, 102].

SUMO isopeptidases show distinct subcellular distributions that limit their activity to specific sets of substrates. Ulp/SENP family members are mainly located in distinct sub-nuclear regions. SENP1 and SENP2, however, also shuttle between the nucleus and the cytoplasm and are concentrated at the nuclear envelope through their interaction with components of the nuclear pore complex [98, 103–106]. SENP1 and SENP2 are excluded from the nucleolus, but can be detected in nuclear foci that show some overlap with PML nuclear bodies. SENP1 and SENP2 redistribute during mitosis from the nuclear envelope to the kinetochore [13]. SENP3 and SENP5 are located in sub-compartments of the nucleolus, where they act on proteins involved in the early steps of ribosome maturation [92, 107–109]. However, a small fraction of SENP3 and SENP5 also reside in the nucleoplasm and the cytoplasm. SENP5 translocates to the mitochondrial surface during the G2/M transition prior to nuclear envelope breakdown [110]. SENP6 and SENP7 mainly exhibit a nucleoplasmic distribution. Desi family members are primarily concentrated in the cytoplasm [110]. USPL1 is a predominantly nuclear protein and co-localizes with coilin in Cajal bodies [100, 102].

3 Methodologies to Study Protein SUMOylation

Due to the expanding interest in the study of protein SUMOylation in distinct fields, methodologies to improve our understanding of the functions regulated by this posttranslational modification are in constant innovation. Collected here are some recent methodologies that have been developed and used by well-recognized SUMO experts. Many of the reviewed techniques/approaches are versatile and can be adapted to different biological models or in cellulo or in vitro systems. The classification of included methods is somewhat practical and refers to one possible application. However, most techniques can be adapted according to the needs of specific projects and used in different in vitro, in cellulo, or in vivo models.

The first section of this book includes in vitro procedures to study protein SUMOylation. Considering the complexity of the SUMOylation analysis in vivo, in vitro procedures provide simplified systems that are more ideally suited to address mechanistic questions. The complete reconstitution of the recombinant RanBP2 SUMO E3 ligase complex proposed by Ritterhoff et al., in Chapter 3 [111], allows for quantitative SUMOylation of RanGAP1 but can be extrapolated to other RanBP2 substrates [112, 113]. A protocol to purify recombinant SUMOylated proteins from bacteria, as outlined by Brockly et al., in Chapter 4 [114], can be used to gain insights into biochemical aspects of specific SUMOylation targets [115–117]. Eisenhardt et al. present in Chapter 5 [118] a fluorescent-SUMO conjugation assay to evaluate E3-mediated chain formation activity in a paralog-specific manner. The application of fluorescent assays to study substrate modification in vitro provides fast procedures to investigate SUMO enzyme activities and mechanistic insights into SUMO chain formation [119]. Once SUMOylated, target proteins are recognized by effector proteins containing functional SIMs. SUMO-SIM interactions are far from being fully understood and deeper exploration is needed to better understand the molecular mechanisms regulating this connection. In Chapter 6, Husnjak et al. [120] describe two complementary approaches to identify SUMOylated proteins and characterize their interactions with SIMs. Their method has been validated and successfully applied to the identification of novel SUMO-binding proteins as well as the characterization of known SUMO-interacting modules [68, 72, 73, 121–123]. A complementary and quantitative method to characterize real-time SUMO-SIM interactions using surface plasmon resonance is outlined by Xolalpa et al. in Chapter 7 [124]. This method can be used to analyze the effect of SUMO or SIM point mutations, or regulatory proteins, on SUMO-SIM interactions. The analysis of SUMOylated proteins can also be carried out using chimeric SIMs arranged in tandem, also known as SUMO-traps

[44, 125, 126]. In the approach proposed by Lang et al. in Chapter 8, [127] a biotinylated version of SUMO-traps is used to analyze SUMO substrates in vitro, but can also be used for in vivo studies. SUMO-SIM interactions are transient, in part due to the action of SUMO-specific proteases. To study the activity of SUMO proteases, Eckhoff and Dohmen have developed a method for fast and economic analysis [128]. The method reported in Chapter 9 [129] was developed for analysis of *S. cerevisiae* Ulp enzymes but can be expanded to SUMO-specific proteases from other species. Since the proteases are key regulatory molecules of protein SUMOylation, their inhibition represents not only a desirable approach to better characterize their functions, but also opens possibilities for clinical intervention.

Protocols for the analysis of SUMOylated proteins in cell cultures are grouped in the second part of this book. Diverse and imaginative approaches use chimeric proteins and other sophisticated strategies to identify SUMO targets and interacting cellular factors. A method proposed by Sahin et al. in Chapter 10 [130] allows detection of protein SUMOylation in situ by the now popular technique of proximity ligation assay (PLA) [131]. Yuasa and Saitoh present in Chapter 11 [132] an alternative technique to detect in situ protein SUMOylation and de-SUMOylation using fluorescence-based assays in permeabilized cells [133]. The analysis of total or individual SUMOylated proteins in cell lysates, but also in vivo, can be performed using tagged SUMO proteins. Some of the most popular tags are biotin [134, 135] and histidine [67, 136], as described by Pirone et al. in Chapter 12 [137] and Hendriks and Vertagaal in Chapter 13 [138]. The detailed protocols can be adapted to detect any SUMOylated target and can also be used for global MS analysis of SUMO conjugation signatures. One of the major bottlenecks to MS analysis of SUMOylated proteins is the enrichment of the SUMO-GG signature peptides, which are longer than those generated by tryptic digestion of ubiquitinylated proteins. Multiple approaches have been developed to address this issue, including the mutation of C-terminal amino acid residues in SUMO to generate shorter tryptic GG-peptide signatures. Alternatively, longer His10-tags have been developed to allow a single-step, high-yield purification of SUMOylated proteins which can then be digested and analyzed by high-resolution MS analysis, as described in Chapter 13 [138].

SIMs in downstream effector proteins function to integrate SUMOylation with specific cellular processes. For this reason, Aguilar-Martínez and Sharrocks used multimeric protein scaffolds to identify novel multi-SUMO-binding proteins, as outlined in Chapter 14 [139]. The isolation and identification of SUMOylated proteins associated specifically with chromatin represents an important

challenge. Bawa-Khalfe report in Chapter 15 [140] a protocol for effectively purifying endogenous SUMOylated proteins from chromatin fractions prepared from cultured cells [141]. This approach has the potential to be used to evaluate chromatin-bound SUMO targets using varying cellular models and biological systems.

The final section of this book includes methods to study protein SUMOylation using distinct biological models. The SUMO pathway often targets protein groups that are functionally and physically connected [142, 143]. Psakhye and Jentsch present a method to identify SUMOylated protein groups in *Saccharomyces cerevisiae* in Chapter 16 [144]. This protocol can be easily adapted for studies of SUMOylation in mammalian cells. *Caenorhabditis elegans* represents another powerful genetic system to study protein SUMOylation, and Pelish and Hay expand in Chapter 17 [145] the existing set of tools to investigate the role of SUMOylation using this nematode [146]. These tools and reagents allow a combination of genetics, imaging, and biochemical approaches that will be useful to gain insights into the biological role of SUMOylation in the context of this multicellular organism [146]. Another attractive system to study protein SUMOylation is the model plant *Arabidopsis thaliana*. Based on the expression of modified SUMOs bearing epitope tags, Rytz and Vierstra combine standard and quantitative MS analysis methods to identify SUMOylated proteins, as detailed in Chapter 18 [147]. The role of protein SUMOylation during multiple human infections, including viral, bacterial, and parasitic infections, also has an increasing interest for microbiologist and immunologists. In Chapter 19 [148], Reiter and Matunis present methods to improve the functional analysis of protein SUMOylation in *Plasmodium falciparum* using antibodies specific for the parasite SUMO [149]. Considering that SUMOylation is essential, a more detailed understanding of its role during the parasite life cycle will be required for the further development of antimalarial drugs targeting SUMOylation. Preclinical studies often use rodent models to validate information collected from other experimental systems. In Chapter 20 [150], Tirad and Brose describe step-by-step methods to purify and analyze SUMO1-modified proteins from His6-HA-SUMO1 knock-in mouse brain based on an anti-HA immunopurification protocol. These methods are generally applicable and can be easily adapted to other cell types and tissues. This His6-HA-SUMO1 mouse line can be crossed into any disease model, thus providing the opportunity to study SUMO1 conjugation in a plethora of disease-relevant processes. These are clear advantages that make using His6-HA-SUMO1 knock-in mice a powerful model system for the analysis of SUMOylation [151].

4 Conclusions and Future Directions

After the first 20 years of study, it has become clear that SUMOylation is linked to virtually all cellular process, including intracellular transport, transcription, DNA replication and repair, chromatin assembly/accessibility and proteolysis, among others. All these processes can be fine-tuned through the action of SUMO-conjugating/de-conjugating enzymes that are activated during physiological and pathological events. How SUMO moieties are integrated into hybrid chains containing ubiquitin or other ubiquitin-like proteins (e.g., NEDD8) is still under intense investigation. One of the major bottlenecks to analysis of hybrid chains is that the available MS technologies do not reveal the order in which individual moieties are integrated into the chains, as these technologies read a single-branched peptide at a time. Thus, new tools and technological implementations will continue to be key for progress in the SUMO and other ubiquitin-related protein fields. In sum, since its original discoveries, the roles for protein SUMOylation have expanded in extraordinary and unanticipated ways. It will surely be fascinating to see how the field develops during the next two decades.

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