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Manuel S. Rodriguez *Editor*

SUMO

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Cover illustration: The image depicts a *C. elegans* embryo in Metaphase of the first mitotic division. Tubulin is seen in green and SUMO in magenta. Image was acquired using 3D structured illumination with an OMX super-resolution microscope. Federico Pelisch and Ronald T. Hay are the authors of the cover image.

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Preface

The Small Ubiquitin-related MOdifier (SUMO) molecules are implicated in the regulation of multiple critical cellular functions and consequently associated with several pathologies. Functions regulated by the different SUMO molecules are diverse and in most cases unrelated to those controlled by ubiquitin, with the enzymes regulating protein modification and de-modification by SUMO molecules being distinct to those regulating other members of the ubiquitin family. However, recent discoveries indicate that SUMO-regulated functions can be more interconnected with those regulated by ubiquitin than initially suspected. In this volume of *SUMO Methods and Protocols*, leading experts propose basic and "state-of-the-art" methodologies to explore biochemical, molecular, and cellular biology aspects of some of the many processes regulated by protein SUMOylation. Chapters highlight relevant aspects of the SUMO biology that should contribute to develop fundamental and translational research in this area.

This volume is organized in four parts, which start with an historical overview of protein SUMOylation and a presentation of the methods included in this book. The first part also includes a review on chromatin regulation by dynamic SUMO modifications. The second part of this volume focuses on in vitro techniques including biochemical methods to study mechanistic aspects of protein SUMOylation. The third part includes protocols to be used with cell cultures, which often are the first approaches used in most laboratories. The final part includes methodologies adapted for the analysis in vivo using distinct model organisms. This volume of *SUMO Methods and Protocols* has been written following the highly successful Methods in Molecular Biology™ series format. Each chapter includes a brief introduction to the subject, a list of necessary materials and reagents, a step-by-step reproducible laboratory protocol, and a Notes section detailing tips on troubleshooting and strategies to avoid known pitfalls. Unique and cutting edge, this SUMO Protocols volume provides the necessary procedures for specialists as well as for researchers not familiar with this vital system.

I would like to extend my deepest gratitude to all contributors of this book. Sharing your know-how with the readers of this book is priceless. I would like to gratefully emphasize the special efforts of Michael Matunis and Mark Hochstrasser who contributed with outstanding reviews that provide relevant scientific background to this book. Special thanks to the PROTEOSTASIS COST Action 1307 and the contribution of its members. We (the SUMO community) are grateful to Humana Press for giving us the opportunity to assemble this book and to John Walker for his help in the edition of this book.

San Sebastian, Spain — *Manuel S. Rodriguez*

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Part I

Introduction

Chapter 1

Concepts and Methodologies to Study Protein SUMOylation: An Overview

Michael J. Matunis and Manuel S. Rodriguez

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

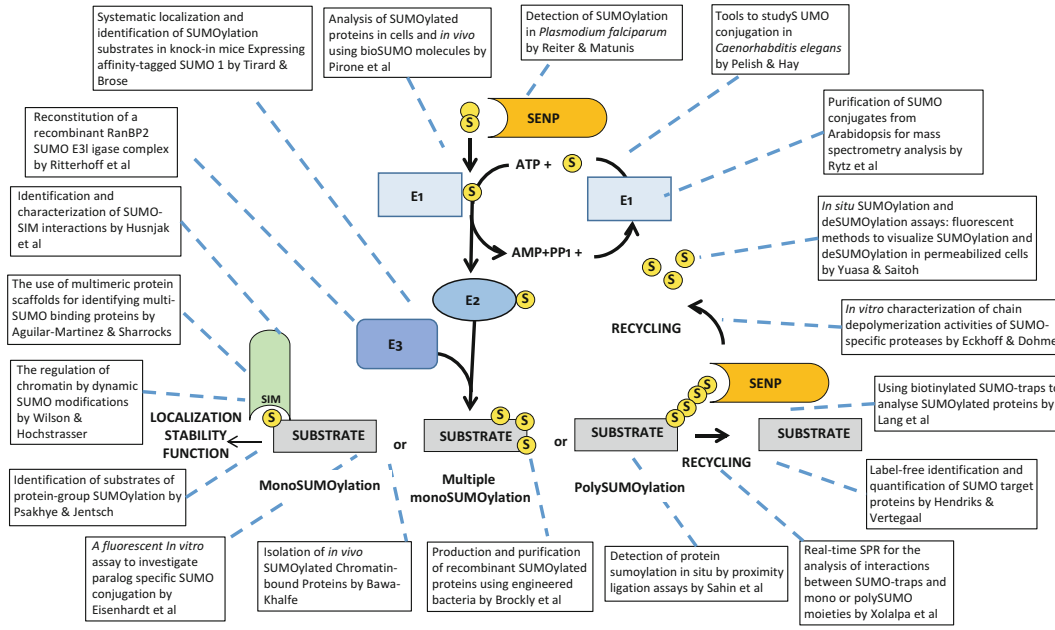

Fig. 1 Regulation of protein SUMOylation and chapters contributing to explore these molecular and cellular events. Multiple steps control the status of SUMOylated proteins and its connection with effector functions. The chapters included in this book contribute to explore some of these events using different 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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Chapter 2

The Regulation of Chromatin by Dynamic SUMO Modifications

Nicole R. Wilson and Mark Hochstrasser

Abstract

Protein modification by the small ubiquitin-related modifier (SUMO) protein regulates numerous cellular pathways and mounting evidence reveals a critical role for SUMO in modulating gene expression. Dynamic sumoylation of transcription factors, chromatin-modifying enzymes, histones, and other chromatin-associated factors significantly affects the transcriptional status of the eukaryotic genome. Recent studies have employed high-throughput ChIP-Seq analyses to gain clues regarding the role of the SUMO pathway in regulating chromatin-based transactions. Indeed, the global distribution of SUMO across chromatin reveals an important function for SUMO in controlling transcription, particularly of genes involved in protein synthesis. These newly appreciated patterns of genome-wide sumoylation will inform more directed studies aimed at analyzing how the dynamics of gene expression are controlled by posttranslational SUMO modification.

Key words Chromatin, Transcription, ChIP-seq, Histones

1 The SUMO Pathway

The small ubiquitin-related modifier (SUMO) protein is a conserved posttranslational modification that alters the binding, conformation, and/or localization of a substrate protein. Protein sumoylation is essential in most eukaryotes and regulates numerous cellular processes including mitochondrial dynamics, ribosome biogenesis, and DNA repair [1]. This review focuses on the recent and exciting body of work connecting protein SUMO modification to chromatin dynamics and transcriptional regulation. Particular emphasis will be placed on several novel high-throughput chromatin immunoprecipitation-DNA sequencing analyses (ChIP-Seq) that have revealed the localization of SUMO across the genome and provide new insight into the role of SUMO in gene expression.

SUMO, like other members of the ubiquitin-like protein (UBL) family, covalently attaches to target proteins in a process

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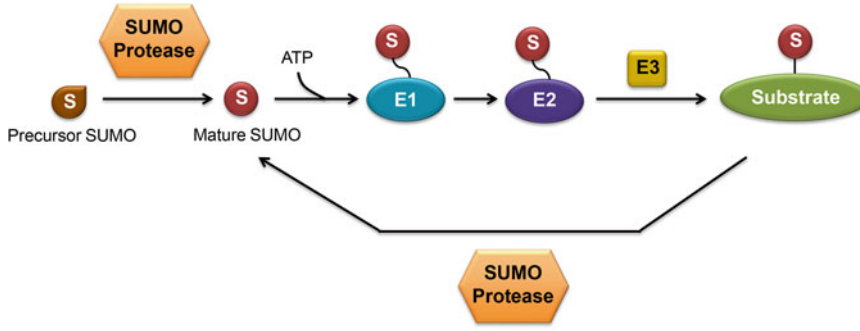

Fig. 1 The SUMO pathway. The small ubiquitin-like modifier protein (SUMO) is first synthesized as an inactive precursor, which is processed by a SUMO protease to create mature, conjugatable SUMO. In an ATP-dependent manner, mature SUMO is activated and subsequently conjugated to the lysine side chain(s) of a substrate protein through the concerted action of E1, E2, and E3 enzymes

similar to ubiquitin-protein conjugation (Fig. 1) [2]. SUMO is synthesized as an inactive precursor with a C-terminal peptide extension. A SUMO protease cleaves after a C-terminal-proximal Gly-Gly motif to form mature, conjugation-competent SUMO. In an ATP-dependent manner, the carboxyl-terminus of mature SUMO is activated by the heterodimeric SUMO-activating enzyme (E1), forming a high-energy thioester bond with the E1. SUMO is then transferred to the active-site cysteine of the E2 SUMO-conjugating enzyme followed by SUMO transfer to a lysine side chain on the target protein. Protein sumoylation is usually assisted by one of a small number of SUMO E3 ligases, which enhance conjugation specificity [2]. Target proteins can be sumoylated on a single lysine (monosumoylation), on multiple lysines with a single SUMO moiety (multisumoylation), or on one or more lysines with an extended SUMO chain (polysumoylation).

The budding yeast *Saccharomyces cerevisiae* expresses one form of SUMO (Smt3), while most vertebrates have three active SUMO isoforms, SUMO-1, SUMO-2, and SUMO-3. The SUMO-2 and SUMO-3 proteins share ~97% sequence identity and are sometimes referred to as SUMO-2/3. The yeast Smt3 protein has the highest sequence identity with human SUMO-1 (~48%); however, while the Smt3 protein can form SUMO chains in vivo, SUMO-1 cannot [3–5]. Instead, SUMO-2 and SUMO-3 are the predominant chain formers in mammalian cells [6]. Protein substrates of sumoylation are often, but not always, modified on a SUMO consensus motif, which is a stretch of four amino acids with the sequence ψ-K-X-D/E (where ψ is a hydrophobic amino acid and X is any amino acid) [7, 8]. Additionally, other posttranslational modifications, notably phosphorylation, can also stimulate sumoylation [9–11].

Sumoylation is a dynamic modification. SUMO proteases specifically cleave the bond between SUMO and substrate. There are

three known classes of SUMO proteases in vertebrates, the SENP/Ulp, DESI, and USPL1 families, with the SENP family being the largest—seven members in humans—and best characterized [12]. In yeast, where the SUMO proteases were first identified, only SENP/Ulp-class SUMO proteases, Ulp1 and Ulp2, have been found to date [13, 14]. As with SUMO conjugation, desumoylation of proteins is highly regulated and plays a crucial role in many cellular pathways [15, 16].

The consequences of protein sumoylation are numerous and include changes in protein localization, altered protein conformation, and either enhanced or impaired protein-protein interactions. Frequently, the assembly and dynamics of large protein complexes are mediated through the interaction of sumoylated proteins with SUMO-interacting motifs (SIMs) of other proteins within the complex. SIMs interact with the β2 strand of SUMO and most are characterized by a core of 3–4 hydrophobic residues (typically Val or Ile) that is often flanked on one or the other side by acidic amino acids (Asp, Glu, or phosphorylated Ser or Thr) [17–19]. Thus, SUMO often acts as a molecular adhesive, bringing protein complexes together in a regulated fashion, as seen with the numerous proteins involved in DNA repair by homologous recombination [20]. Consequently, assessing the effects of protein sumoylation is often challenging because removal of one sumoylation site within a protein complex rarely produces an observable phenotype. Therefore, identifying most or all sites of sumoylation within a protein assembly is often required to understand the precise role of SUMO regulation for a particular cellular process.

2 SUMO and Transcription: The Example of the Yeast Tup1-Ssn6 Corepressor

Sumoylation contributes broadly to the regulation of gene expression, with many studies finding that SUMO inhibits transcription [21]. However, in budding yeast sumoylation is required for efficient RNA polymerase II recruitment to constitutively expressed genes, as well as for coordinating the proper activation and inactivation kinetics of several inducible genes [22, 23]. Furthermore, several genome-wide ChIP studies have shown that SUMO is present at the promoters of many constitutive genes, including ribosomal protein genes, in both yeast and humans [24–26]. SUMO modification of proteins involved in transcription has been reviewed extensively [27–30], and thus we will focus on recent insights into the consequences of sumoylation of the yeast general transcriptional corepressors Tup1 and Ssn6 as an illustration of how these modifications alter gene expression.

In *S. cerevisiae*, transcription of the galactose-inducible *GAL* genes is tightly regulated by carbon source [31]. Full repression of the *GAL* genes, as occurs when cells are grown in glucose, is

mediated by the Mig1 transcriptional repressor along with the corepressors Tup1 and Ssn6 [32, 33]. When cells are shifted from glucose to galactose growth media, the *GAL* genes are slowly derepressed before full activation. Derepression of the *GAL1* gene (glucose to galactose) requires the SUMO protease Ulp1; however, Ulp1 is not required for *GAL1* activation from an inactive but not fully repressed state (for example, by switching from raffinose to galactose) [34]. When Ulp1 is untethered from the nuclear pore complex (NPC), where it normally concentrates, or when the catalytic domain of Ulp1 is artificially tethered to the *GAL1* locus, *GAL1* derepression kinetics are enhanced compared to wild-type yeast [34]. The corepressors Ssn6 and Tup1 are both sumoylated in vivo and are Ulp1 substrates. Mutation of Ssn6 SUMO consensus attachment sites results in faster *GAL1* derepression, indicating that Ssn6 desumoylation is likely required for proper *GAL1* upregulation upon a shift from glucose to galactose. The model proposed from this study is that *GAL1* gene activation involves recruitment of the repressed *GAL1* locus to the NPC, and consequent Ssn6 desumoylation by NPC-localized Ulp1, enabling subsequent recruitment of transcriptional activators through an undetermined molecular mechanism. This mode of regulation was also observed for the glucose-repressed gene *HXK1*, indicating a potentially general role for Ulp1-mediated desumoylation in inducible gene activation.

Tup1 sumoylation also represses gene expression, particularly in response to various stress conditions [35]. Tup1 is normally recruited to the promoters of the inducible genes *ARG1* and *CPA2* following amino acid starvation, as revealed by time-course ChIP analysis. Using a "SUMOless" mutant of Tup1, Ng et al. [35] found that initial Tup1 recruitment to the promoter regions is not hampered following amino acid starvation. However, the maintenance of Tup1 association with promoter regions is reduced with the "SUMOless" mutant, leading to an extended period of RNA polymerase II occupancy at the promoters and a consequent increase in *ARG1* and *CPA2* mRNA levels. These results suggest that Tup1 sumoylation helps to deactivate inducible genes following an initial period of stress-induced transcription. Taken together, these studies highlight an important function for SUMO in fine-tuning inducible gene expression.

3 SUMO Localization Across the Genome

To gain a deeper understanding of SUMO function in transcription and chromatin dynamics, it is often essential to dissect the consequences of sumoylation on one particular protein substrate or group of proteins [20]. In recent work, however, researchers have also zoomed out to analyze the broad distribution of SUMO

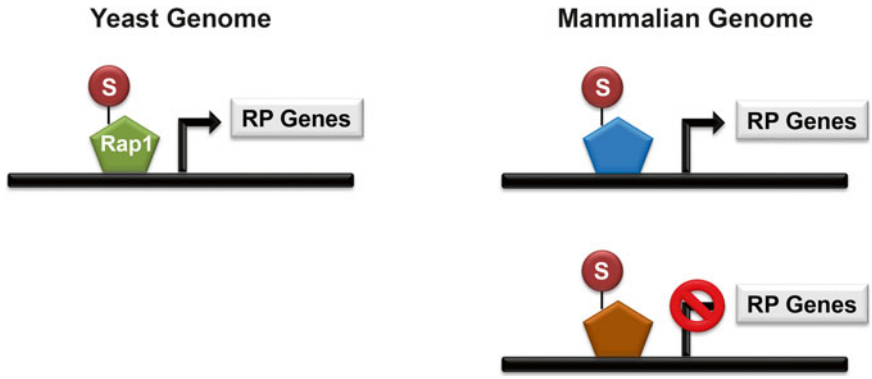

Fig. 2 SUMO localization across the genome. SUMO ChIP-Seq studies revealed the pattern of SUMO across the yeast and mammalian genomes. In *Saccharomyces cerevisiae* (*left panel*), sumoylation of the transcription factor Rap1 localizes it to the promoters of ribosomal protein (RP) genes. Sumoylated Rap1 recruits RNA polymerase II to these gene sites, and stimulates their transcription. Similarly, in mammalian cells (*right panel*), SUMO-1 and SUMO-2/3 are primarily concentrated at the promoter regions of RP genes. However, SUMO localization at these regions has been shown to correlate with both activation and repression of RP gene transcription

across the genome, with the aim of determining a more global and general role for SUMO modification in gene expression. Using chromatin immunoprecipitation followed by high-throughput DNA sequencing (ChIP-Seq), several groups have monitored the pattern of SUMO binding to chromatin in both mammalian and yeast cells (Fig. 2) and under normal growth conditions and during periods of stress [24–26, 36–38]. Interestingly, these studies reveal SUMO enrichment in areas of active gene transcription, particularly within the upstream promoter region of many constitutively expressed genes. Furthermore, sumoylation appears to control the expression of many genes involved in protein synthesis, thereby linking SUMO with the transcriptional regulation of cell growth and proliferation.

4 SUMO Across the Mammalian Genome

In the first ChIP-Seq study of SUMO localization along mammalian chromosomes, Liu et al. analyzed the pattern of SUMO-1 localization on HeLa cell genomic DNA as a function of cell-cycle stage [26]. The genome-wide distribution of SUMO-modified substrates was assessed by next-generation sequencing of the protein-bound DNA fragments. Contrary to studies showing association of SUMO-1 with repressive elements, the authors found SUMO-1 to be enriched at promoters of active genes, particularly during interphase (namely G1 through late S phase). This association of SUMO-1 with active gene promoters decreased during mitosis, the least transcriptionally active cell-cycle stage. The promoter

regions marked by SUMO-1 included many housekeeping genes, particularly ribosomal protein (RP) genes and other factors involved in translation. Further correlating SUMO with active transcription, approximately 70% of promoters marked with SUMO-1 were enriched for histone H3 trimethylated on lysine-4 (H3K4me3), an active transcriptional mark, while only 9% of SUMO-1 peaks overlapped with the repressive H3K27me3 mark.

To confirm a positive role for SUMO-1 in gene activation, Liu et al. [26] depleted SUMO-1 from HeLa cells using siRNA-mediated depletion of the *SUMO-1* mRNA, and changes in gene expression were monitored by RNA-Seq. In total, 357 genes were differentially expressed compared to wild-type cells, with 199 of these genes being down-regulated in the SUMO-1-depleted cells. Gene Ontology (GO) analysis of these down-regulated genes showed a significant enrichment in genes involved in translation, thus indicating a positive role for SUMO-1 in transcriptional activation of protein synthesis genes. On the other hand, some transcript levels increased following SUMO-1 depletion; therefore, these transcripts are normally repressed by SUMO-1 in some way. It is possible that the effects of SUMO-1 siRNA on the transcription of some genes are indirect, or that SUMO-1 affects other steps in mRNA production. To further complicate matters, mRNA levels of several ribosomal protein genes, as determined by RT-qPCR (reverse transcription-quantitative polymerase chain reaction), were differentially regulated in cells depleted of SUMO-1 or Ubc9, the E2 SUMO-conjugating enzyme. Several transcripts down-regulated in SUMO-1 knockdown cells were instead up-regulated in Ubc9-depleted cells. It is possible that SUMO-2/3, which is also conjugated to proteins by Ubc9, antagonistically affects transcription of these particular ribosomal protein genes. These findings imply a more intricate role for SUMO-1 modification of transcription factors and chromatin-associated proteins, with some specific genes being activated by SUMO while others are repressed.

A mass spectrometry analysis for SUMO-1-modified proteins from S-phase HeLa cell lysates identified the DNA-binding protein scaffold-associated factor B1/2 (SAFB1/2), a multifunctional protein that interacts with both RNA polymerase II and RNA-processing proteins [36]. Knockdown of SAFB1/2 in HeLa cells reduced SUMO-1 occupancy of the promoter regions of RP genes, suggesting that SAFB1/2 or SAFB1/2 interactors are the major proteins sumoylated at these promoters. Additionally, RNA polymerase II occupancy at RP gene promoters was diminished in SAFB siRNA-treated cells. Liu et al. [36] also monitored pre-mRNA splicing of two RP genes, *RPL26* and *RPL27a*, which are regulated by SAFB1/2. Neither SUMO-1 nor SAFB1/2 depletion affected unspliced primary transcript levels of *RPL26* and *RPL27a*;

however, their knockdown did reduce spliced mRNA levels, revealing a novel effect of SAFB1/2 sumoylation on ribosomal protein gene expression, potentially through a dual—and possibly coupled—function in RNA polymerase II recruitment and pre-mRNA splicing.

Another recent report has also connected SUMO modification of chromatin factors with the regulation of RP gene transcription [24]. Neyret-Kahn et al. used ChIP-Seq to perform an in-depth analysis of SUMO-1, SUMO-2/3, Ubc9, and PIASY (a mammalian SUMO E3 ligase) binding to chromatin in proliferating human fibroblasts. As with the previous study, it was found that SUMO-1, and also SUMO-2/3, localizes to the transcription start site of many active gene promoters. SUMO-1 and SUMO-2/3 have similar association patterns (~two-thirds overlap of binding peaks); however there are genomic regions that only bind to one or the other paralog, indicating potentially unique roles for SUMO-1 and SUMO-2/3 in gene expression regulation. A strong correlation in genomic localization was observed in fibroblasts among SUMO, RNA polymerase II, and H3K4me3, paralleling the SUMO-1 patterns observed in HeLa cells [26].

Fibroblast mRNA-Seq results revealed that 67% of the genes marked by SUMO-1 or SUMO-2/3 were significantly expressed. Moreover, occupancy ranking of genes marked by SUMO-1 and SUMO-2/3 showed enrichment for histone genes, as well as genes involved with translation (e.g., RP genes), RNA Pol III-transcribed tRNA genes, and RNA Pol I-transcribed rRNA genes. These results again suggest an integral function for SUMO modifications at chromatin sites that regulate expression of genes important for protein synthesis.

Based on work in fibroblasts and HeLa cells, the precise roles for SUMO in regulating protein synthesis genes might well depend on cell type, as SUMO has been found to both positively and negatively regulate expression of specific RP genes. Moreover, the expression levels of several RNA Pol III transcripts (*RNA5S*, *RN7SL1*, and *tRNA-Tyr*), rRNA, and ribosomal protein genes *RPL26* and *RPS14* were modestly increased in both *UBC9* and *SUMO* (*SUMO-1*+*SUMO-2/3*) knockdown fibroblasts [24]. These results suggest a repressive role for sumoylation in the regulation of certain genes involved in translation, contrary to the results observed in HeLa cells [26]. Interpretation of the results in HeLa cells is confounded by the observation that mRNA levels of several ribosomal protein genes were higher in Ubc9-depleted cells but lower in SUMO-1-depleted cells. It is possible that either all the SUMO paralogs together or the E2-conjugating enzyme Ubc9 must be knocked down in order to observe loss of the repressive effects of sumoylation on RP gene expression. Nevertheless, in their follow-up paper, Liu et al. [36] identified the chromatin

scaffold protein SAFB1 as a sumoylated factor whose sumoylation correlates with enhanced pol II recruitment and pre-mRNA splicing of several RP genes. Thus, it is likely that SUMO-mediated control of gene expression is complex and that SUMO-dependent expression changes will depend on cell type, growth conditions, or other experimental factors.

5 SUMO Across the Yeast Genome

The global localization of SUMO (Smt3) on *S. cerevisiae* chromatin is similar to its localization in human cell lines in that SUMO clusters near the transcription start sites of many RP genes and tRNA genes [25]. Of the 395 unique SUMO ChIP-Seq peaks, 246 were at RNAPIII-transcribed tRNA genes, 110 at RP genes (out of 138 RP genes), 12 at non-ribosomal protein-coding genes, and 27 at genes for noncoding RNAs. A genome-wide RNA-Seq analysis of a temperature-sensitive *ubc9-1* mutant, which is severely impaired for SUMO ligation, revealed no effect on global transcription, but showed that expression of nearly all RP genes was decreased [25]. Thus, Ubc9, presumably through SUMO conjugation to specific gene-proximal chromatin components, stimulates yeast RP gene expression.

The SUMO-binding sites within the RP gene promoters were found to be similar to the consensus DNA-binding motif of the transcription factor Rap1 [25]. Rap1 ChIP-Seq analysis revealed strong colocalization of Rap1 and SUMO at RP gene promoters. Rap1 is a SUMO target, and blocking Rap1 sumoylation by mutating nine of its lysines to arginine, Rap1-K9R, reduced SUMO enrichment at RP gene promoters (but not Rap1 binding) and resulted in reduced RP gene expression. RNA polymerase II and TFIID (an RNA polymerase II preinitiation complex factor) binding to the RP gene promoters was reduced in *ubc9-1* cells, suggesting that Rap1 sumoylation may be required for recruitment of RNA polymerase II and TFIID to RP gene promoters. Indeed, cells expressing the "SUMOless" Rap1-K9R have decreased binding of RNA polymerase II and TFIID at these promoters.

This study by Chymkowitch et al. [25] highlights a novel role for SUMO in regulating RP gene expression in yeast through modification of the conserved Rap1 transcription factor, thereby connecting the SUMO pathway to a regulatory mechanism of cell proliferation. It also opens up many avenues of study regarding SUMO dynamics and the control of transcription, including the role of SUMO in tRNA transcription and the molecular mechanism of TFIID recruitment to RP gene promoters by sumoylated Rap1.

6 SUMO-Chromatin Interactions During Heat Shock

Global sumoylation increases dramatically following heat shock, and targeted proteins include the heat-shock factors 1 and 2 (HSF1 and HSF2), other transcription factors, and numerous chromatin-associated proteins [39–49]. Recent genomic studies by both Niskanen et al. and Seifert et al. investigated the changes in chromatin-bound SUMO in response to heat shock [37, 38]. Both studies found that global SUMO-2/3 patterns change drastically across the mammalian genome in response to heat shock. During the stress, SUMO-2/3 accumulated at the promoter regions of actively transcribed genes. However, SUMO-2/3 does not simply stimulate transcription at these gene regions during heat shock. In leukemia and prostate cancer cells, knockdown of Ubc9 or PIAS1 by RNA interference, which causes a general drop in protein sumoylation, led to increased expression of heat-shock genes with normally SUMO-2/3-enriched promoters [37], suggesting that SUMO-2/3 ligation normally represses heat shock-induced gene expression, possibly to prevent hyperactivation of heat-shock genes during acute temperature stress. Contrary to the above study, Seifert et al. [38] found that SUMO-2/3 accumulation at actively transcribed regions during heat shock did not alter gene expression at these sites, but did likely influence the stability of protein complexes bound to the chromatin.

These genome-wide ChIP-Seq studies revealed the dynamic nature of sumoylation across the chromatin landscape in response to heat shock. The mechanism of regulating this massive shift in sumoylation likely involves the coordination of multiple signals and factors. For example, SUMO proteases might need to remove SUMO from chromatin-bound factors that are no longer sumoylated during heat shock, although it is possible that the sumoylated protein conjugate is removed intact by other mechanisms. The mammalian SUMO protease SENP6 is recruited to transcriptionally active DNA regions during heat shock [38]. Further work aimed at studying factors that affect the dynamics of SUMO on chromatin in response to different stresses will provide greater insight into the function of SUMO in regulating gene expression during heat shock and other changes in the cellular environment.

7 SUMO and Histones

Many chromatin-associated proteins are sumoylated, as discussed above. In addition to transcription factors, chromatin remodelers, and other chromatin-modifying enzymes, the central components of chromatin, the histone proteins, are also sumoylated.

In budding yeast, all four core histone proteins and the histone variant H2A.Z can be detected in SUMO-modified forms [50, 51], while only sumoylated histones H3 and H4 have been identified in mammalian cells [46, 52]. Sumoylated histones have been discovered in a diverse range of organisms, including plants [53] and apicomplexan parasites (*Plasmodium falciparum*) [54], implicating histone-SUMO conjugation as a conserved and significant chromatin posttranslational modification. Thus, the already complicated and diverse histone code is now revealed to be even more complex, with a vast number and combination of possible histone posttranslational modifications. Understanding the interplay of these varied modifications will be necessary for elucidating the intricacies of gene regulation by histone alteration.

8 Histone Sumoylation Regulates Transcription

The four core histone proteins, H2A, H2B, H3, and H4, are subject to various posttranslational modifications (PTMs), including acetylation, methylation, and ubiquitylation [55]. SUMO modifies lysine side chains of histones. First identified in mammalian cells in 2003, histone sumoylation appears to play a repressive role in transcription [52]. Histone H4 sumoylation in human cells recruits the histone deacetylase HDAC1 and heterochromatin protein 1 (HP1) to DNA, two factors involved in repressing transcription and maintaining silenced regions of the genome. All four yeast histone proteins were found to be sumoylated in vivo, and as seen in mammalian cells, SUMO-modified histones dampen transcriptional activity [50]. One possible mode of gene repression through histone sumoylation is by opposing activating histone modifications (i.e., acetylation). Indeed, yeast histone H2B sumoylation followed an inverse trend as compared to acetylation at the *GAL1* locus upon activation. Since only a small fraction of bulk histone H2B is sumoylated, this repressive mechanism would require either a localized buildup of SUMO-H2B or SUMO-induced changes to H2B that persist after SUMO deconjugation that inhibits acetylation. More generally, histone sumoylation may have multiple modes of transcriptional regulation, including recruiting transcriptional repressors to gene promoters and blocking activating histone modifications.

Interestingly, a recent report by Hendriks et al. suggests that histone acetylation in human (HeLa) cells may stimulate sumoylation at a nearby lysine residue [46]. Human histone H3 was found by mass spectrometry to be simultaneously modified by SUMO, at Lys19, and by an acetyl group, at Lys24. When HeLa cells were treated with the histone deacetylase inhibitor trichostatin A, histone H3 sumoylation also increased. Conversely, histone

acetyltransferase inhibition (curcumin) led to a corresponding decrease in H3 sumoylation. These results highlight a new example of cross talk between two different histone modifications, the function of which remains to be determined.

A recent in vitro study suggests a role for histone sumoylation in modulating chromatin structure and long-range chromatin interactions [56]. Dhall et al. created a disulfide-linked SUMO-3-histone H4 (at Lys-12) conjugate, showing that the SUMO-H4 readily incorporates into histone octamers and 12-mer nucleosome arrays. However, histone H4 sumoylation thwarted nucleosome compaction. Förster resonance energy transfer (FRET) measurement of internucleosomal interactions showed that histone H4 sumoylation reduces the affinity between two adjacent mononucleosomes. The basic N-terminal tail of histone H4 is important for establishing chromatin compaction, and accordingly, modification of residues within this region leads to changes in chromatin structure and organization [57]. Whether the mechanism of chromatin rearrangement by H4 Lys-12 sumoylation holds true in vivo remains to be determined; however, these findings suggest an additional potential mode of chromatin regulation mediated by histone sumoylation.

Despite these recent studies examining the function of histone sumoylation, our understanding of histone sumoylation remains limited. For example, the distribution of site-specific histone sumoylation across the genome has not been resolved. Furthermore, the dynamics of SUMO modification of histone proteins, including the possible regulated desumoylation by SUMO proteases, have yet to be worked out.

9 SUMO and Chromatin-Modifying Enzymes

SUMO also functions indirectly to modulate other histone PTMs by altering the activity of chromatin-modifying enzymes. Sumoylation of chromatin modifiers has varying consequences for the function and stability of the targeted enzyme. For example, sumoylation can enhance activity, as observed with SUMO-1 modification of the histone deacetylase HDAC4 [58]. Conversely, sumoylation of the histone lysine methyltransferase JARID1B/KDM5B leads to its RNF4-mediated degradation, both during cell cycle progression and in response to DNA damage [59, 60]. RNF4 is a SUMO-targeted ubiquitin ligase (STUbL).

Frequently, SUMO modification of a transcription factor leads to recruitment of histone deacetylases, and thus repression of gene expression [61–63]. Sumoylation of the transcription factor Elk-1 recruits HDAC-2 to chromatin, which in turn reduces histone acetylation and consequently dampens transcription [61]. Intriguingly, SUMO modification both enhances and inhibits the

interaction between chromatin factors and the histone methyltransferase SETDB1, depending on the identity of the modified protein [64, 65]. Conjugation of SUMO-1 to methyl-CpG-binding domain protein 1 (MBD1) reduced binding to SETDB1 and hence failed to repress transcription through histone methylation [64]. Conversely, sumoylation of the KAP1 corepressor recruited SETDB1 to chromatin, stimulated SETDB1 methyltransferase activity, and decreased gene expression [65]. SUMO modification appears to alter chromatin status through multiple mechanisms, and often the molecular consequences of these changes are target and context dependent.

A recent report by Nayak et al. has identified a role for dynamic desumoylation in the regulation of *HOX* gene expression, as controlled by the MLL1/MLL2 histone methyltransferase complexes [66]. *HOX* genes encode homeobox (HOX)-containing transcription factors crucial for vertebrate development. Transcriptional activation of these genes is tightly regulated. RbBP5, one of the four regulatory subunits of the MLL1/MLL2 complexes, is desumoylated by the SUMO protease SENP3. Removal of SUMO-2 from RbBP5 in turn recruits the MLL components Ash2L and menin to a subgroup of *HOX* genes. The fully assembled MLL1/MLL2 complexes trimethylate H3K4 and recruit RNA polymerase II to promoter regions of the *HOX* genes, thereby turning on gene expression. These findings link SENP3 and the SUMO pathway to transcription-mediated modulation of a key developmental program.

10 Conclusions and Future Directions

The SUMO pathway is intimately linked to the control of gene expression. Covalent SUMO modification of transcription factors, chromatin-remodeling enzymes, and various other chromatin-related factors regulates transcription, not only in a repressive manner as originally observed (Fig. 3), but at times also in a stimulatory capacity. Novel ChIP-Seq investigations have shed light on the distribution of SUMO across the genomes of both budding yeast and mammalian cells. SUMO was found primarily at the promoter region of actively transcribed genes, most notably those of ribosomal protein genes. However, whether SUMO functions to activate or repress transcription at these genomic regions remains to be conclusively established, and the answer may depend on cell type, organism, and/or environmental conditions.

Nonetheless, the results of these genomic studies have uncovered an unanticipated role for SUMO in regulating genes involved in protein synthesis and have implicated the SUMO pathway in controlling cell growth and the nutrient response. Several chromatin-binding proteins were found to be sumoylated when localized to ribosomal protein gene promoters, namely SAFB1/2

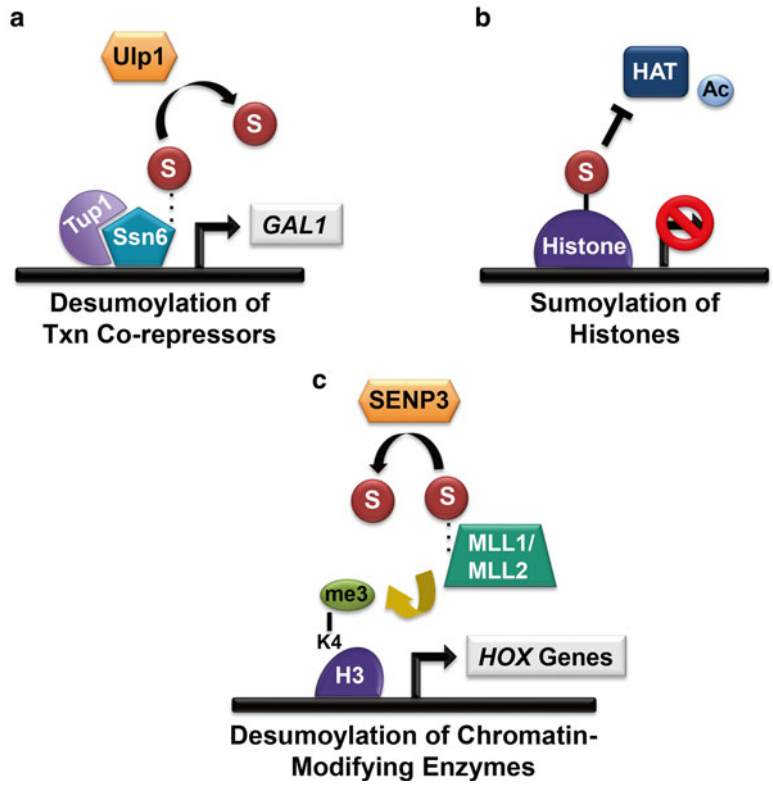

Fig. 3 Dynamic SUMO modification regulates transcription. (**a**) Desumoylation of the yeast transcriptional co-repressor Ssn6 by the SUMO protease Ulp1 results in activation of the *GAL1* gene following a shift to galactose-containing medium. (**b**) Sumoylation of histone core proteins, namely H2B and H4, represses transcription of yeast genes. One possible mechanism for transcriptional repression by histone-SUMO modification is through blocking sites of histone acetylation, an activating histone mark catalyzed by histone acetyltransferases (HATs). (**c**) Activation of mammalian histone methyltransferase complexes MLL1/MML2 requires desumoylation of the RbBP5 subunit by SENP3. Once activated and fully assembled, MLL1/MLL2 methylate lysine-4 of histone H3 that is present at *HOX* gene promoters, which leads to RNA polymerase II recruitment and transcriptional activation

in mammalian cells and Rap1 in yeast. It will be important to determine if there are additional specific factors sumoylated at RP promoters in order to understand the exact role of SUMO at these sites.

The dynamic nature of SUMO-protein modification, through the action of SUMO proteases, allows for fine-tuning of cellular pathways controlled by sumoylation. The role of SUMO proteases in regulating expression of RP genes and other actively transcribed genes under stress conditions has yet to be studied. There are likely functions for SUMO proteases at these specific chromatin regions, particularly when the stimulatory or inhibitory effect of SUMO on transcription is no longer required.

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Part II

Procedures to Study Protein SUMOylation In Vitro

Chapter 3

Reconstitution of the Recombinant RanBP2 SUMO E3 Ligase Complex

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Abstract

One of the few proteins that have SUMO E3 ligase activity is the 358 kDa nucleoporin RanBP2 (Nup358). While small fragments of RanBP2 can stimulate SUMOylation in vitro, the physiologically relevant E3 ligase is a stable multi-subunit complex comprised of RanBP2, SUMOylated RanGAP1, and Ubc9. Here, we provide a detailed protocol to in vitro reconstitute the RanBP2 SUMO E3 ligase complex. With the exception of RanBP2, reconstitution involves untagged full-length proteins. We describe the bacterial expression and purification of all complex components, namely an 86 kDa His-tagged RanBP2 fragment, the SUMO E2-conjugating enzyme Ubc9, RanGAP1, and SUMO1, and we provide a protocol for quantitative SUMOylation of RanGAP1. Finally, we present details for the assembly and final purification of the catalytically active RanBP2/RanGAP1*SUMO1/Ubc9 complex.

Key words RanBP2, Ubc9, SUMO, RanGAP1, RanBP2 complex, In vitro SUMOylation, SUMO E3 ligase, Complex reconstitution

1 Introduction

Reconstitution of SUMOylation reactions in vitro is a valuable method for the biochemical characterization of enzymes involved in the SUMOylation cascade, identification, or validation of SUMO targets and their SUMO acceptor sites as well as the generation of SUMOylated proteins for functional studies. Among the few proteins that have been described as bona fide SUMO E3 ligases is the nucleoporin RanBP2/Nup358 [1]. It is a large multi-domain protein [2, 3] with essential functions in nucleocytoplasmic transport processes and during mitosis [4, 5]. Throughout the cell cycle, RanBP2 is stably associated with the Ran GTPase-activating protein RanGAP1. Prerequisites for this interaction are the modification of RanGAP1 with SUMO1 [6, 7] and the presence of the SUMO E2-conjugating enzyme Ubc9 [8]. The RanBP2 E3 ligase region is a natively unfolded stretch consisting of two short homologous

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repeats, named IR1 and IR2 (Fig. 1a) [9]. While IR1 is largely responsible for catalysis in isolated RanBP2 fragments, it also serves as the platform for the assembly of the stable RanBP2/RanGAP1*SUMO1/Ubc9 complex [10]. In this complex, Ubc9 does not serve as catalytic E2 enzyme but stabilizes the interaction between SUMOylated RanGAP1 and RanBP2. Upon complex formation, IR1 is masked by this interaction and IR2 functions as the catalytic center [11] (*see* Fig. 1a). Here we describe the in vitro reconstitution of this multisubunit E3 ligase. This includes protocols for the purification of untagged SUMO1, untagged Ubc9, untagged RanGAP1, a His-tagged 86 kDa RanBP2 fragment ranging from RanGTP-binding domains 3 and 4 (RanBP2-BD3-4), and a protocol for quantitative SUMOylation of RanGAP1. Protocols for SUMO1 and Ubc9 have been published previously [12], but have now been slightly modified. The final step in the reconstitution is the formation and separation of the very stable, catalytically active RanBP2/RanGAP1*SUMO1/Ubc9 complex (with RanGAP1*SUMO1 and Ubc9 bound to IR1 in a 1:1:1 ratio) from an unwanted side product, an inactive complex having additional RanGAP1*SUMO1 and Ubc9 more loosely attached to IR2 (RanBP2/RanGAP1*SUMO1/Ubc9 complex in a 1:2:2 ratio).

The recombinant complex will serve as an essential tool to investigate catalytic mechanism and substrate specificity of the RanBP2 SUMO E3 ligase complex and to study the interplay between SUMOylation and the Ran GTPase cycle. Examples for applications are given in [11, 13].

2 Materials

2.1 Purification of Complex Components

2.1.1 General Expression and Purification Supply

1. LB medium.
2. 1 M $MgCl_2$, autoclaved.
3. 20% w/v glucose, sterile filtered, stored at −20 °C.
4. 100 mg/mL Ampicillin, sterile filtered, stored in 1 mL aliquots at −20 °C.
5. 1 M Isopropyl-β-D-thiogalactoside (IPTG), sterile filtered, stored in aliquots at −20 °C.
6. 1 M DTT (1000-fold stock), stored in aliquots at −20 °C. Added freshly.
7. 1 mg/mL Aprotinin (1000-fold stock) in 20 mM HEPES pH 7.4, stored in aliquots at −20 °C. Added freshly.
8. 1 mg/mL Leupeptin and pepstatin (1000-fold stock) in DMSO, stored in aliquots at −20 °C. Added freshly.
9. Transport buffer (TB): 20 mM HEPES pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 1 mM DTT, 1 μg/mL of each aprotinin, leupeptin, pepstatin.

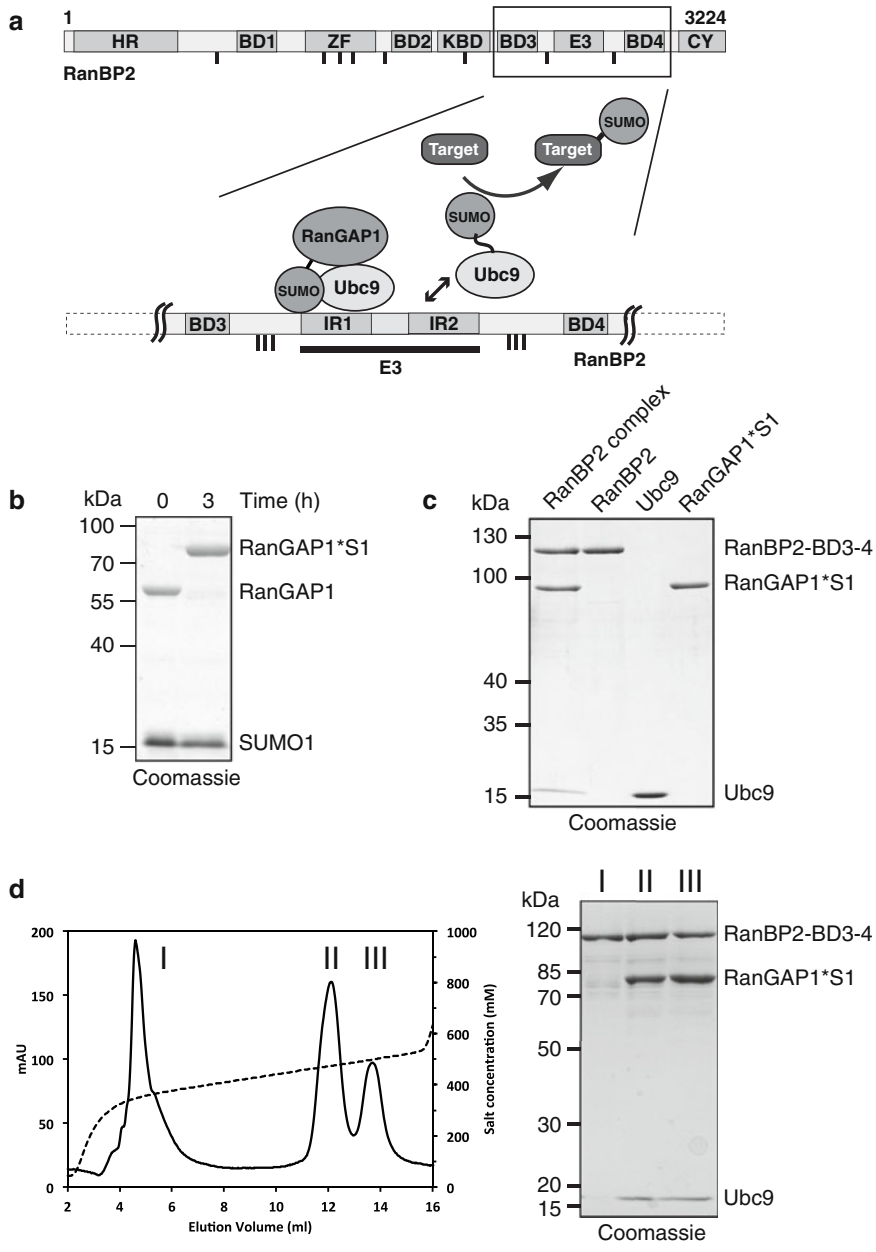

Fig. 1 In vitro reconstitution and purification of the RanBP2 complex. (**a**) Domain structure of RanBP2 with α-helical region (HR), four RanGTP-binding domains (BD1-4), the zinc finger domain (ZF), kinesin-binding domain (KBD), SUMO E3 ligase region (E3), cyclophilin domain (CY), and several FG repeats (dashes). The RanBP2-BD3-4 complex is shown in more detail. The IR1 repeat serves as a platform for assembly of the stable RanBP2 complex. In the context of the complex, IR2 transiently interacts with the Ubc9-SUMO thioester and thereby mediates SUMOylation of targets. (**b**) Quantitative SUMOylation of RanGAP1. Samples were taken before and at the end of the reaction and analyzed on a 5–20 % SDS gel stained with Coomassie. (**c**) 2 μg of each purified protein was analyzed on a 5–20 % SDS gel stained with Coomassie. (**d**) RanBP2 complex was reconstituted and applied to a MonoQ column as described in Chapter 3.2. The elution profile is shown and peak fractions were visualized by SDS-PAGE and Coomassie staining. The desired RanBP2-BD3-4/RanGAP1*SUMO1/Ubc9 complex in a 1:1:1 stoichiometry elutes at about 440 mM NaCl (peak II). Peak I at about 230 mM NaCl represents free RanBP2-BD3-4 and a RanBP2-BD3-4/Ubc9 complex, and peak III at about 480 mM NaCl is an unwanted, catalytically inactive complex in a 1:2:2 stoichiometry

10. Shift assay buffer (SAB): TB buffer supplemented with 0.05 % v/v Tween20.
11. EmulsiFlex (Avestin, Canada) or similar device for bacterial lysis.
12. MonoQ 5/50 GL anion-exchange column (1 mL bed volume), HiLoad 26/60 Superdex 200 prep grade (320 mL bed volume), HiLoad 26/60 Superdex 75 prep grade (320 mL bed volume), and Superdex 200 10/300 GL (24 mL bed volume) gel filtration columns (GE Healthcare).
13. Centrifugal concentrators (10, 5, and 3 kDa MWCO).
14. PonceauS and nitrocellulose.
15. Recombinant purified SUMO E1 enzyme [12].
16. ATP: 100 mM ATP in 20 mM HEPES pH 7.4, 100 mM magnesium acetate, pH adjusted with NaOH, stored in aliquots at −80 °C.

2.1.2 Expression Constructs

1. pET11a-SUMO1 (SMT3C): The coding region was amplified by PCR using the primers CGGCTTAAATGAAT CCTAACCCCCCGTTTG and GGTTCCGCGTGGACATA TGTCTGACCAGG and ligated into *Nde*I/*Bam*HI sites of pET11a.
2. pET23a-Ubc9: The coding region was amplified by PCR from EST clone No. IMAGp998A061122 using the primers CATATGTCGGGGATCGCCCTCAGCCGC and GGATCC TTATGAGGGGGCAAACTTCTTCGC and was ligated into the *Nde*I/*Bam*HI sites of pET23a. It encodes for mouse Ubc9, which is identical to human Ubc9 in amino acid sequence.
3. pET11a-RanGAP1: Human RanGAP1 was amplified by PCR and ligated into NdeI/BamHI sites of pET11a.
4. pET23a-RanBP2-BD3-4: The region from Ran-binding domain 3–4 of human RanBP2 (amino acids 2304–3062) was amplified by PCR and ligated into *Nde*I/*Xho*I sites of pET23a, resulting in a C-terminal 6x His-tag.

2.1.3 Purification of SUMO1

1. DEAE-Sepharose (Sigma-Aldrich).
2. SUMO lysis buffer: 50 mM Tris–HCl pH 7.5, 50 mM NaCl, 2 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.
3. DNaseI (lyophilized, e.g., from Roche).

2.1.4 Purification of Ubc9

1. SP-Sepharose (Fast Flow, Sigma-Aldrich).
2. Ubc9 lysis/wash buffer: 50 mM Na-phosphate pH 6.5.
3. Ubc9 elution buffer: 50 mM Na-phosphate pH 6.5, 300 mM NaCl, 1 mM DTT, 1 μg/mL of each aprotinin, leupeptin, pepstatin.

2.1.5 Purification of RanGAP1

1. Douncer (50 mL, Sartorius).
2. Q-Sepharose (Fast Flow, Sigma-Aldrich).
3. Dialysis bag (14 kDa MWCO, Spectra/Por).
4. RanGAP1 lysis buffer: 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.
5. RanGAP1 wash buffer I: 50 mM Tris–HCl pH 8.0, 1% v/v TritonX100, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.
6. RanGAP1 wash buffer II: 50 mM Tris–HCl pH 7.4, 2 M urea, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.
7. RanGAP1 solubilization buffer: 50 mM Tris–HCl pH 7.4, 8 M urea, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.
8. RanGAP1 dialysis buffer: 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.
9. RanGAP1 wash buffer III: 50 mM Tris–HCl pH 7.4, 300 mM NaCl, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.
10. RanGAP1 elution buffer: 50 mM Tris–HCl pH 7.4, 1 M NaCl, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.

2.1.6 Purification of RanBP2-BD3-4

1. Nickel beads (e.g., Ni-NTA, Qiagen).
2. RanBP2-BD3-4 lysis buffer: 50 mM Sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole pH 8.0, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.
3. RanBP2-BD3-4 wash buffer: 50 mM Sodium phosphate pH 8.0, 300 mM NaCl, 20 mM imidazole pH 8.0, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.
4. RanBP2-BD3-4 elution buffer: 50 mM Sodium phosphate pH 8.0, 300 mM NaCl, 250 mM imidazole pH 8.0, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.

2.2 Reconstitution and Purification of the RanBP2 Complex

1. MonoQ buffer I: 50 mM Tris–HCl pH 8.0, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.
2. MonoQ buffer II: 50 mM Tris–HCl pH 8.0, 1 M NaCl, 1 mM DTT, and 1 μg/mL of each aprotinin, leupeptin, pepstatin.

3 Methods

3.1 Purification of the Complex Components

3.1.1 Purification of SUMO1

1. Transform the *E. coli* strain BL21(DE3) with the pET11a-SUMO1 plasmid and grow a single colony overnight in 5 mL LB medium containing 100 μg/mL ampicillin, 1 mM $MgCl_2$, and 0.1 % w/v glucose.
2. Harvest the bacteria by centrifugation, resuspend them in 500 mL of fresh medium, and grow them at 37 °C with vigorous shaking. At an OD_{600} of 0.6, induce protein expression by adding 1 mM IPTG and continue growth at 37 °C for 3–4 h.
3. Harvest the cells by centrifugation (5000 × *g* for 15 min), resuspend the pellet in 20 mL SUMO lysis buffer, flash freeze, and store at −80 °C until use.
4. Thaw the cell suspension, and add protease inhibitors (1 μg/mL of each aprotinin, leupeptin, pepstatin) as well as 1 mM DTT while thawing. Lyze the bacteria by two passages through an EmulsiFlex (*see* **Note 1**).
5. Supplement the suspension with 1 mM $MgCl_2$, 50 μM $CaCl_2$, as well as several crumbs of DNase I and incubate at 4 °C for 45 min. Clear the lysate by centrifugation (100,000 × *g*, 4 °C, 1 h).
6. Incubate the supernatant with gentle agitation at 4 °C for 1–2 h with 2.5 mL DEAE-Sepharose equilibrated in SUMO lysis buffer containing protease inhibitors and DTT.
7. Remove the DEAE-Sepharose by centrifugation at 250 × *g* for 15 min in a swing-out rotor. SUMO remains soluble in the supernatant during this pre-clearing step.
8. Concentrate the supernatant to a final volume of 3–5 mL using a centrifugal concentrator with a 3 kDa MWCO. Please note that concentrating is very slow at this step.
9. Clear the sample by centrifugation (20,000 × *g*, 15 min, 4 °C, in tabletop centrifuge) or by filtration (0.2 μm low-protein-binding non-pyrogenic filter) to remove small amounts of precipitated protein.
10. Run the sample over a HiLoad 26/60 Superdex 200 prep grade column equilibrated in TB buffer collecting 5 mL fractions. The elution profile is quite dirty with a large void volume peak; SUMO1 elutes at about 250 mL, but in this purification step not as a symmetric peak. Analyze 15 μL of each fraction around this elution volume by SDS-PAGE (15 % gel—Coomassie staining, *see* **Note 2**).
11. Pool all fractions containing SUMO and concentrate again to a final volume of 3–5 mL using a centrifugal concentrator with a 3 kDa MWCO.

12. Run the sample over a HiLoad 26/60 Superdex 75 prep grade column equilibrated in TB buffer collecting 5 mL fractions. SUMO1 elutes at about 190 mL. Analyze 15 μL of each fraction around this elution volume by SDS-PAGE (15% gel—Coomassie staining, *see* **Note 2**).
13. Pool fractions containing pure SUMO1; if necessary, concentrate using a centrifugal concentrator with a 3 kDa MWCO, flash freeze in aliquots, and store at −80 °C.

This protocol can be used to purify untagged SUMO1 as well as SUMO2/3. The expected yield is 15–20 mg SUMO per liter *E. coli* culture.

3.1.2 Purification of Ubc9

1. Transform the *E. coli* strain BL21(DE3) with the plasmid pET23a-Ubc9; inoculate 5 mL LB containing 100 μg/mL ampicillin, 1 mM $MgCl_2$, and 0.1% w/v glucose with a single colony, and grow at 37 °C overnight.
2. Harvest the bacteria by centrifugation at 5000 × *g*, resuspend them in 500 mL fresh medium, and grow at 37 °C. At OD_{600} of 0.6, induce protein expression by adding 1 mM IPTG and grow the culture at 37 °C for another 3–4 h.
3. Harvest the cells by centrifugation (5000 × *g*, 15 min), resuspend the pellet in 20 mL Ubc9 lysis/wash buffer, flash freeze, and store at −80 °C until use.
4. Thaw the cell suspension, and add protease inhibitors (1 μg/mL of each aprotinin, leupeptin, pepstatin) and 1 mM DTT while thawing. Lyze the bacteria by two passages through an EmulsiFlex (*see* **Note 1**) and clear the lysate by centrifugation (100,000 × *g*, 4 °C, 1 h).
5. Apply the supernatant to a 2.5 mL SP-Sepharose column equilibrated in Ubc9 lysis/wash buffer containing protease inhibitors and DTT.
6. Wash the column with the same buffer, until no protein can be detected in the flow-through (test, e.g., by spotting onto nitrocellulose and staining with PonceauS solution).
7. Elute Ubc9 using Ubc9 elution buffer, until no protein can be detected (test, e.g., by spotting onto nitrocellulose and staining with PonceauS solution); collect 1 mL fractions.
8. Pool protein-containing fractions and concentrate to 3–5 mL using a centrifugal concentrator with a 5 kDa MWCO.
9. Clear the sample by centrifugation (20,000 × *g*, 15 min, 4 °C, in tabletop centrifuge) or by filtration (0.2 μm low-protein-binding non-pyrogenic filter) to remove small amounts of precipitated protein.

10. Run the sample over a HiLoad 26/60 Superdex 75 prep grade column equilibrated in TB buffer collecting 5 mL fractions. Ubc9 elutes at about 185 mL. Analyze 15 µL of each fraction around this elution volume by SDS-PAGE (15% gel—Coomassie staining).
11. Pool the Ubc9 peak fractions; if necessary, concentrate using a centrifugal concentrator with a 5 kDa MWCO, flash freeze in aliquots, and store at −80 °C.

This protocol results in about 20–25 mg untagged Ubc9 per liter *E. coli* culture (*see* **Note 3** and Fig. 1c). Although Ubc9 can be thawed and frozen several times, we prefer to thaw aliquots only once to guarantee the reproducibility of the specific activity from experiment to experiment.

3.1.3 Purification of RanGAP1

1. Transform the *E. coli* strain BL21(DE3)-Rosetta2 with the plasmid pET11a-RanGAP1; inoculate 20 mL LB containing 100 µg/mL ampicillin, 1 mM $MgCl_2$, and 0.1% w/v glucose with a single colony; and grow overnight at 37 °C.
2. Harvest the bacteria by centrifugation at 5000 × *g*, resuspend them in 2 L fresh medium, and grow at 37 °C. At OD_{600} of 0.6, induce protein expression by adding 1 mM IPTG and grow the culture at 37 °C for another 3–4 h.
3. Harvest the cells by centrifugation (5000 × *g*, 15 min), resuspend the pellet in 50 mL RanGAP1 lysis buffer, flash freeze, and store at −80 °C.
4. Thaw the cell suspension, and add protease inhibitors (1 µg/mL of each aprotinin, leupeptin, pepstatin) and 1 mM DTT while thawing. Lyze the bacteria by two passages through an EmulsiFlex and clear the lysate by centrifugation (100,000 × *g*, 4 °C, 1 h).
5. Discard the supernatant (RanGAP1 forms inclusion bodies) and wash the pellet by homogenizing it in 50 mL RanGAP1 wash buffer I using a douncer. Clear the suspension by centrifugation (100,000 × *g* 4 °C, 20 min).
6. Repeat **step 5**.
7. Repeat **step 5** with 50 mL RanGAP1 wash buffer II. Important: Complete this step quickly to avoid premature solubilization of RanGAP1 in wash buffer II (contains 2 M urea).
8. Repeat **step 5** with 50 mL RanGAP1 solubilization buffer (*see* **Note 4**) and centrifugation at room temperature (8 M urea, which is used in this step to solubilize RanGAP1, precipitates at 4 °C).
9. After the last round of centrifugation, collect the supernatant and dialyze it four times for at least 2 h against 2 L of RanGAP1 dialysis buffer at 4 °C. Important: For the first dialysis step, the

buffer should have room temperature; for subsequent dialysis steps the buffer should be precooled at 4 °C.

10. Apply the dialysate to a 20 mL Q-Sepharose column equilibrated in RanGAP1 dialysis buffer.
11. Wash the column with RanGAP1 wash buffer III until no protein can be detected in the flow-through (test, e.g., by spotting onto nitrocellulose and staining with PonceauS solution).
12. Elute RanGAP1 using RanGAP1 elution buffer, until no protein can be detected (test, e.g., by spotting onto nitrocellulose and staining with PonceauS solution); collect 5 mL fractions.
13. Pool protein-containing fractions and concentrate to 3–5 mL using a centrifugal concentrator with a 10 kDa MWCO.
14. Clear the sample by centrifugation (20,000 × *g*, 15 min, 4 °C, in tabletop centrifuge) or by filtration (0.2 μm low-protein-binding non-pyrogenic filter) to remove small amounts of precipitated protein.
15. Run the sample over a HiLoad 26/60 Superdex 200 prep grade column equilibrated in TB buffer collecting 5 mL fractions. RanGAP1 elutes at about 185 mL (*see* **Note 4**). Analyze 15 μL of each fraction around this elution volume by SDS-PAGE (5–20% gradient gel—Coomassie staining, *see* Fig. 1b).
16. Pool the properly folded RanGAP1 fractions; if necessary, concentrate using a centrifugal concentrator with a 10 kDa MWCO, flash freeze in aliquots, and store at −80 °C.

This protocol results in about 3–5 mg untagged RanGAP1 per liter *E. coli* culture.

3.1.4 Purification of RanBP2-BD3-4

1. Transform the *E. coli* strain BL21(DE3)-Rosetta2 with the plasmid pET23a-RanBP2-BD3-4, inoculate 10 mL LB containing 100 μg/mL ampicillin with a single colony, and grow overnight at 37 °C.
2. Harvest the bacteria by centrifugation at 5000 × *g*; resuspend them in 1 L fresh LB containing 20 mM Hepes pH 7.4, 100 μg/mL ampicillin, 1 mM $MgCl_2$, and 0.1% w/v glucose; and grow at 37 °C. At OD_{600} of 0.6, briefly place culture on ice to cool it down to 25 °C, supplement the medium with 3% v/v ethanol, induce protein expression by adding 400 μM IPTG, and grow the culture at 25 °C for another 6 h.
3. Harvest the cells by centrifugation (5000 × *g*, 15 min), resuspend the pellet in 50 mL RanBP2-BD3-4 lysis buffer, flash freeze, and store at −80 °C.
4. Thaw the cell suspension, and add protease inhibitors (1 μg/mL of each aprotinin, leupeptin, pepstatin) and 1 mM DTT while thawing. Lyze the bacteria by two passages through an EmulsiFlex and clear the lysate by centrifugation (100,000 × *g*, 4 °C, 1 h).

5. Apply the supernatant to 10 mL nickel beads equilibrated in RanBP2-BD3-4 lysis buffer containing protease inhibitors and DTT.
6. Wash the column with RanBP2-BD3-4 wash buffer, until no protein can be detected in the flow-through (test, e.g., by spotting onto nitrocellulose and staining with PonceauS solution).
7. Elute RanBP2-BD3-4 using RanBP2-BD3-4 elution buffer, until no protein can be detected (test, e.g., by spotting onto nitrocellulose and staining with PonceauS solution); collect 1 mL fractions.
8. Pool protein-containing fractions and concentrate to 3–5 mL using a centrifugal concentrator with a 10 kDa MWCO.
9. Clear the sample by centrifugation (20,000 × *g*, 15 min, 4 °C, in tabletop centrifuge) or by filtration (0.2 μm low-protein-binding non-pyrogenic filter) to remove small amounts of precipitated protein.
10. Run the sample over a HiLoad 26/60 Superdex 200 prep grade column equilibrated in TB buffer collecting 5 mL fractions. RanBP2-BD3-4 elutes at about 150 mL. Analyze 15 μL of each fraction around this elution volume by SDS-PAGE (5–20% gradient gel—Coomassie staining, *see* **Note 5** and Fig. 1c).
11. Pool the RanBP2-BD3-4 peak fractions; if necessary, concentrate using a centrifugal concentrator with a 10 kDa MWCO, flash freeze in aliquots, and store at −80 °C.

This protocol results in about 10–15 mg C-terminally His-tagged RanBP2-BD3-4 per liter *E. coli* culture.

3.1.5 Quantitative SUMOylation of RanGAP1

1. Set up a 5 mL in vitro SUMOylation reaction containing 30 μM SUMO1, 10 μM RanGAP1, 125 nM SUMO E1 enzyme, and 125 nM Ubc9 in SAB buffer.
2. Start the reaction by adding 5 mM ATP and incubate for 3 h at 30 °C. Take a 15 μL sample before the addition of ATP and after 3 h and analyze by SDS-PAGE (5–20% gradient gel—Coomassie) to ensure that RanGAP1 has been quantitatively SUMOylated (*see* **Note 6** and Fig. 1b).
3. Concentrate the reaction to 500 μL using a centrifugal concentrator with a 10 kDa MWCO (*see* **Note 7**).
4. Clear the sample by centrifugation (20,000 × *g*, 15 min, 4 °C, in tabletop centrifuge) or by filtration (0.2 μm low-protein-binding non-pyrogenic filter) to remove small amounts of precipitated protein.
5. Run the sample over a Superdex200 10/300 GL column equilibrated in TB buffer collecting 500 μL fractions.

RanGAP1*SUMO1 elutes at about 12 mL (*see* **Note 8** and Fig. 1c). Analyze fractions around this elution volume by SDS-PAGE (5–20% gradient gel—Coomassie stain).

6. Pool the RanGAP1*SUMO1 fractions; if necessary, concentrate using a centrifugal concentrator with a 10 kDa MWCO, flash freeze in aliquots, and store at −80 °C.

This protocol results in about 2 mg untagged RanGAP1*SUMO1 (approximately 60% yield of the reaction) and can be scaled up by increasing the volume of the reaction.

3.2 Reconstitution and Purification of Complex

1. Set up a 500 µL complex forming reaction containing 24 µM Ubc9, 20 µM RanGAP1*SUMO1, and 24 µM RanBP2-BD3-4 in SAB buffer (*see* **Notes 9** and **10**).
2. Incubate the complex forming reaction on ice overnight (*see* **Note 11**).
3. Clear the sample by centrifugation (20,000 × *g*, 15 min, 4 °C, in tabletop centrifuge) or by filtration (0.2 µm low-protein-binding non-pyrogenic filter) to remove small amounts of precipitated protein.
4. Load the sample onto MonoQ 5/50 GL anion-exchange column equilibrated in MonoQ buffer I and wash with at least 3 column volumes. Free Ubc9 will elute from the column during washing.
5. Elute the complex by applying a linear gradient of 30–55% of MonoQ buffer II (equivalent to 300–550 mM NaCl) over 13 column volumes while collecting 500 µL fractions. The desired RanBP2-BD3-4/RanGAP1*SUMO1/Ubc9 complex in a 1:1:1 stoichiometry elutes at about 440 mM NaCl. A catalytically inactive complex in a 1:2:2 stoichiometry elutes at about 480 mM NaCl. Free RanBP2-BD3-4 and a RanBP2-BD3-4/Ubc9 complex elute at about 230 mM NaCl (*see* Fig. 1d).
6. Pool the fractions containing the 1:1:1 RanBP2 complex and concentrate to 500 µL using a centrifugal concentrator with a 10 kDa MWCO. If possible, avoid pooling fractions containing residual free RanGAP1 (*see* **Note 12**).
7. Clear the sample by centrifugation (20,000 × *g*, 15 min, 4 °C, in tabletop centrifuge) or by filtration (0.2 µm low-protein-binding non-pyrogenic filter) to remove small amounts of precipitated protein.
8. Run the sample over a Superdex200 10/300 GL column equilibrated in TB buffer collecting 500 µL fractions. The 1:1:1 RanBP2 complex elutes at about 10 mL (*see* **Note 12**).
9. Pool the fractions containing the RanBP2 complex; if necessary, concentrate using a centrifugal concentrator with a 10 kDa MWCO, flash freeze in small aliquots, and store at −80 °C.

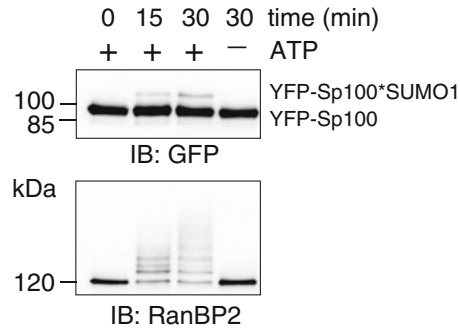

Fig. 2 The purified RanBP2 complex is an active SUMO E3 ligase. 20 μL reactions containing 500 nM YFP-Sp100 as substrate were incubated with 100 nM E1, 50 nM E2, and 25 nM RanBP2 complex as E3 ligase without or with 5 mM ATP for the indicated times at 30 °C in SAB buffer supplemented with 0.2 mg/mL ovalbumin. Reactions were stopped by the addition of Laemmli buffer. SUMOylation of YFP-Sp100 and autoSUMOylation of RanBP2 were detected by immunoblotting against GFP and RanBP2

This protocol results in about 0.5 mg of the 1:1:1 RanBP2 complex (approximately 25–30% yield) and can be scaled up by increasing the volume of the reaction. This RanBP2 complex is an active E3 ligase (*see* Fig. 2)

4 Notes

1. Do not use lysozyme for bacterial lysis as it will not be separated from SUMO and Ubc9 during the purification procedure.
2. Whereas SUMO is 11 kDa in molecular weight, it runs at 20 kDa in SDS-PAGE (Fig. 1b).
3. Ubc9 is a small protein interacting with the E1, E3, SUMO, and the target protein; tags at the N- or C-terminus not only impair Ubc9's function, but also drastically decrease the yield of the purification protocol.
4. While RanGAP1 can be solubilized in as little as 5 mL solubilization buffer, efficient refolding requires concentrations <0.1 mg/mL. The efficiency of refolding can be checked during gel filtration, where misfolded RanGAP1 elutes in the void volume, whereas properly folded protein elutes at about 185 mL from the indicated column.
5. The RanBP2-BD3-4 fragment is largely unfolded and hence has a smaller elution volume in gel filtration than expected for an 86 kDa protein. Likewise, it runs at about 120 kDa in SDS-PAGE.

6. Because SUMOylated and non-SUMOylated RanGAP1 cannot be separated by gel filtration, it is important to ensure quantitative SUMOylation.
7. Direct concentration of the in vitro SUMOylation reaction sometimes led to partial protein precipitation. This never happened when the sample was dialyzed against TB prior to concentration, indicating that ATP may be the culprit. If optimal yields are required, we recommend to include the dialysis step.
8. In gel filtration, the peak of RanGAP1*SUMO1 may show a shoulder at its beginning; it contains a stable complex of RanGAP1*SUMO1 and (the catalytic amounts of) Ubc9 [10, 14]. If SUMOylated RanGAP1 is to be used for RanBP2 complex formation, this fraction can be pooled with fractions containing pure RanGAP1*SUMO1.
9. Due to the fact that free RanGAP1*SUMO1 and the RanBP2 complex elute at similar salt concentrations from the MonoQ column, the complex forming reaction is set up with limiting amount of RanGAP1*SUMO1.
10. Since RanGAP1 displays some degree of stickiness to plastic surfaces, it is advisable to first add Ubc9 to the complex forming reaction, then RanGAP1*SUMO1, and finally RanBP2-BD3-4.
11. Appropriate stoichiometry of components and overnight incubation are important to ensure homogenous complex formation. Three possible complexes can form: a complex with a 1:1:1 stoichiometry on IR1 (desired RanBP2 complex), a complex with a 1:1:1 stoichiometry on IR2, and a complex with a 1:2:2 stoichiometry (on IR1 and on IR2). However, the stability of complexes formed in vitro on IR1 or on IR2 differs dramatically: assembly on IR2 is reversible, while assembly on IR1 is not [11]. Given enough time, most complexes will end up on IR1. While 1:1:1 complexes on IR1 or IR2 cannot be distinguished by chromatography, an indicator for proper assembly is resistance of complex-bound SUMOylated RanGAP1 towards SUMO isopeptidases [11].
12. Residual amounts of free RanGAP1*SUMO1 elute on MonoQ between the 1:1:1 and the 1:2:2 RanBP2 complex (at about 460 mM NaCl); they can be identified by their low A_{280}/A_{230} ratio (record chromatography profiles at 280 and 230 nm; RanGAP1 has a characteristically low absorption coefficient at 280 nm; $A_{280}/A_{230} = \sim 0.06$ for RanGAP1*SUMO1; $A_{280}/A_{230} = \sim 0.15$ for other proteins). If the 1:1:1 RanBP2 complex is not perfectly separated from residual free RanGAP1*SUMO1 on the MonoQ column, contaminations of the latter can be separated during the final gel filtration step (identification of RanGAP1*SUMO1 via A_{280}/A_{230}).

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Chapter 4

Production and Purification of Recombinant SUMOylated Proteins Using Engineered Bacteria

Frédérique Brockly, Marc Piechaczyk, and Guillaume Bossis

Abstract

SUMO is a ubiquitin-like protein that is covalently conjugated to numerous cellular proteins to modify their function and fate. Although large progresses have been made in the identification of SUMOylated proteins, the molecular consequences of their SUMOylation are generally unknown. This is, most often, due to the low abundance of SUMOylated proteins in the cell, usually less than 1 % of a given protein being modified at steady state. To gain insights into the role of specific SUMOylation targets, SUMO conjugation can be reconstituted in vitro using purified proteins. However, for most substrates, the efficiency of in vitro SUMOylation is too low to obtain sufficient amounts of their SUMOylated forms for biochemical studies. Here, we describe a detailed protocol to purify large amounts of recombinant SUMOylated proteins using bacteria modified to express His-tagged SUMO as well as the SUMO-activating and -conjugating enzymes.

Key words SUMOylation, Protein purification, Biochemistry

1 Introduction

SUMO is a posttranslational modifier of the ubiquitin family. SUMOylation consists in the covalent conjugation of SUMO-1, -2, or -3 on lysines ε-side chain via isopeptide bond formation. More than 1000 SUMOylated proteins, involved in most cellular pathways, have already been identified [1]. SUMOylation affects target protein function in a variety of ways. In particular, it can hide existing interaction surfaces, therefore preventing the interaction of the target protein with some of its partners. This is, for example, the case for the E2 ubiquitin-conjugating enzyme E2-25k, the interaction of which with the E1 of ubiquitin pathway is inhibited upon SUMOylation [2]. Another important consequence of SUMOylation is the recruitment of SUMO-binding proteins containing so-called SUMO-interaction motifs (SIM) made up of a core of 3–4 hydrophobic residues (usually valine or isoleucine) flanked by an acidic region. SUMO/SIM interactions

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have been involved in many processes such as recruitment of transcriptional co-repressor complexes to SUMOylated transcription factor, ubiquitylation of SUMOylated proteins by StUbL (SUMO-targeted-ubiquitin ligases), or recruitment of DNA repair complexes to sites of DNA damage [3].

SUMOylation is highly dynamic and concerns a small fraction of each target protein at steady state (typically 0.1–1%), which renders the identification of its role on the regulation of target proteins with its partners particularly difficult. For such studies, the use of recombinant SUMOylated proteins is particularly useful. In particular, it is possible to preform in vitro SUMOylation reaction using recombinant SUMO, the dimeric SUMO-activating enzyme Aos1-Uba2, and the SUMO-conjugating enzyme Ubc9. In the presence of ATP, SUMO can be transferred to protein substrates added to the reaction. In vitro SUMOylation is however generally not highly efficient, although it can be improved by the addition of recombinant SUMO E3s, such as the catalytic fragment of the RanBP2 ligase (IR1+M) or PIAS proteins [4, 5]. To produce higher yields of recombinant SUMOylated proteins, a tri-cistronic vector expressing SUMO-1 or SUMO-3, as well as Ubc9 and a fusion between Uba2 and Aos1, was generated [6]. When co-expressed with a protein of interest in the same bacteria, this system allows SUMOylation of such a substrate. If the protein contains a tag (His, GST, or MBP, for example), it can be purified and used for biochemical assays. However, although this system is highly efficient for some targets such as RanGAP, it leads to low levels of SUMOylation for most other substrates. Therefore, the purified protein is a mixture of a small amount of SUMOylated and a large amount of non-SUMOylated target, which renders subsequent analysis difficult.

Here, we describe a modified version of this bacterial SUMOylation system and a detailed protocol to easily purify large amounts of SUMOylated proteins with no or little contamination from the non-modified protein. To this aim, we inserted a His_6 tag at the N-terminus of SUMO to allow for the specific purification of SUMO-modified substrates and their separation from the non-modified target. Finally, the SUMOylated target is further purified with an MBP tag present on the target before cleavage of the tag with the TEV protease to release the purified SUMOylated protein (Fig. 1).

Fig. 1 (continued) MBP-tagged-SUMOylated target. The MBP-tagged is then removed with the TEV protease. SUMOylation also occurs at low rate on the MBP tag. A second purification can be performed to remove the SUMOylated-MBP from the purified SUMOylated target

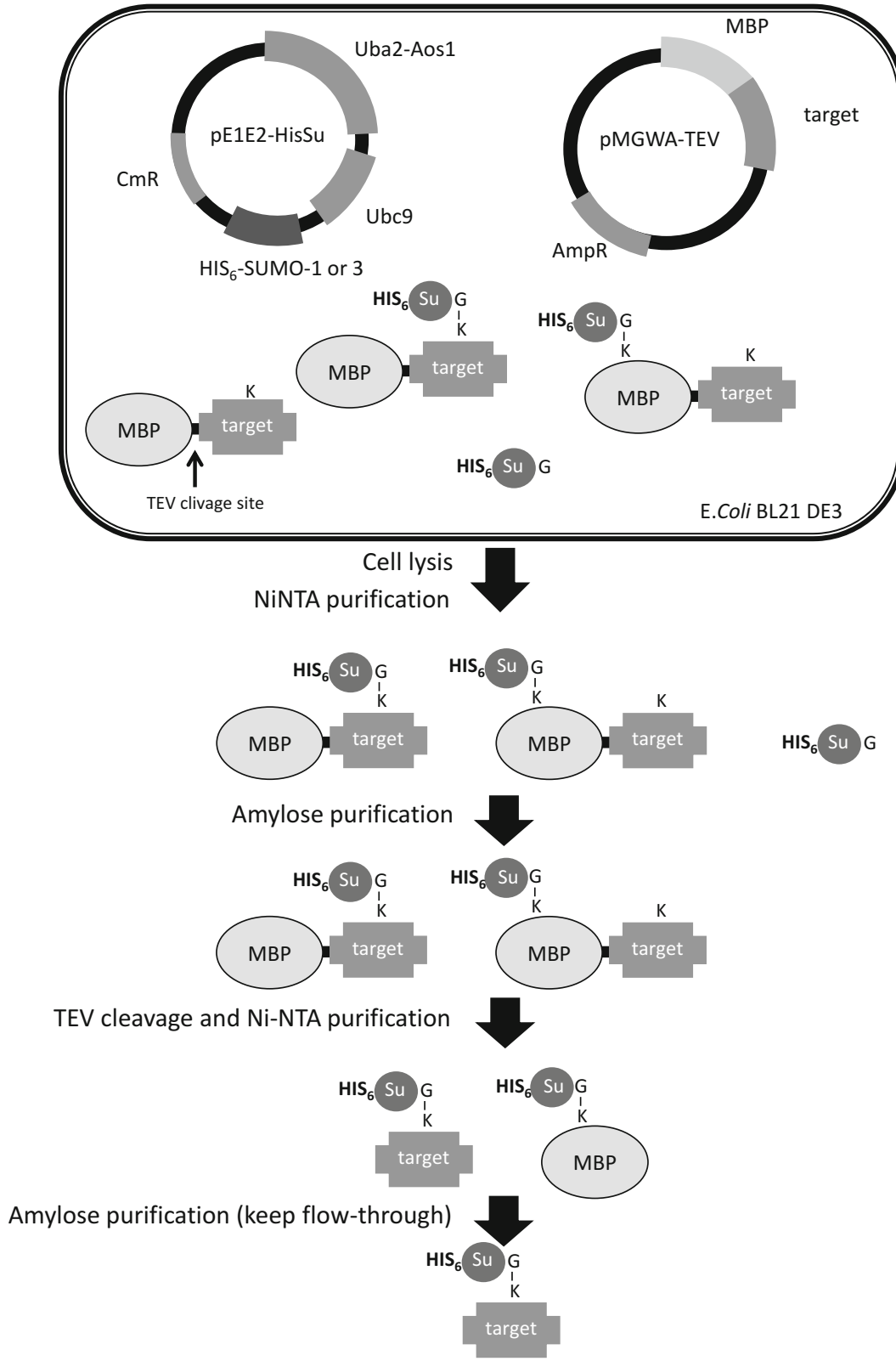

Fig. 1 Outline of the purification strategy. BL21 bacteria expressing the pE1E2-HisSU1 or pE1E2-HisSU3 together with the pMGWA-TEV-target plasmids are induced to produce the SUMOylated target protein. The bacterial lysate is then purified on Ni-NTA to isolate SUMOylated proteins and on amylose resin to isolate the

2 Materials

2.1 Expression Constructs

pTE1-E2-HisSU1 and *pTE1-E2-HisSU3*: These plasmids are derived from pTE1E2S1 and pTE1E2S3 [6] (*see* **Note 1**). A 6-histidine tag was inserted in the SUMO-1 or SUMO-3 sequence after their fifth amino acid (*see* **Note 2**) by insertion mutagenesis using the following oligonucleotides for SUMO-1 (5′-CATCACCATCACCATCACGCAAAACCTTCAACT-3′ and 5′-GTGATGGTGATGGTGATGCTCCTGCTGAGACAT-3′)and for SUMO-3 (5′-CATCACCATCACCATCACCCCAAGGAAG-GAGTC-3′ and 5′-GTGATGGTGATGGTGATGCTTTTCGT-CGGCCAT-3′).

pMGWA-TEV: This plasmid is derived from pMGWA [7], which is a Gateway-based destination vector allowing the generation of MBP-tagged proteins using the Gateway recombination technology (Life Technology). The GenBank accession number for pMGWA is EU680844.1. To allow for the cleavage of MBP from the protein of interest, we have inserted at TEV cleavage site between the MBP sequence and the attR1 recombination sequence by insertion mutagenesis using the following oligonucleotides (5′-GAAAATCTATACTTCCAGTCTACAAGTTTGTACAAAAA AGCTGAACGAGAAACG-3′ and 5′-AGACTGGAAGTATAGA-TTTTCGATGTAAGATCCGGTACCAGTCTGCGCGTC TTTCAG-3′). An Acc1 restriction site was inserted in the oligonucleotides to screen the positive clones.

pRK1043: This plasmid is available from Addgene plasmid 8835 (www.addgene.org/8835) and encodes the catalytic domain of tobacco etch virus (TEV) protease with an N-terminal MBP tag [8]. The TEV protein produced is an autolysis-resistant S219V mutant. It also carries a polyarginine tag at its C-terminus.

2.2 Components

1. Isopropyl β-D-1-thiogalactopyranoside (IPTG) is prepared at 1 M, pH 7.8 in H_2O, filtered (0.2 μm filters), and stored at −20 °C.
2. Chicken white egg lysozyme is prepared at 50 mg/mL in H_2O, aliquoted, and stored at −20 °C.
3. Imidazole is prepared at 2 M in H_2O and kept at room temperature in the dark.
4. NiNTA agarose beads.
5. Amylose resin.
6. SP-Sepharose fast.
7. Methyl-alpha-D-glucopyranoside (*see* **Note 3**).
8. BL-21 DE3 Gold competent bacteria (Stratagene).

2.3 Buffers

1. Resuspension buffer: 50 mM Tris–HCl pH 8.6, 500 mM NaCl, 50 mM $MgSO_4$.
2. Ni-NTA wash buffer: 50 mM Tris–HCl pH 8.6, 500 mM NaCl, 50 mM $MgSO_4$, 8 mM β-mercaptoethanol, 0.5% Triton X-100, 10 mM imidazole, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin.
3. Ni-NTA elution buffer: 50 mM Tris–HCl pH 8.6, 500 mM NaCl, 50 mM $MgSO_4$, 250 mM imidazole, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin.
4. Amylose wash buffer: 50 mM Tris–HCl pH 8.6, 150 mM NaCl, 50 mM $MgSO_4$, 8 mM β-mercaptoethanol, 0.5% Triton X-100, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin.
5. Amylose elution buffer: 50 mM Tris–HCl pH 8.6, 150 mM NaCl, 50 mM $MgSO_4$ 1 M methyl-alpha-D-glucopyranoside.
6. MBP-TEV resuspension buffer: 50 mM Hepes pH 7.5, 200 mM NaCl, 1 mM EDTA.
7. MBP-TEV wash buffer: 50 mM Hepes pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin.
8. MBP-TEV elution buffer: 50 mM Hepes pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 M methyl-alpha-D-glucopyranoside.
9. SP-Sepharose buffer: 50 mM Hepes pH 8.2, 5 mM DTT, 1 mM EDTA.

3 Methods

1. *Bacteria transformation*. BL21 competent bacteria are transformed with both pE1-E2-His-SU1 (or pE1-E2-His-SU3) and pMGWA-TEV-target. The transformed bacteria are plated on LB-agar plates with chloramphenicol and ampicillin (*see* **Note 4**). One colony is then picked up and grown overnight in 50 mL of LB supplemented with ampicillin and chloramphenicol.
2. *Bacteria induction*. The preculture is diluted in 1 L of LB with antibiotics (*see* **Note 5**) and grown with strong agitation (210 rpm) at 37 °C until the OD reaches 0.4–0.6 (*see* **Note 6**). Protein expression is induced by adding 1 mM IPTG for 6 h at 25 °C (*see* **Note 7**). The bacteria are harvested by centrifugation at 1800 × *g* for 20 min. The pellet is resuspended in 30 mL of resuspension buffer, frozen in liquid nitrogen, and stored at −80 °C (*see* **Note 8**).
3. *Nickel affinity chromatography purification*. All steps are performed at 4 °C with precooled buffers. The resuspended bacteria are thawed. Lysozyme (1 mg/mL), 8 mM

β-mercaptoethanol, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin are added and incubated for 1 h on ice before centrifugation at 100,000 × *g* for 1 h (4 °C). The supernatant is collected. 5 mL of Ni-NTA resin is then poured in a column (we use 20 mL columns) and equilibrated with 10 mL of Ni-NTA wash buffer. The supernatant is loaded on the column, and the flow through is collected and loaded a second time on the column. The column is washed three times with 10 mL of Ni-NTA wash buffer and eluted with 15 mL of Ni-NTA elution buffer. 2 mL Fractions are collected. 1 μL of each fraction is spotted on a nitrocellulose membrane and the membrane is stained with Ponceau Red to identify the positive fractions. All positive fractions are combined (*see* **Note 9**).

4. *Amylose affinity chromatography purification*. All steps are performed at 4 °C with precooled buffers. 0.5 mL of amylose resin is poured in a column (*see* **Note 10**). The column is equilibrated with 3 mL of amylose wash buffer. Positive fractions from the Ni-NTA purification are loaded on the column. The flow-through is collected and loaded again on the column. The column is washed two times with the amylose wash buffer and eluted with 3 mL of amylose elution buffer. A first 300 μL fraction is collected and then three to four 400 μL fractions (*see* **Note 11**). 1 μL of each fraction is spotted on a nitrocellulose membrane and the membrane is stained with Ponceau Red to identify the positive fractions. The SUMOylated protein should elute in fraction 2 and occasionally 3. Positive fractions are combined (*see* **Note 12**).
5. *TEV production and purification*. The pRK1043 plasmid is transformed in chemically competent BL21-Arg and an overnight preculture is done in LB + ampicillin + kanamycin. The preculture is diluted in 1 L of LB + ampicillin + kanamycin and grown at 37 °C under agitation (210 rpm) until it reaches an OD of 0.6. Protein expression is then induced with 1 mM IPTG for 4 h at 30 °C. After centrifugation, the bacterial pellet is resuspended in 30 mL of MBP-TEV resuspension buffer and frozen in liquid nitrogen. The pellet is then kept at −80 °C or purification is continued. After thawing, lysozyme (1 mg/mL), 8 mM β-mercaptoethanol, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin are added and the mixture is left on ice for 1 h and then centrifuged for 1 h at 100,000 × *g* (4 °C). The supernatant is then loaded on a column containing 5 mL amylose beads pre-equilibrated in the MBP-TEV wash buffer. The column is washed with 10 mL of the same buffer and eluted with 10 mL MBP-TEV elution buffer. 500 μL Fractions are collected. 1 μL of each fraction is spotted on a nitrocellulose membrane and stained with Ponceau Red to identify the positive fractions. All positive

fractions are combined, diluted ten times with the SP-Sepharose buffer and loaded on a column containing 5 mL SP-Sepharose beads equilibrated with the same buffer. The column is then eluted with 5 mL of SP-Sepharose buffer supplemented with increasing concentrations of NaCl (50, 100, 150, 200, 250 mM). Aliquots from each fraction are then analyzed by SDS-PAGE and staining of gels with Coomassie Brilliant Blue. The fraction containing the MBP-TEV protease, which should be the one eluted at 200 mM NaCl, is aliquoted, frozen in liquid nitrogen, and kept at −80 °C. The typical concentration is 1 mg/mL.

6. *Cleavage by TEV*. SUMOylated proteins purified in **step 4** (400 µL eluted fraction) are supplemented with 1 mM EDTA and 20 µg of MBP-TEV (*see* **Note 13**) and incubated at 4 °C overnight. Cleaved proteins are then bound to Ni-NTA agarose beads (250 µL) equilibrated in Ni-NTA wash buffer, washed with 3 mL of the same buffer, and eluted with 2 mL Ni-NTA elution buffer. A first 150 µL fraction is collected and then 400 µL fractions. The eluted SUMOylated proteins should be in the second fraction. Some SUMOylation can also occur on the MBP part of the fusion protein. This SUMOylated MBP can then be eliminated by loading the eluate on a mini-column containing 400 µL of amylose resin equilibrated in the resuspension buffer (*see* **Note 14**). The column is then centrifuged in an Eppendorf microtube to recover SUMOylated proteins.

3.1 Application of the Protocol to the Production of SUMOylated c-Jun

c-Jun is a transcription factor of the AP-1 family involved in the regulation of numerous cellular processes. c-Jun is SUMOylated mainly on K226 and, in the presence of SUMO E3, on K254. c-Jun SUMOylation limits its transcriptional activity, although the underlying mechanisms have not been defined yet [9, 10]. We have used the protocol described here to purify SUMOylated c-Jun. pMGWA-TEV-c-Jun (*see* **Note 15**) and the pTE1E2-HisSU1 or pTE1E2-His-SU3 were co-expressed in bacteria. SUMOylated proteins, including SUMOylated c-Jun, were then purified on nickel beads and on amylose beads. The cleavage with the TEV protease released SUMOylated c-Jun. Importantly, mutants of K226, the main SUMO acceptor site, prevented c-Jun SUMOylation and were not recovered in the purification procedure (Fig. 2). C-Jun being rather insoluble when produced in bacteria, the amount of SUMOylated c-Jun recovered was low, around 50 µg from 1 L of bacteria.

pE1E2-HisSU1 or pTE1E2-His-SU3 can be used in combination with tags other than MBP. However, we prefer MBP over GST, as we usually obtained higher yields of SUMOylation using MBP. This might be due to the fact that GST can form dimers, which could limit SUMOylation efficiency.

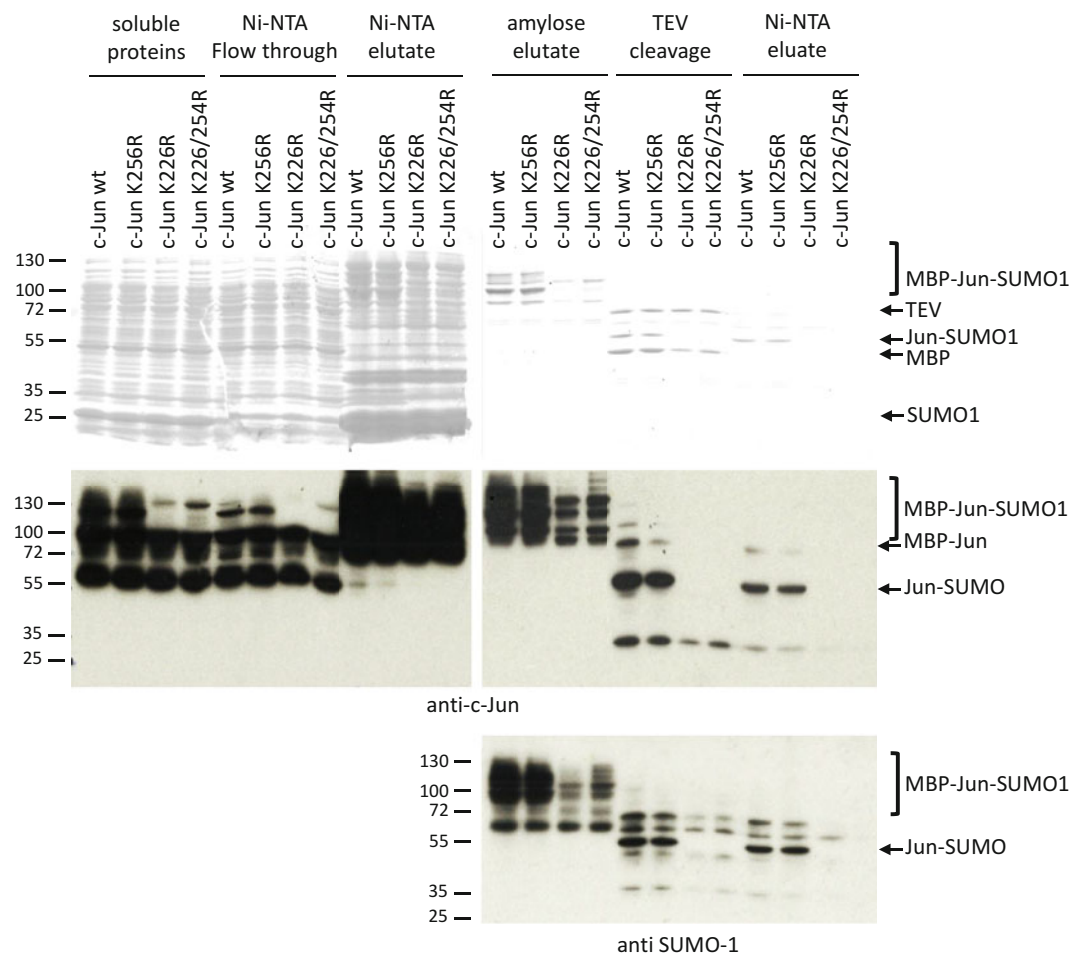

Fig. 2 Purification of SUMOylated c-Jun. MBP-tagged c-Jun (wild type, K226R, K254R, or K226/254R) was co-expressed with pTE1E2-HisSU1 and the purification procedure was followed as described in the protocol. An aliquot of the different steps of the purification was run on SDS-PAGE. The membranes were colored with Amidoblack (*top panel*) and immunoblotted with c-Jun (*middle panel*) or SUMO-1 (*lower panel*) antibodies

Two similar systems using SUMOylation in bacteria with His-tagged SUMO have been described recently. In particular, these approaches were used to purify recombinant SUMOylated IkB-α [11], TDG, and XRCC1 [12]. Our detailed protocol can be applied using the vectors described in both of these publications. In all cases, it was proven that these systems are highly efficient for producing large amounts of recombinant SUMOylated proteins and that SUMOylation occurs on the right lysines on the target proteins. These proteins can then be used for multiple purposes aiming at characterizing the role of their SUMOylation. In particular, they can be used to identify specific interactors of the SUMOylated/non-SUMOylated forms in pull-down experiments using cell extracts followed by mass spectrometry analysis of pulled

down proteins. The identification of such interactors is critical to decipher the molecular consequences of specific protein SUMOylation.

4 Notes

1. In its initial description, pTE1E2S3, which contains SUMO-3 (Smt3B), was named pTE1E2S2 and described as expressing SUMO-2 [6]. Like many colleagues in the SUMO field, we follow the nomenclature proposed by Hinchey and Saitoh [13]. Their assignment was consistent with the original description of mammalian SUMO genes. According to this, mature SUMO-2 (Smt3A) is 92 amino acid long and mature SUMO3 (Smt3B) consists of 93 amino acids.
2. The His_6 tag could not be inserted at the N-terminus because the sequence present before the ATG is repeated three times in the vector. However, the N-terminal part of SUMO-1 and SUMO-3 being unstructured, we assume that the insertion of the His_6 tag inside the sequence does not affect the properties of SUMO or their conjugation.
3. In most protocols describing MBP-tagged protein purification, maltose is used for elution from amylose beads. However, maltose binds irreversibly to MBP, which prevents its further binding to amylose (*see* **step 6** of the purification procedure).
4. Once we have transformed the pE1E2-HisSU1 or pE1E2-HisSU3, we make chemically competent bacteria. These bacteria can then directly be transformed with the vector encoding the protein of interest.
5. The protocol is described for 1 L of bacteria, for a protein that is not highly soluble and the SUMOylation rate of which is around 10%. The quantities of bacteria should therefore be adapted to each protein. The rate of SUMOylation and the solubility of the protein should be tested on a small scale (50 mL) by performing **steps 1**, **2**, and **3** (stopping after the ultracentrifugation). Samples from bacteria, before and after induction, after lysis and after ultracentrifugation, are then fractionated by SDS-PAGE and, then, immunoblotted with antibodies specific of proteins of interest.
6. The bacteria should be in exponential growing phase. If the OD reaches more than 0.6, dilute the cells 3–4 times so that the OD is below 0.2 and wait that it reaches 0.4.
7. The temperature and the time of induction should be adapted to each protein. For proteins that are highly soluble, induction can be performed at 37 °C for 4–5 h. For proteins with low solubility, the temperature should be decreased to 25 °C or even 16 °C but the time of induction should be increased.

8. The protocol can be continued directly without storing the lysate at −80 °C. However, the liquid nitrogen freezing step is required for efficient lysis of the bacteria.
9. It is best to continue the protocol after this step. However, it is possible to keep the eluates at 4 °C and proceed on the following day.
10. If the protein is highly soluble and the yield of SUMOylation is good, use more beads.
11. Decrease the amount of amylose beads and the elution volumes if the SUMOylated form is in low abundance to avoid its dilution.
12. The protocol can be stopped here and MBP-tagged SUMOylated proteins used for further experiments. However, it should be noted that some SUMOylation can occur on the MBP tag, which will remain if the tag is not cleaved.
13. The cleavage efficiency has to be tested for each preparation of MBP-TEV. This can be done on the MBP-tagged target used for the SUMOylation and purified from bacteria using the protocol described here, without the Ni-NTA purification (**steps 1**, **2**, **4**).
14. Centrifuge the column in an Eppendorf microtube before loading the SUMOylated protein to remove the liquid.
15. This construct was obtained through Gateway recombination of c-Jun c-DNA in the pMGWA vector. The TEV cleavage site was added upstream of c-Jun c-DNA by insertion mutagenesis on recombined pMGWA-c-Jun and not by direct recombination in the pMGWA-TEV vector, as described above because this vector was not yet available.

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Chapter 5

A Fluorescent In Vitro Assay to Investigate Paralog-Specific SUMO Conjugation

Nathalie Eisenhardt, Viduth K. Chaugule, and Andrea Pichler

Abstract

Protein modification with the small ubiquitin-related modifier SUMO is a potent regulatory mechanism implicated in a variety of biological pathways. In vitro sumoylation reactions have emerged as a versatile tool to identify and characterize novel SUMO enzymes as well as their substrates. Here, we present detailed protocols for the purification and fluorescent labeling of mammalian SUMO paralogs for their application in sumoylation assays. These assays provide a fast readout for in vitro SUMO chain formation activity of E3 ligases in a paralog-specific manner. Finally, we critically analyze the application of fluorescent SUMO proteins to study substrate modification in vitro revealing also the drawbacks of the system.

Key words SUMO, SUMO chains, E3 ligase, RanBP2, PIAS1, ZNF451, SUMO protease, In vitro sumoylation, Sp100

1 Introduction

SUMOylation is a reversible posttranslational modification, which is essential in most organisms, and results in the covalent linkage of the small ubiquitin-related modifier (SUMO) protein to a lysine residue within the target protein. The covalent attachment of SUMO to its target protein (sumoylation) is mediated by a hierarchical enzymatic cascade [1–3]. The heterodimeric E1 enzyme Aos1/Uba2 activates SUMO in an ATP-consuming step. Next, SUMO is transferred to the sole E2-conjugating enzyme Ubc9, which is able to directly transfer SUMO to an acceptor lysine of a substrate. However, such E3-independent SUMO conjugation is usually inefficient but often occurs in the presence of high E2 enzyme concentrations in vitro. Typically, SUMO E3 ligases catalyze the transfer of the modifier from the E2 to the substrate at substoichiometric concentrations and are thought to provide substrate specificity to the system [1, 2, 4, 5]. To reverse the modification, SUMO proteases remove the modifier from the substrate [6–8].

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Mammalian cells express four different SUMO paralogs: SUMO 1–4. SUMO 2 and 3 are almost identical sharing 97% sequence identity and differ only in three amino acids. Therefore, they cannot be distinguished from each other by specific antibodies. SUMO1 shares approximately 50% sequence identity with SUMO 2/3. Biological differences between the different SUMO paralogs are starting to emerge and surprisingly only SUMO3 (following the nomenclature introduced by Saitoh and Hinchey [9]) is essential for viability in mice [10]. In contrast to SUMO1, SUMO2 and SUMO3 contain a SUMO consensus motif (SCM: ΨKxD/E, where Ψ is a large hydrophobic amino acid and x any amino acid) within their flexible N-termini, which is important for SUMO chain formation [11, 12]. However, albeit less frequently, other lysines in SUMO 2/3 can be SUMO-conjugated as well and also SUMO1 can efficiently form SUMO chains [5, 13–15]. On the enzyme level, proteases and E3 ligases display SUMO paralog-specific preferences [7, 8, 16–18].

Here, we describe an in vitro sumoylation assay using fluorescently labeled SUMO paralogs. Due to the fluorescent readout, no immunoblots are required and enzymatic activities can be directly compared between the individual SUMO paralogs. Such direct fluorescence gel scans do not only save time but also entail the advantage of circumventing inefficient transfer of high-molecular-weight SUMO conjugates. Thus, the described assay provides an efficient readout to analyze the enzymatic activity of recombinant E3 ligases in SUMO chain assembly in a paralog-specific manner. Moreover, the assay can be used with all kinds of functional SUMO mutants in order to determine the sequence requirements in SUMO for chain formation, paralog specificity, and enzymatic activity or in other mechanistic studies [18]. Another likely approach of the assay is the reversed application to analyze SUMO proteases for their enzymatic activity on SUMO-modified substrates or preformed SUMO chains to investigate protease activity and paralog specificity. By contrast, it is unfavorable to use the fluorescent readout to study substrate modifications due to high chain formation and automodification activities of E3 ligases, which take place in the background. Exceptions of this are substrates, which are efficiently modified at low enzyme concentrations, like RanGAP1 [5, 19, 20]. In addition, the fluorescently labeled SUMO species are less efficiently conjugated to substrates than the unlabeled equivalent SUMO paralogs in vitro. To investigate SUMO protease activities on specific substrates, this problem can be overcome by purification of the sumoylated substrate species. Of note, SUMO labeling might influence protease activity [21].

Altogether, our fluorescent in vitro assay provides a fast and helpful tool to investigate SUMO enzyme activities and the mechanism of SUMO chain formation in many directions of interest.

2 Materials

2.1 Purification of SUMO Species

1. Competent *E. coli* cells, e.g., strain Bl21(DE3).
2. SUMO1, SUMO2, or SUMO3 cloned into pET11a expression vector [5, 22].
3. LB medium (self-made or custom).
4. Ampicillin: 100 mg/ml in H_2O.
5. IPTG: 1 M in H_2O.
6. Resuspension buffer: 50 mM Tris–HCl pH 8.8, 25 mM NaCl, 1 mM DTT.
7. Protease inhibitor cocktail: 1 mg/ml Leupeptin + 1 mg/ml pepstatin in DMSO, 1 mg/ml aprotinin in 20 mM Hepes pH 7.4, and 100 mM PMSF in ethanol.
8. DNaseI: 5 mg/ml in 20 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$, 50% glycerol.
9. DTT: 1 M in H_2O.
10. Q Sepharose Fast Flow agarose beads (GE Healthcare).
11. Liquid chromatographic glass column, e.g., from Sigma.
12. Q Sepharose wash buffer: 50 mM Tris–HCl pH 8.8, 50 mM NaCl, 1 mM DTT.
13. Elution buffer: 50 mM Tris–HCl pH 7.5, 500 mM NaCl, 1 mM DTT.
14. Centrifugal concentrator, e.g., Vivaspin 2 (MWCO: 5 kDa).
15. Sephadex 200 16/60 preparative column (GE Healthcare).
16. Gel filtration buffer: 1× Transport buffer (TB: 20 mM Hepes pH 7.3, 110 mM KOAc, 2 mM $Mg(OAc)_2$), 150 mM NaCl, 1 mM DTT.
17. Mono Q buffer: 50 mM Tris–HCl pH 10, 25 mM NaCl, 1 mM DTT.
18. Desalting column, e.g., PD midiTrap™ G-25 (GE Healthcare).
19. Mono Q 5/50 GL column (GE Healthcare).
20. Mono Q elution buffer: 50 mM Tris–HCl pH 10, 500 mM NaCl, 1 mM DTT.
21. Standard SDS-PAGE equipment.
22. Protein concentration determination reagent, e.g., BCA assay.

2.2 Labeling of SUMO Proteins

1. DTT: 1 M in H_2O.
2. Desalting column, e.g., PD midiTrap™ G-25 (GE Healthcare).
3. Phosphate buffer: 100 mM Phosphate buffer pH 7.2, 150 mM NaCl, 1 mM EDTA.

4. Fluorescent dye, e.g., DyLight 800 Maleimide (Thermo Scientific).
5. Dimethylformamide (DMF).
6. SUMO storage buffer: 1× TB (*see* Subheading 2.1), 150 mM NaCl, 1 mM TCEP, and 0.05% NaN_3.
7. Slide-A-Lyzer MINI Dialysis Device (MWCO: 7 kDa, Thermo Scientific).
8. Standard SDS-PAGE equipment.
9. Odyssey Infrared Imaging System (Li-Cor).

2.3 In Vitro SUMO Chain Formation Assay

1. Recombinant E1, E2, and E3 enzymes [5, 18, 22].
2. Fluorescent SUMO (*see* Subheadings 2.2 and 3.2).
3. ATP: 100 mM in 20 mM Hepes pH 7.4, 100 mM $Mg(OAc)_2$.
4. SUMO assay buffer (SAB): 1× TB (*see* Subheading 2.1), 0.2 mg/ml ovalbumin, 0,05% Tween (v/v), 0.5 mM TCEP.
5. LDS loading buffer: 3× NuPAGE LDS sample buffer (Thermo Scientific), 75 mM EDTA, 50 mM TCEP.
6. 9.5% SDS Tris-glycine gel with pH 9.2 resolving gel.
7. Standard SDS-PAGE equipment.
8. Odyssey Infrared Imaging System (Li-Cor).

2.4 In Vitro Substrate Modification Using Fluorescent SUMO

1. Recombinant E1, E2, and E3 enzymes (e.g., RanBP2 IR1 + M, RanBP2ΔFG, MBP-PIAS1, MBP-ZNF-N) and recombinant substrate (e.g., GST-Sp100) [18, 22–24].
2. Fluorescent and unlabeled SUMO (*see* Subheadings 2.2 and 3.2) [22].
3. ATP: 100 mM in 20 mM Hepes pH 7.4, 100 mM $Mg(OAc)_2$.
4. SAB: *See* Subheading 2.3.
5. LDS loading buffer: *See* Subheading 2.3.
6. 7% SDS Tris-glycine gels (or other suitable percentage depending on the substrate to be tested).
7. Standard SDS-PAGE equipment.
8. Odyssey Infrared Imaging system (Li-Cor).
9. Nitrocellulose membrane.
10. Standard western blot transfer equipment and antibodies.

3 Methods

3.1 Purification of SUMO Species

Fluorescent labeling of SUMO species requires proteins of high purity to reduce fluorescent background. Therefore, in comparison to conventional SUMO purification protocols an additional ion-exchange chromatography step is included (Fig. 1).

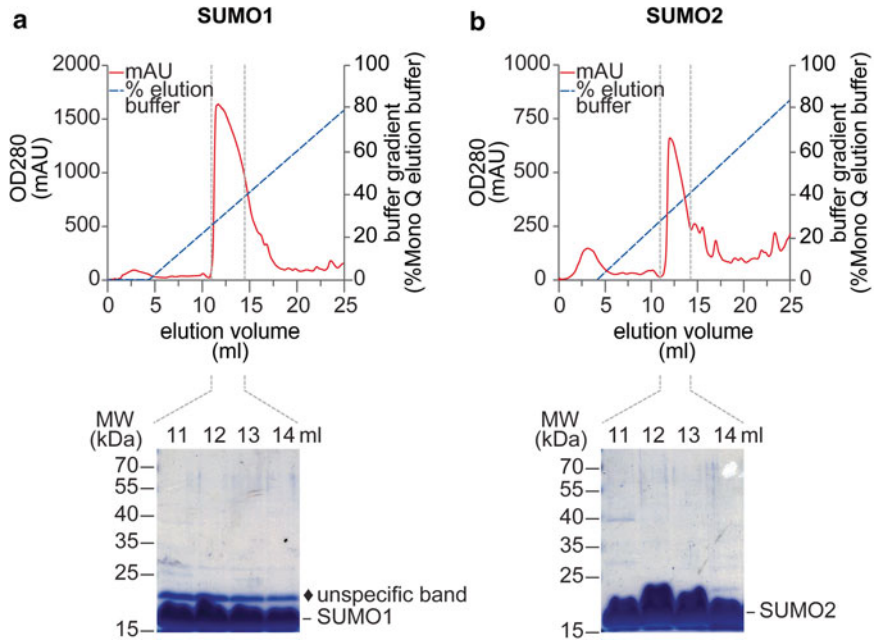

Fig. 1 Purification of highly pure SUMO1 and SUMO2. Elution profile of SUMO1 (**a**) and SUMO2 (**b**) proteins that were subjected to an additional anion-exchange chromatography step and eluted by a continuous salt gradient (*upper panel*). Highly pure SUMO species elute around 200 mM of NaCl, which corresponds to approximately 25–50 % elution buffer. Coomassie gels of eluted SUMO species (*lower panel*) were analyzed by SDS-PAGE using a 9.5 % SDS gel pH 9.2 followed by staining with Coomassie. *Black diamond* marks unspecific band

1. Transform *E. coli* BL21 (DE3) cells with a pET11a SUMO expression vector and set up a primary culture in LB media containing 100 μg/ml ampicillin. Allow the culture to grow overnight at 37 °C.
2. Dilute primary culture 50× into a 2 × 1 l expression culture in LB media containing 100 μg/ml ampicillin and grow at 37 °C until $OD_{600} = 0.6$. Induce protein expression with 1 mM IPTG and maintain for 5–6 h at 37 °C.
3. Harvest cells by centrifugation at 4 °C and 4000 × *g* and resuspend each liter of cell pellet in 10–15 ml resuspension buffer. Add protease inhibitors (e.g., a mixture of 1 μg/ml leupeptin, pepstatin, aprotinin, and 100 μM PMSF).
4. Lyse bacteria by freeze-thawing and brief sonication in an ice bath (three cycles with 10 s on and 15 s off at 80 μm amplitude, *see* **Notes 1** and **2**).
5. Add 5 μg/ml of DNaseI, fresh protease inhibitors, and 1 mM DTT and clarify cell lysates by centrifugation at 19,000 × *g* for 30 min at 4 °C.
6. Equilibrate 4 ml of Q Sepharose beads in a glass column with resuspension buffer.

7. Passage the clarified supernatant through the Q Sepharose column followed by a wash with 10 column volumes (cvs) of Q Sepharose wash buffer. Repeat bind/wash cycle 3–4 times.
8. Elute bound protein with 8 × 1 cv elution buffer.
9. Concentrate eluate to 5 ml (*see* **Note 3**) using a centrifugal concentrator (MWCO: 5 kDa).
10. Purify concentrated eluate over a high-grade Sephadex 200 16/60 preparative column pre-equilibrated with gel filtration buffer. Collect 2.5 ml fractions and analyze by SDS-PAGE.
11. Pool SUMO-containing fractions and concentrate the protein using a centrifugal concentrator.
12. Buffer exchange the protein into MonoQ buffer using a desalting column.
13. Apply sample to a MonoQ 5/50 GL column pre-equilibrated with MonoQ buffer (*see* **Note 4**).
14. Wash MonoQ column with 10 cvs MonoQ buffer to remove unbound protein.
15. Elute SUMO with a continuous salt gradient from 25 to 500 mM NaCl over 25 cvs by using MonoQ buffer (25 mM NaCl) and MonoQ elution buffer (500 mM NaCl, Fig. 1 upper panel, *see* **Note 5**).
16. Analyze fractions by SDS-PAGE (Fig. 1 lower panel) and pool pure SUMO fractions.
17. Determine protein concentration (*see* **Note 6**) and continue with labeling (Subheading 3.2, *see* **Note 7**).

3.2 Labeling of SUMO Proteins

All mammalian SUMO paralogs contain a single internal cysteine residue (Cys52 in SUMO1, Cys 47 in SUMO2 and Cys 48 in SUMO3) in helix 1 of the conserved β-grasp fold. This cysteine can be targeted for labeling resulting in a single, site-specific incorporation of a fluorescent dye (Fig. 2).

1. Reduce SUMO protein at a concentration of at least 150 μM with 5 mM DTT for 30 min at 37 °C.
2. Buffer exchange the sample into phosphate buffer using a desalting column and adjust the final volume to 1 ml.
3. Dissolve DyLight 800 Maleimide (Thermo Scientific) in dimethylformamide (DMF) and immediately add it to the protein sample at 10- to 12-fold molar excess. Maintain the labeling reaction for 3–4 h at 20 °C in the dark.
4. Quench the reaction with 3 mM DTT at 37 °C for 5–10 min.
5. Dialyze the sample into SUMO storage buffer using a Slide-A-Lyzer MINI Dialysis Device (MWCO: 7 kDa, *see* **Note 8**).

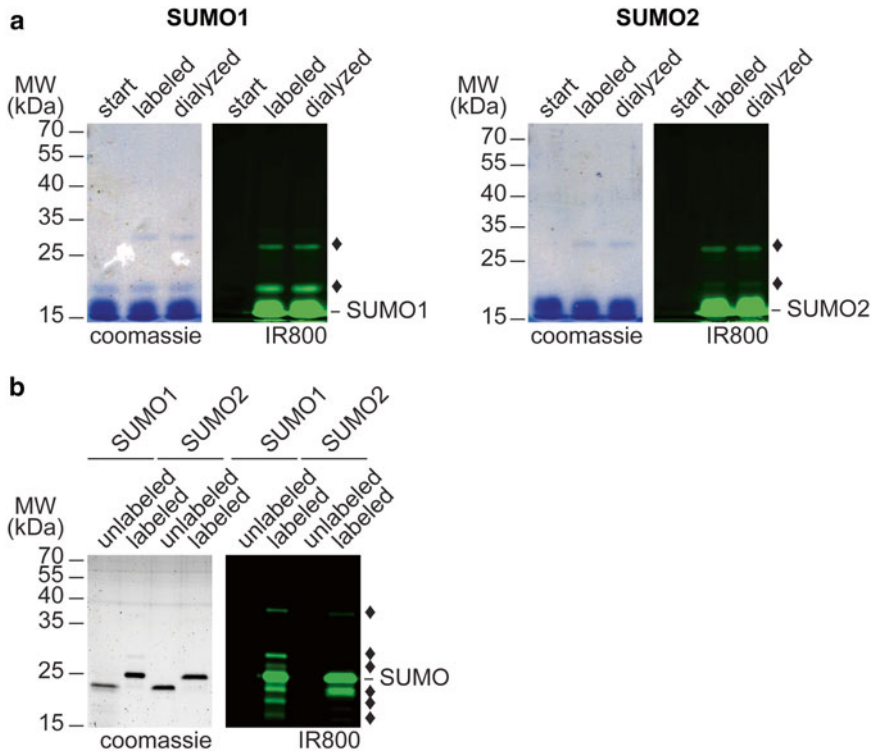

Fig. 2 Fluorescent labeling of SUMOs. Coomassie gels and direct fluorescence scans of SUMO proteins labeled with DyLight 800 Maleimide, finally dialyzed to remove excess of free dye and subsequently separated by SDS-PAGE using a 9.5 % SDS gel pH 9.2 (**a**). Coomassie gel and direct fluorescence scan of a comparison of labeled and unlabeled SUMO1 and SUMO2 on the same 13.5 % SDS gel pH 8.8 show a similar labeling efficiency when done in parallel but different unspecific by-products (*black diamond*, **b**)

6. Analyze the labeling by SDS-PAGE (Fig. 2) and fluorescence scanning (*see* **Note 9**).
7. Determine the protein concentration (*see* **Note 6**).
8. Aliquot the sample, flash freeze in liquid nitrogen and store at −20 °C (*see* **Note 10**).

3.3 Paralog-Specific In Vitro SUMO Chain Formation Assay

SUMO E3 ligases efficiently catalyze the transfer of the donor SUMO molecule on Ubc9 to a substrate at substoichiometric enzyme concentrations. In vitro, most E3 ligases readily form polymeric SUMO chains. Such SUMO chain formation assays allow insights into the trans conjugation activity of E3 ligases in a paralog-specific manner (Fig. 3, *see* **Note 11**).

1. Mix 60 nM E1, 100 nM Ubc9, a dilution series of different concentrations of the E3 enzyme, and 2 μM of fluorescent SUMO (*see* Subheading 3.2) and adjust with SAB buffer to a total volume of 19 μl. Add 1 μl of ATP to reach a final concentration of 5 mM to start the reaction (*see* **Note 12**).

2. Incubate at 30 °C for 60 min.
3. Stop the reaction by adding 10 µl LDS loading buffer and immediate heat denaturation for 5 min at 95 °C.
4. Run 6 µl of the sample on a 9.5% SDS gel pH 9.2 (*see* **Note 13**).
5. Rinse the gel briefly for 3 × 2 min in H_2O.
6. Scan the gel with an appropriate fluorescence scanner (e.g., Odyssey infrared imaging system, Fig. 3).

3.4 In Vitro Substrate Modification Using Fluorescent SUMO

Direct comparison of fluorescently labeled and unlabeled SUMO species demonstrates that labeling impairs conjugation efficiency (Fig. 4, *see* **Note 14**).

1. Mix 60 nM E1, different concentrations of the E2 enzyme (50, 250 nM), 200 nM of the substrate (GST-Sp100), and 2 µM of fluorescent SUMO (*see* Subheading 3.2) and adjust the total volume with SAB buffer to 19 µl. Add 1 µl of ATP to reach a final concentration of 5 mM to start the reaction (*see* **Note 12**).

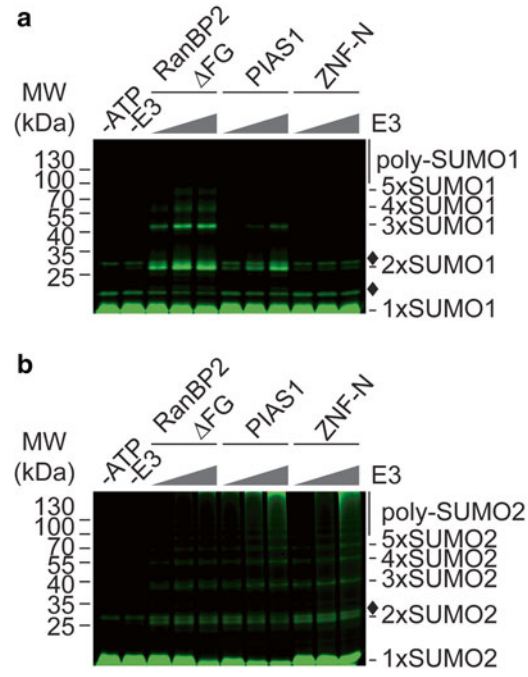

Fig. 3 In vitro SUMO chain formation assay using fluorescent SUMO paralogs. Direct fluorescence gel scans of in vitro sumoylation reactions using 60 nM E1, 100 nM E2, and 2 µM Dylight 800-labeled SUMO1 (**a**) or SUMO2 (**b**) and increasing concentrations of RanBP2ΔFG (5, 20, 80 nM), MBP-PIAS1 (10, 40, 160 nM), or MBP-ZNF-N (aa 2-246, 15, 60, 240 nM) or without an E3 ligase for 60 min at 30 °C in the presence or absence of 5 mM ATP. *Black diamond* marks unspecific bands

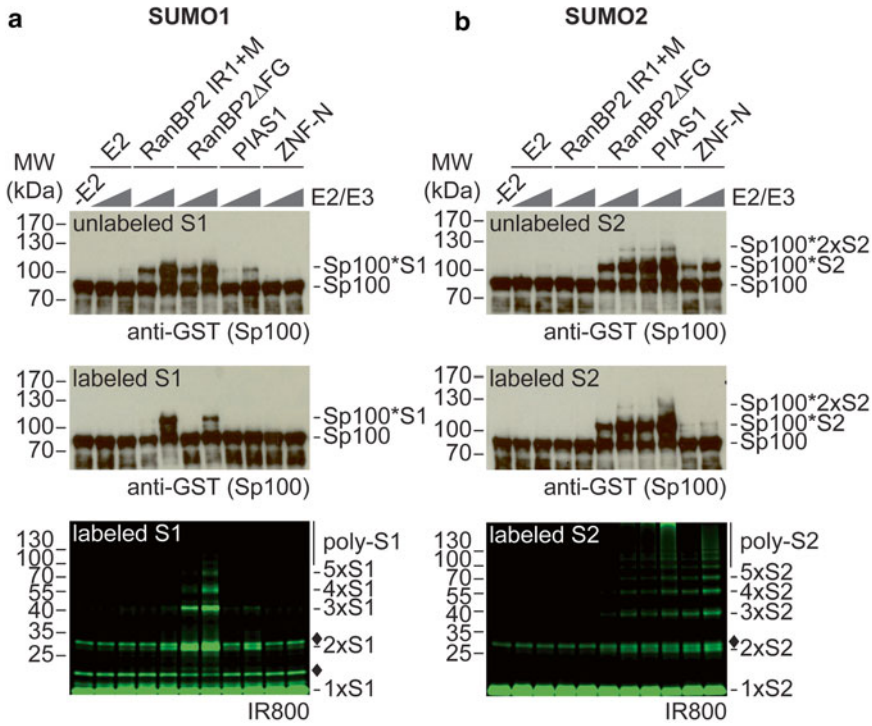

Fig. 4 SP100 modification using unlabeled or fluorescently labeled SUMO paralogs. Immunoblots and direct fluorescence gel scans of in vitro sumoylation reactions using 60 nM E1, 200 nM GST-Sp100, and 2 μM Dylight 800-labeled SUMO1 (**a**) or SUMO2 (**b**). E3-independent reactions were performed with increasing concentrations of E2 (0, 50, and 250 nM) and E3-dependent reactions with 50 nM E2 and increasing concentrations of RanBP2 IR1 + M (4 and 20 nM), RanBP2ΔFG (2 and 10 nM), MBP-PIAS1 (10 and 50 nM), or MBP-ZNF-N (40 and 200 nM) for 30 min at 30 °C in the presence of 5 mM ATP. Sp100 was detected by immunoblotting using GST antibodies. *Black diamond* marks unspecific bands

For E3-dependent reactions use 50 nM Ubc9 and different concentrations of the E3s: RanBP2 IR1 + M at 4 and 20 nM, RanBP2ΔFG at 2 and 10 nM, MBP-PIAS1 at 10 and 50 nM, or MBP-ZNF-N at 40 and 200 nM (*see* **Note 15**).

2. Incubate at 30 °C for 30 min.
3. Stop the reaction by adding 10 μl LDS loading buffer and immediate heat denaturation for 5 min at 95 °C.
4. Run 10 μl of the sample on a 9.5% SDS gel pH 9.2 (*see* **Note 13**).
5. Rinse the gel briefly for 3 × 2 min in H_2O.
6. Scan the gel by an appropriate fluorescence scanner for fluorescent detection (e.g., Odyssey infrared imaging system, Fig. 4).
7. Transfer 10 μl of the samples after separation with a 7% SDS Tris-glycine gel onto nitrocellulose membranes by standard semidry protein transfer.
8. Detect the substrate and the modification by immunoblotting using substrate-specific antibodies.

4 Notes

1. Resuspended cells can be flash frozen in liquid nitrogen and stored at −80 °C. After thawing, fresh protease inhibitors have to be added.
2. Other methods to lyse the bacteria can be employed, like French press.
3. Spot 2 µl of eluted fractions on nitrocellulose membrane and stain with Ponceau to check for protein content; pool only protein-containing fractions.
4. For fluorescent labeling of SUMO species material of high purity is required. Thus, the SUMO proteins are subjected to an additional anion-exchange chromatography step.
5. SUMO paralogs usually elute between 25 and 50% of MonoQ elution buffer (around average salt concentration of 200 mM) which approximately corresponds to the peak between 11 and 15 ml in the elution profiles. Higher salt concentrations result in another peak, which can be discarded.
6. Protein concentration can be determined by BCA, Bradford, Lowry assay, or any other suitable technique if the sample is pure. We usually determine the protein concentration by comparison to a known standard protein of similar size (e.g., lact-albumin) in Coomassie-stained SDS-PAGE gels.
7. Avoid freeze-thawing of SUMO proteins intended for labeling. Instead, try to proceed immediately and store these samples on ice if necessary. Aliquot residual SUMO protein into small aliquots, flash freeze in liquid nitrogen, and store at −80 °C.
8. Dialysis not only exchanges the SUMO protein to TB buffer, which is the basis for future sumoylation reactions, but also removes the free unconjugated dye.
9. For comparison of SUMO paralogs, labeling reactions should be carried out in parallel to obtain comparable labeling efficiencies.
10. Once thawed, avoid freezing of the labeled protein and store at 4 °C instead. The labeled protein is stable for a couple of weeks.
11. Another reverted application of this assay can be used to study SUMO protease activity on preformed fluorescent SUMO chains as start material. Of note, SUMO labeling might influence protease activity [21].
12. Typically, SUMO chain formation assays are done in 20 µl total volume but can be upscaled if necessary. Always dilute protein stocks in an appropriate volume using SAB. Always perform control reactions without ATP or E2 and without E3 ligases.
13. In order to separate an unspecific fluorescent band from diSUMO conjugates, Tris-glycine SDS gels with pH 9.2 of the resolving gel have to be prepared. These gels run similar to

12–12.5 % Tris-glycine gels pH 8.8. In order to observe mono-SUMO, gels have to be stopped immediately once the loading dye reaches the bottom; otherwise it will be run out.

14. Although substrate modification with labeled SUMO is less efficient it can be used as start material to study SUMO protease activity in a similar but reverted assay. In order to reduce background, SUMO-modified substrates have to be purified before protease analysis. Of note, SUMO labeling might influence protease activity [21].
15. We prefer using dilution series of enzymes over single concentrations as this more reliably illustrates E3-specific substrate modifications.

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Chapter 6

Identification and Characterization of SUMO-SIM Interactions

Koraljka Husnjak, Jan Keiten-Schmitz, and Stefan Müller

Abstract

The covalent attachment of SUMO to lysine residues of cellular proteins serves as an important mechanism for the dynamic control of protein networks. SUMO conjugates typically mediate selected protein-protein interactions by binding to specific recognition modules. Identification of SUMO-binding proteins and the characterization of the binding motifs are key to understanding SUMO signaling. Here we describe two complementary approaches that are used to tackle these questions.

Key words SUMO, SUMO-interacting motif, Yeast two-hybrid, Protein interaction

1 Introduction

Posttranslational modifications (PTMs) of distinct amino acids with small chemical groups, such as phosphate, methyl, or acetyl moieties, provide a versatile way to regulate the dynamics of protein-protein interactions. As a general principle the modified residues provide docking sites for specific binding modules of an interaction partner [1]. The conjugation of entire polypeptides, like ubiquitin, to lysine (K) residues of proteins expands the repertoire of PTMs. Ubiquitin can be attached as a monomer or as a lysine-linked or linear (head-to-tail) polymeric chain. This generates structurally distinct interaction surfaces that are recognized by specific binding modules, thus allowing a highly diversified control of protein interactions [2, 3]. Similarly, conjugation of the ubiquitin-related SUMO modifier to target proteins provides a platform for protein-protein interactions and has a central role in a variety of cellular signaling pathways [4, 5]. SUMO conjugation typically coordinates protein interaction networks and is particularly important for the ordered assembly of multiprotein complexes[6, 7]. In humans, three SUMO forms (SUMO1, SUMO2, and SUMO3) can be attached to lysine residues of

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target proteins, whereas in lower eukaryotes, such as *Saccharomyces cerevisiae* or *Drosophila melanogaster*, only one SUMO form (also termed Smt3) is found. All SUMO/Smt3 forms are translated as precursor proteins that have to be carboxy-terminally processed to liberate a double glycine (GG)-motif, which is essential for conjugation. Like ubiquitin, SUMO2/3 (which in humans only differ in two amino acids and therefore are treated here as a single entity) can form polymeric chains, preferentially via K11 [8]. Modification of proteins by SUMO typically earmarks them for recognition by specific SUMO-binding or SUMO-interacting motifs termed SBMs or SIMs [2, 4, 5, 9]. PolySUMOylation recruits distinct interaction partners, such as E3 ubiquitin ligases RFN4 or RNF111, that bind to polySUMO chains through tandem SIMs [10, 11]. In contrast to the ubiquitin system, where more than 20 autonomously folded recognition domains have been defined, only a very limited set of SUMO-binding modules has been described. The best characterized canonical SBMs/SIMs are linear motifs of around 10–15 amino acids, which are all characterized by a stretch of hydrophobic residues with a loosely conserved consensus sequence ([V/I]-x-[V/I]-[V/I] or [V/I]-[V/I]-x-[V/I]). Based on this consensus motif several subtypes have been defined [12]. Two subtypes of SIMs, termed SIMa and SIMr, are defined by acidic amino acids in an amino-terminal (SIMa) or carboxy-terminal (SIMr) position to the hydrophobic core of SIMs. The third SIM subtype, which resembles SIMa, but is slightly shorter, was termed SIMb. In a subgroup of SIMs, defined as phosphoSIMs, phosphoserine/phosphothreonine residues are found adjacent to the acidic stretches [13–15]. The available crystal structures of distinct SUMO-SIM and SUMO-phosphoSIM complexes illustrate how these combinatorial sequence features contribute to binding [16–21]. As a typical example the interaction of the phosphoSIM region of PML with SUMO1 is shown in Fig. 1a, b [22]. SIMs typically form a β-strand aligning in parallel (SIMa and SIMb) or antiparallel orientation (SIMr) to the β2 strand of SUMO. The hydrophobic SIM residues contact amino acids in a hydrophobic pocket of SUMO formed between strand β2 and helix α1. Critical hydrophobic residues in SUMO1 are H35, F36, and V38 (corresponding to Q35, F36, and I38 in SUMO2). Accordingly, canonical SIM interactions are disrupted by alanine substitutions of these residues [23]. In addition to hydrophobic forces, electrostatic interactions are in many cases involved in the formation of SUMO-SIM interactions. Negative charges in SIM regions that are provided by aspartic/glutamic acid or phosphoserine/phosphothreonine residues contact a positively charged basic interface on SUMO comprised of K37, K39, and K46 in SUMO1 or the corresponding K33, K35, and K42 in SUMO2 [13–15, 22].

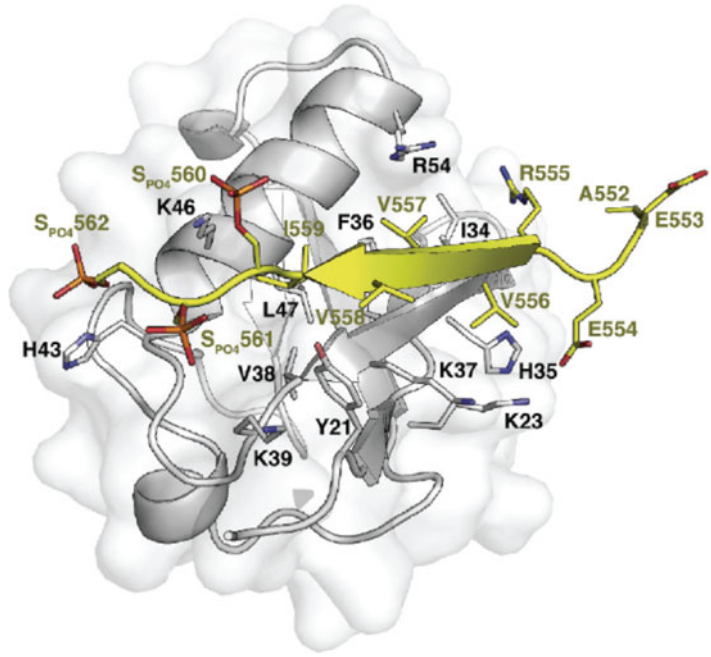

Fig. 1 Structure of a SUMO-SIM interaction as exemplified for binding of SUMO1 to the phosphoSIM of PML. An N-terminally truncated form of SUMO1 encompassing residues 17–97 was crystallized in complex with a phosphoSIM peptide of PML. The phosphoSIM peptide (*yellow*) adopts a β-strand conformation aligning in parallel to the β2 strand of SUMO. The hydrophobic SIM residues contact amino acids in the hydrophobic pocket of SUMO, including F36 and V38. In addition, phosphoserine residues S560, 561, and 562 of the phosphoSIM form hydrogen or electrostatic bonds with K46, K39, and H43 of SUMO1 (PDB: 4JWN). Figures from [22], with permission from Elsevier

Importantly, the electrostatic interactions can be modulated through PTMs on either SIM or SUMO, thereby regulating the dynamics and specificity of their interactions. Reversible phosphorylation/dephosphorylation of the serine or threonine residues in phosphoSIMs dramatically enhances the affinity of SUMO binding (Fig. 2a, b). Biophysical measurements of selected interactions revealed a phospho-dependent shift of the dissociation constant K_D from around 50 to 1.5 μM [13–15, 22]. Structural data show that this is due to the formation of additional salt bridges with distinct residues in the basic patch of SUMO (Fig. 1) [22]. Accordingly, replacement of basic residues by alanine abolishes SUMO-SIM interactions. Similarly, the neutralization of these basic charges through acetylation modulates selected SUMO-SIM interactions (Fig. 3a, b) [15, 24]. This strengthens the idea that PTMs provide a specificity code for SUMO-SIM interactions.

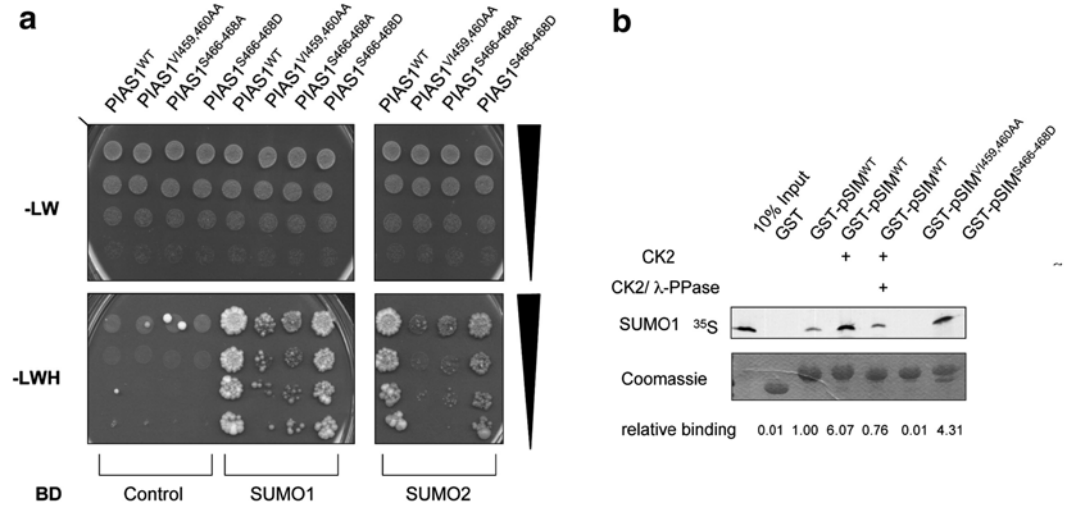

Fig. 2 Phosphorylation enhances SUMO-SIM interactions as exemplified for the binding of PIAS1 to SUMO paralogs. (**a**) Wild-type PIAS1 and the indicated mutants were tested for interaction with SUMO1 or SUMO2 by Y2H. Here, SUMO2.ΔGG was fused to the GAL4 DNA-binding domain (BD) and PIAS1 to the GAL4 activation domain (AD). In the PIAS1^{VI459,460AA} mutant two critical hydrophobic residues in the SIM region are replaced by alanine. In the PIAS1$^{S466-468A}$ mutant three serine residues that are targeted by phosphorylation are replaced by alanine, while in the PIAS1$^{S466-468D}$ mutant these residues were replaced by glutamic acid to mimic phosphorylation. To visualize the strength of interaction, serial dilutions (1:5) were spotted. Under non-selective conditions growth of yeast was followed on control plates lacking Leu and Trp (–LW). To monitor interaction, plates additionally lacking His (–LWH) were used. The yeast strain used here is auxotroph for His and contains a HIS3 reporter under the control of GAL4. Growth on –LWH is therefore indicative for interaction. (**b**) GST or the respective GST-phosphoSIM versions of PIAS1 were immobilized on glutathione sepharose beads as described under Subheading 3.2.2. The GST-phosphoSIM module used here encompasses residues 444–493 of human PIAS1. It was either used unphosphorylated or prephosphorylated in vitro by CK2. A prephosphorylated/phosphatase-treated sample served as a control. GST-phosphoSIM$^{S466-468D}$ and GST-phosphoSIM$^{S466-468A}$ correspond to a phospho-mimicking or phospho-deficient variant of the phosphoSIM. The different baits were incubated with ^{35}S-labeled in vitro-transcribed/translated SUMO1, which can be used as a rapid alternative to recombinant expression in *E. coli*. The GST pull-down experiment was done as described in Subheading 3.2.3. Bound SUMO1 was visualized by autoradiography and bait proteins by staining with *Coomassie blue*. Figures from [15], with permission from Elsevier

Identifying and characterizing SUMO-SIM interactions are of central importance for the understanding of SUMO signaling. Predicting SIMs by bioinformatics tools, however, remains a challenging task [25]. Therefore, there is a need for experimental methods enabling the reliable identification and characterization of SIMs. Here we present two complementary methods that allow the assessment of canonical as well as potential atypical SUMO-binding entities.

The yeast two-hybrid (Y2H) technique is a sensitive and robust method for the identification and confirmation of direct protein-protein interactions, including interactions that involve ubiquitin- and ubiquitin-like modifiers. There are different

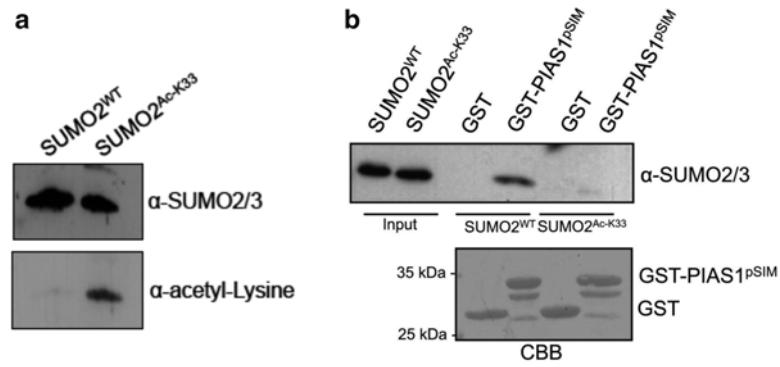

Fig. 3 Acetylation of SUMO affects SUMO-SIM interactions as exemplified for the binding of the phosphoSIM of PIAS1 to SUMO2. (**a**) Test for acetylation of recombinantly expressed SUMO2. Unacetylated and acetylated SUMO2 were expressed as described under Subheading 3.2.1. Acetylation was verified by the use of an acetyl-specific antibody. (**b**) GST or the phospho-mimicking variant of the phosphoSIM module of PIAS1 fused to GST was immobilized on glutathione sepharose beads and incubated with recombinantly expressed wild-type SUMO2 or K35-acetylated SUMO2. The GST pull-down experiment was done as described in Subheading 3.2.3. Bound SUMO2 was visualized by anti-SUMO2 immunoblotting and by *Coomassie blue* (CBB) staining. Figures from [24], with permission from Elsevier

variations of the method, including split-ubiquitin membrane-based Y2H for the detection of membrane-protein interactions [26], but we here focus only on the Y2H approach suitable for the identification of non-membrane-embedded, non-DNA-binding protein-protein interactions (Fig. 4). The method has already been successfully applied for the identification of novel SUMO-binding proteins as well as the characterization of known SUMO-interacting modules [14, 15, 27–30]. The major strength of Y2H is the possibility to detect relatively weak protein-protein interactions, which are characteristic for ubiquitin- and ubiquitin-like binding modules [31]. Another main advantage of the yeast system is the presence of PTMs on the potential interaction partners, which is a critical determinant for strength and specificity of the interaction as outlined above [14, 15]. Moreover, compared to affinity chromatography followed by mass spectrometry, Y2H has the advantage of preferentially capturing direct binding partners rather than entire protein complexes. Despite theses advantages, the gold standard for the characterization of a defined SUMO-SIM interaction is binding experiments with purified protein. We will provide state-of-the-art protocols for this technique with a special focus on the involvement of PTMs in the control of SUMO-SIM interactions.

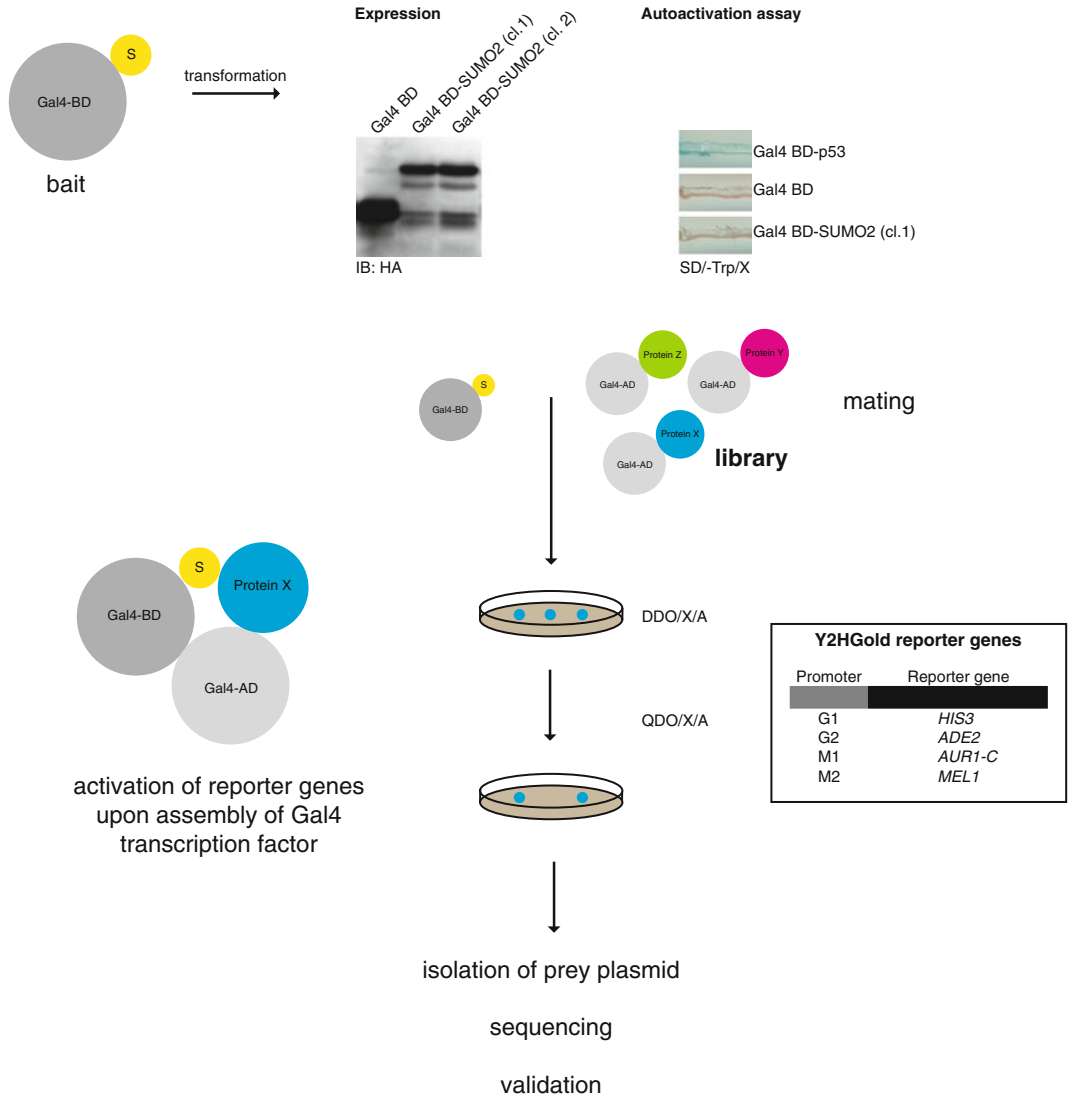

Fig. 4 Basic principle of the Y2H approach. Bait cDNA (encoding full-length proteins, an isolated protein domain or fragment) is cloned into bait vector under the control of lacZ promoter and downstream of the DNA sequence encoding the Gal4 BD. The library is constructed in a way that a mixture of cDNAs (from certain tissue, organ, or various combinations of it) is cloned into prey vector and expressed under the control of lacZ promoter, downstream of the DNA sequence encoding the Gal4 AD. Bait and prey proteins are therefore expressed as C-terminal fusions of Gal4 BD and Gal4 AD, respectively. When yeast is co-transformed with both vectors, and only if bait and prey proteins interact, two GAL4 domains come in close proximity and form an active transcription factor. This in turn, by binding to GAL1 upstream activating sequence (UAS), activates transcription of GAL4-responsive genes, enabling activation of reporter genes, growth on selective plates, and subsequent clone selection

2 Materials

2.1 Media, Buffers, and Other Material Needed for the Y2H

2.1.1 Yeast Strains (See Note 1)

2.1.2 Plates for Cultivating Yeast

1. YPAD.
2. SDO agar (SD/-Leu).
3. SDO agar (SD/-Trp).
4. SDO/X agar (SD/-Trp, X-α–Gal).
5. SDO/X/A agar (SD/-Trp, X-α–Gal, Aureobasidin A).
6. DDO agar (SD/-Leu/-Trp).
7. DDO/X agar (SD/-Leu/-Trp, X-α–Gal).
8. DDO/X/A agar (SD/-Leu/-Trp, X-α–Gal, Aureobasidin A).
9. QDO/X/A agar (SD/-Ade/-His/-Leu/-Trp, X-α–Gal, Aureobasidin A).

2.1.3 Yeast Media

1. YPAD.
2. SDO (SD/-Trp).
3. DDO (SD/-/Leu/-Trp).
4. YPAD medium

   Dissolve 20 g peptone, 10 g yeast extract, and 18 g agar (for plates only) in ddH_2O, and fill in to 935 ml. Autoclave (121 °C, 20 min), cool down to 55 °C, and add 50 ml of 40% sterile glucose and 15 ml of 0.2% adenine hemisulfate (0.003% final). It is recommended to add glucose only to the aliquot of YPAD medium fresh before the use, to avoid potential contamination!

   Please note: 0.5× and 2× YPAD have 0.5× or 2× amounts of ingredients in comparison to 1× YPAD.
5. Minimal SD agar base (*Clontech*)

   Dissolve 46.7 g in 1 l ddH_2O, add appropriate dropout (DO) medium supplement (see below), autoclave (121 °C, 15 min), cool down to 55 °C, and pour plates. If necessary, add Aureobasidin A or X-α-Gal before pouring the plates (when temperature drops to 55 °C).
6. Minimal SD base (*Clontech*)

   Dissolve 26.7 g minimal SD base in 1 l ddH_2O, add appropriate DO medium supplement (see below), autoclave (121 °C, 15 min), and cool down to 55 °C.
7. Yeast synthetic DO medium supplement (choice depends on the type of plates/media).
   - Leu
   - Trp
   - Leu/-Trp
   - Ade/-His/-Leu/-Trp

2.1.4 Buffers, Antibiotics, and Other Solutions

1. 1 M Lithium acetate (LiAc)
   Dissolve 5.1 g lithium acetate dihydrate in 50 ml sterile ddH_2O, autoclave, and store at RT.
2. 50% Polyethylene glycol (PEG)
   Dissolve 50 g PEG 3350 in 30 ml sterile ddH_2O on a stirring plate. Adjust the volume to 100 ml, autoclave, and store at RT.
3. Aureobasidin A
   Dissolve 1 mg Aureobasidin A in 2 ml absolute ethanol (stock solution 500 μg/ml). Store at 4 °C and use at final concentration 200 ng/ml.
4. X-α-Gal
   Dissolve X-α-Gal at 20 mg/ml in dimethylformamide (DMF). Store at –20 °C in the dark. Use at final concentration of 40 μg/ml.
5. Single-stranded carrier DNA
   Dissolve 100 mg salmon sperm DNA in 50 ml sterile TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Denature aliquots (95 °C, 5 min), put on ice (5 min), and store at –20 °C (stock solution 2 mg/ml). Alternatively, purchase sheared, denatured salmon sperm DNA. It is recommended to denature carrier DNA before each use (95 °C, 5 min; ice, 5 min).
6. Lysis buffer for yeast DNA isolation
   0.9 M d-Sorbitol.
   0.1 M EDTA, pH 8.0.
   50 mM DTT.
   200 U Lyticase per sample (VFIN = 300 μl).
   Autoclave 2 M sorbitol and 0.5 M EDTA solution, and keep at 4 °C. Prepare appropriate amount of the buffer before each use.

2.1.5 Mini-prep

Use any DNA mini-prep kit.

2.1.6 Bait Proteins

SUMO-encoding cDNAs (SUMO1.ΔGG, SUMO2.ΔGG, or SUMO3.ΔGG, either wild-type or distinct mutants) are cloned into appropriate bait plasmids and the constructs are verified by sequencing. Typically, Gal4 BD is N-terminally fused to SUMO in bait plasmid (*see* **Note 2**).

2.2 Buffers and Materials for Investigating SUMO-SIM Interactions by Reconstituted In Vitro Binding Experiments

1. His-purification lysis buffer
   50 mM NaH_2PO_4, 150 mM NaCl, 1% (v/v) Triton X-100, 10 mM imidazole, 1 mM PMSF. Adjust pH to 8.0 using NaOH. When expressing acetyl-SUMO variants add 50 mM nicotinamide (NAM) (*see* **Note 3**).
2. His-purification wash buffer

2.2.1 Buffers

Same as lysis buffer, supplemented with 20 mM imidazole.

3. His-purification elution buffer

   Same as lysis buffer, supplemented with 250 mM imidazole.

4. Dialysis buffer

   PBS

   Add 20 mM NAM when expressing acetyl-SUMO variants.

5. GST-purification lysis buffer

   PBS, 1% (v / v) Triton X-100, 1 mM DTT, 1 mg/ml lysozyme, 1 mM PMSF.

6. GST-purification wash buffer

   Same as GST-purification lysis buffer, but without lysozyme.

7. GST-binding buffer (for GST-pull-down)

   50 mM HEPES (pH 7.5), 120 mM NaCl, 0.1% (v/v) NP-40, 1 mM DTT, 1 mM PMSF.

8. GST-wash buffer 1 (for GST-pull-down)

   Same as GST-binding buffer.

9. GST-wash buffer 2

   GST-binding buffer supplemented with 1% (v/v) Triton X-100.

10. 2× SDS-PAGE sample buffer

    10% (v/v) Glycerol, 5% (v/v) 2-mercaptoethanol, 3% (w/v) SDS, 0.1 M Tris–HCl (pH 8.0), 10 mg/ml bromophenol blue.

3 Methods

3.1 Screening a Yeast cDNA Library for SUMO-Interacting Proteins

3.1.1 Transformation of Yeast with the Bait Construct

1. Inoculate yeast into 25 ml YPAD medium and shake overnight at 200 rpm, 30 °C.
2. Measure OD_{600} of a 1:10 dilution of the overnight (O/N) culture ($OD_{600} = 0.1$ of the undiluted yeast culture corresponds to 1×10^6 cells/ml).
3. Add 2.5×10^8 cells to pre-warmed (30 °C) YPAD medium to a final volume of 50 ml in a 250 ml flask (i.e., 5×10^6 cells/ml).
4. Incubate the diluted culture at 30 °C (200 rpm) for 3–4 h to obtain $1–2 \times 10^7$ cells/ml.
5. Centrifuge (1000 × *g*, 5 min, RT), gently remove supernatant, and resuspend the cell pellet in 25 ml ddH_2O. Repeat centrifugation, resuspend the cell pellet in 900 µl of sterile ddH_2O, and transfer to a 1.5 ml tube.
6. Pellet the cells (13,000 × *g*, 1 min, RT), remove supernatant, resuspend in 700 µl of 100 mM LiAc, and incubate at 30 °C for 10 min.

7. For each transformation reaction aliquot 100 µl of the cell suspension into a new 1.5 ml tube, centrifuge (13,000×*g*, 1 min, RT), and remove supernatant.
8. Prepare the transformation mixture in the following order:
   240 µl 50% PEG.
   36 µl 1 M LiAc.
   50 µl Denatured single-stranded DNA (stock 2 mg/ml).
   1 µg Plasmid DNA encoding GalBD-SUMO variants.
   34 µl ddH2O (*minus* DNA volume).
9. Mix, add to the cell pellet, and vortex vigorously to fully resuspend the pellet. Incubate at 30 °C for 30 min with occasional gentle mixing first, and then heat shock at 42 °C for 30 min with occasional gentle mixing.
10. Pellet the cells at 13,000×*g* for 1 min at RT, remove supernatant, add 200 µl sterile ddH_2O, and resuspend by gentle pipetting.
11. Plate on ∅100 mm SD/-Trp plate and incubate the plates wrapped with parafilm at 30 °C for 2–4 days until colonies appear.
12. Pick 4–6 colonies and streak them onto new ∅100 mm SD/-Trp plates (make 3–4 cm long, thin, separated lines) (i.e., **master plate**).

3.1.2 Verification of Protein Expression

1. Transfer a small amount of each yeast colony from SD/-Trp plate into 5 ml SD/-Trp medium and shake overnight with 200 rpm at 30 °C.
2. Next day centrifuge (1000×*g*, 5 min, RT), gently remove supernatant, and add 5 ml of YPAD medium. Incubate again by shaking at 200 rpm for 6 h at 30 °C. Centrifuge to collect yeast cells (1000×*g*, 5 min, RT), and gently remove supernatant.
3. Add 200 µl 1× SDS-PAGE buffer with 5% β-mercaptoethanol, denature (95 °C, 30 min), and run 30 µl of sample on 12% SDS-PAGE.
4. Depending on bait plasmid, incubate with appropriate primary antibody (usually anti-HA or anti-myc; if plasmid lacks tag use Gal4 BD-specific antibody). Please note: The size of Gal4 BD is 22 kDa.

3.1.3 Testing of Bait Autoactivation (See Note 4)

1. Streak small amount of each colony from SD/-Trp plate in a thin layer onto ∅100 mm SD/-Trp/X and ∅100 mm SD/-Trp/X/A plates.
2. Monitor the appearance of blue yeast color within 24 h. Only yeast that does not develop blue color (i.e., not autoactivated) can be used further. Please note: Extended growth or thick streaked yeast will lead to the appearance of blue color generating false-positive autoactivation results.

3.1.4 Transformation of Bait-Containing Yeast with cDNA Library

1. Inoculate the verified bait-containing yeast colony from master plate into 50 ml SD/-Trp medium (in a 500 ml flask), and shake (200 rpm, 16–20 h or until OD_{600} reaches 0.8, 30 °C).
2. The next day centrifuge (1000 × *g*, 5 min, RT) to collect yeast, remove the supernatant, and resuspend yeast pellet to cell density $>1 \times 10^8$ cells/ml in 4 ml SD/–Trp medium.
3. Thaw one aliquot (1 ml) of the "Universal human (normalized) Mate & Plate™ Library" (*Clontech*) at RT (water bath) (*see* **Note 5**).

   Remove 10 µl to titer on ∅100 mm SD/–Leu agar plates (see below).
4. Combine 1 ml of the Mate & Plate™ Library with 4 ml of the yeast from **step 2** in a sterile 1 l flask, rinse cells from library vial with 2xYPAD medium (total volume of 2xYPAD should be 45 ml), and incubate at 30 °C (20–24 h, 30 rpm).
5. After 20–24 h analyze one drop of yeast culture under microscope (put it between coverslip and glass slide) to observe efficiency of yeast mating. Please note: If zygotes are not observed, prolong mating for 4 h.
6. Rinse the flask with 50 ml 0.5× YPAD medium, combine with yeast culture.
7. Centrifuge (1000 × *g*, 10 min, RT), and remove supernatant.
8. Resuspend yeast cells in 10 ml 0.5× YPAD medium. Measure the total cell volume (it will be around 12 ml)!
9. Remove 100 µl of mated culture for titering on ∅100 mm SD/-Leu, SD/-Trp and DDO agar plates (see later).
10. Plate equal aliquots of yeast culture on 60 ∅150 mm DDO/X/A agar plates, and wrap plates with parafilm to prevent drying in the incubator.
11. Incubate at 30 °C for 3–6 days (colonies should be blue, with bluish "halo" in the agar surrounding the colony).

3.1.5 Determination of Transformation and Mating Efficiency

Before plating library, take aliquot (100 µl) of mated culture and prepare serial dilutions in ddH_2O (1/10, 1/100, 1/1000, and 1/10,000 dilutions), streak 100 µl each dilution onto ∅100 mm SD/-Trp, ∅100 mm SD/-Leu and ∅100 mm DDO agar plates, and incubate at 30 °C for 3–5 days.

1. Calculate the efficiency of transformation by using the following equation:

   (a) Calculate the number of screened clones (diploids) by counting the colonies from ∅100 mm DDO plates after 3–5 days.

   (b) Number of screened clones = cfu/ml of diploids × resuspension volume (ml) (cfu = colony-forming unit).

2. Calculate mating efficiency by using the following equation:

   (a) Mating efficiency = number of cfu/ml of diploids (DDO)/number of cfu/ml of limiting partner × 100 (limiting partner = strain with lower viability).

3.1.6 Restreaking of Yeast on QDO/X/A Plates and Isolation of Plasmids

1. Restreak positive clones on ∅150 mm QDO/X/A plates, and allow to grow for 3–5 days at 30 °C.
2. For isolation of plasmid DNA pick individual yeast clones that grew as big blue colonies on QDO/X/A to 5 ml DDO medium and shake (200 rpm, O/N, 30 °C).
3. Centrifuge (1000 × *g*, 5 min, RT), wash pellet in 1 ml ddH_2O, transfer in new 1.5 ml tube, spin down (13,000 × *g*, 1 min, RT), and mix pellet with 300 μl yeast lysis buffer. Incubate for 3 h (or O/N) at 37 °C.
4. Centrifuge (1000 × *g*, 10 min, RT), gently remove supernatant, and isolate yeast DNA by using Mini-prep kit.
5. Transform around 40% Mini-prep to competent DH5α bacteria and isolate plasmid DNA by using Mini-prep kit.

3.1.7 Sequencing and Analysis of Sequencing Results

Sequence prey plasmid (primer choice will depend on library type, for pGADT7-based library primer is T7).

3.1.8 Analyze the Obtained Sequencing Results by Using BLAST Software (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

Make sure that insert is in the same reading frame as Gal4 BD.

3.2 Validation of SUMO-SIM Interactions by GST Affinity Interaction Studies

3.2.1 Expression of His-SUMO and Acetyl-His-SUMO Variants

1. For the expression of His-SUMO1, 2 or 3 *E. coli* transform BL21 (DE3) cells with appropriate plasmids encoding His-tagged versions of the respective SUMO forms (*see* **Note 6**). For the purification of site-specific acetylated SUMO variants co-transform *E. coli* BL21 (DE3) with plasmids pAcKRS-3 and pCDF PylT-1. pCDF PylT-1 carries the ORF for the respective SUMO paralog with an amber codon (TAG) at the position of the desired acetyl-lysine residue (*see* **Note 7**).
2. Supplement 100 ml of LB media with the appropriate antibiotics, inoculate with a single bacterial colony, and grow overnight by shaking at 37 °C. *E. coli* transformed with pQE-30 SUMO require 50 μg/ml ampicillin. The pAcKRS-3/pCDF-PylT1 pair requires 50 μg/ml spectinomycin and 50 μg/ml kanamycin.
3. Inoculate 500 ml of media with O/N culture in order to get an OD_{600} of 0.1 and incubate at 37 °C. For expression of unacetylated SUMO induce protein expression by addition of

0.5 mM IPTG when OD_{600} of 0.7 is reached. Incubate at 37 °C for another 3 h with shaking. In case of expressing acetyl-SUMO variants add 50 mM NAM and 10 mM acetyl-lysine (AcK) to the culture when an OD_{600} of 0.7 is reached. After 30 min induce protein expression by the addition of 0.5 mM IPTG and incubate for 3 h with shaking.

4. Harvest cells by centrifugation (20 min, 6000 × *g*, 4 °C) and resuspend in 20 ml cold PBS (supplemented with 50 mM NAM in case of acetyl-SUMO purification). Repeat centrifugation (20 min, 6000 × *g*, 4 °C) to collect bacteria.
5. Resuspend the cell pellet in 15 ml His-purification lysis buffer and subject the suspension to two repeated freeze-thaw cycles (freezing at –80 °C and thawing at 37 °C). Sonicate (3 × 1 min at 70% power) for complete lysis. Centrifuge lysate (60 min, 25,000 × *g*, 4 °C).
6. Pre-equilibrate and wash Ni-NTA agarose beads (corresponding to 500 µl packed beads) twice in 5 ml cold lysis buffer. Mix washed beads with supernatant and incubate for 3 h at 4 °C with end-over-end mixing.
7. Centrifuge (5 min, 500 × *g*, 4 °C) and remove supernatant. Wash three times for 10 min in 10 ml of cold His-purification wash buffer at 4 °C under rotation.
8. Elute bound proteins with 2.5 ml of His-purification elution buffer twice.
9. Dialyze eluted proteins (5 ml) overnight at 4 °C against 2.5 l precooled PBS (supplemented with 20 mM NAM in case of acetyl-SUMO purification), followed by two additional dialysis steps (2 h, 4 °C, each against 2.5 l precooled PBS, supplemented with 20 mM NAM in case of acetyl-SUMO purification).
10. Determine protein concentration, adjust to ≈0.5–1 mg/ml, and store aliquots at –80 °C. If necessary, concentrate proteins by centrifugation.
11. Load an aliquot of the purified protein on a 12% SDS-PAGE for quality control. In case acetylated versions of SUMO are expressed, acetylation should be verified by the use of acetyl-specific antibodies or by mass spectrometry (*see* Fig. 3a).

3.2.2 Expression and Purification of GST-Fusion Proteins Used as Affinity Baits

1. For the expression and subsequent purification of GST-SIM or GST-phosphoSIM fusion proteins, clone the respective cDNAs in suitable vectors, transform plasmid DNA into *E. coli* BL21 (DE3) (*Stratagene*) cells, and plate on LB/agar supplemented with 50 µl/ml ampicillin (*see* **Note 8**).
2. For expression, inoculate a single bacterial colony in 25 ml LB media with appropriate antibiotic and incubate overnight at 37 °C with shaking.

3. The following day inoculate O/N culture to 500 ml LB media to a starting OD_{600} of 0.1 and grow to a final OD_{600} of 0.6–0.8 at 37 °C. Induce protein expression by addition of 0.5 mM IPTG and incubate for additional 3 h at 37 °C with shaking.
4. Harvest cells by centrifugation (20 min, 6000 × *g*, 4 °C). Resuspend pellet in 20 ml cold PBS and pellet again (20 min, 6000 × *g*, 4 °C). Resuspend the washed pellet in lysis buffer. Subject the cells to three repeated freeze-thaw cycles (freezing at −80 °C and thawing at 37 °C). Sonicate (3 × 1 min at 70% power) for complete lysis.
5. Centrifuge lysate (60 min, 25,000 × *g*, 4 °C) to remove any non-lysed bacteria and cell debris.
6. Incubate supernatant with glutathione sepharose beads (corresponding to 500 µl packed beads), previously equilibrated and washed in cold lysis buffer for 2 h at 4 °C. Wash beads three times with 10 ml of ice-cold GST-purification wash buffer.
7. After the final wash step resuspend beads in 800 µl of PBS. Remove an aliquot of 20 µl, add 20 µl 2× SDS PAGE buffer, and load on an SDS-PAGE.
8. After running incubate the gel with stain the protein with Coomassie staining solution for quality control (*see* **Note 9**).
9. For storage of the bead-bound proteins add 20% (v / v) glycerol and store aliquots at −20 °C.

3.2.3 GST Pull-Down Experiments Using GST-SIM Proteins

1. Set up the number of 1.5 ml tubes according to the number of interactions you wish to test. In addition to the set of GST-fusion proteins used as affinity matrices an additional sample with the GST-only control is needed (*see* **Note 10**). Remove 20–40 µl (corresponding to 10 µg of protein) of the glutathione sepharose bead suspension from the above purification (from Subheading 3.2.2, **step 7**) (*see* **Note 11**).
2. Add the bead suspension to 1 ml of GST-binding buffer and equilibrate for 5 min followed by centrifugation for 2 min at 700 × *g* and 4 °C. Remove supernatant by aspiration and resuspend the beads again in a final volume of 500 µl GST-binding buffer.
3. Take 10–20 µl (≈10 µg) of purified His-tagged SUMO proteins (from Subheading 3.2.1, **step 8**) and dilute to a final volume of 50 µl with GST-binding buffer. Separate 1/10th of this mix (5 µl) and add 5 µl 2× SDS-PAGE buffer. Denature at 95 °C for 5 min. This sample will serve as input. Add the remaining 45 µl to the resuspended beads from **step 2**.
4. Incubate tubes for 3 h at 4 °C with end-over-end mixing.
5. Wash the beads twice with 1 ml ice-cold GST wash buffer 1 and twice with 1 ml ice-cold GST wash buffer 2. For each wash

incubate for 10 min with end-over-end mixing followed by centrifugation (2 min, 700 × *g*, 4 °C). Remove supernatant by gentle aspiration.

6. Elute bound proteins by the addition of 40 µl 2× SDS sample buffer and boil at 95 °C for 5 min.
7. For analysis load 20 µl of the eluted proteins as well as 5 µl of the input sample (from Subheading 3.2.3, **step 3**) on a 12% SDS-PAGE. Bound SUMO can be monitored by immunoblotting using anti-RGS-His (*Qiagen*) or anti-SUMO antibodies. Hybridoma cell lines producing mouse monoclonal antibodies anti-SUMO1 (Clone 21C7) or anti-SUMO2 (Clone 8A2) are available from the Developmental Studies Hybridoma Bank at the University of Iowa. Alternatively, in a purified form both antibodies are commercially available (*Abcam*). Bait proteins (GST and GST-fusion proteins) can be detected by anti-GST antibodies (*GE Healthcare*) (*see* **Note 12**). Alternatively, the detection can be done on the membrane by Ponceau S staining or on a separate gel stained with Coomassie staining solution, which was loaded with the second half of the sample (*see* Figs. 2b and 3b).

4 Notes

1. Various reporter host strains (*S. cerevisiae*) can be used for Y2H, such as Y190, Y187, and Y2HGold (*Clontech*). These yeast strains are deficient for Trp and Leu and cannot grow in a minimal medium lacking these amino acids, unless functional TRP1 and LEU2 genes are introduced. Therefore, the bait vector contains TRP1 and the prey vector has LEU2 in its sequences. The use of Y2HGold strain enables stringent screening conditions, due to the presence of sensitive Aureobasidin A antibiotic resistance gene (AUR1-C reporter) as one of its four reporters (besides ADE2, HIS3, and MEL1), minimizing the false-positive protein interactions and background during the Y2H. Aureobasidin A is toxic to yeast at low concentrations (0.1–0.5 µg/ml) and inhibits yeast enzyme inositol phosphoryl ceramide synthase.

   Yeast strains are long-term stored at −80 °C (in YPAD medium with 25% glycerol). To prepare working stock plates, streak a small portion of the yeast onto a YPAD agar plate and incubate at 30 °C until yeast colonies reach 2 mm in diameter (2–4 days). For short-term storage streak yeast on YPAD agar plates, and after yeast is grown store plates at 4 °C (yeast needs to be restreaked to a fresh plate every few weeks). It is recommended to use freshly restreaked yeast for each transformation.

2. The SUMO bait proteins can be cloned into pYTH9, pGBKT7 (*Clontech*), or any other commercially available or generated vector containing TRP1 marker and GAL4 DNA BD. Yeast transformed with pYTH9 vector grows on SD/-Trp plates and constitutively expresses Gal4 BD-fusion protein from *ADH1* (alcohol dehydrogenase) promoter. With its size of 10 kDa SUMO paralogs are ideally suited for Y2H. Since the aim is to detect only non-covalent SUMO-SIM interacts, covalent conjugation of Gal4-BD-SUMO has to be avoided. Therefore, the C-terminal GG residues that are essential for conjugation are removed from the sequence. Alternatively, the second Gly residue can be replaced by alanine. SUMO mutants that harbor specific mutations within its β2 strand in the SIM-interacting region can be used as negative controls, when studying canonical SIMs. In order to increase the avidity and even to a certain extent "mimic" extended SUMO chains, tandem SUMO proteins can also be used as bait.
3. When expressing and purifying acetylated SUMO paralogs in *E. coli* the sirtuin inhibitor NAM is added to all buffers to inhibit deacetylation. In *E. coli*, only one lysine deactylase, the sirtuin CobB, has been reported.
4. Autoactivation describes the ability of bait to trigger activation of reporter genes in the absence of an interacting protein containing Gal4 AD. In the above-described Y2H subtype (based on Gal4 AD and Gal4 BD), autoactivation is common for proteins that bind DNA. Wild-type SUMO paralogs are typically not prone to autoactivation. However, for any protein autoactivation can occasionally occur when mutants or partially unfolded protein fragments are used as bait. Therefore, whenever uncharacterized mutants of SUMO are used as baits a test for autoactivation is necessary, but is highly recommended for all the yeast clones before starting the screen.
   After confirming bait expression and lack of autoactivation, a known interaction partner should be used to monitor proper functioning of the components. One suitable prey for controlling SUMO-SIM interactions is PIAS family members. For these directed interaction experiments yeast transformed with bait vector should be additionally transformed with prey plasmid according to the protocol for the transformation of yeast with the bait construct with the following modifications. Because yeast is already transformed with bait plasmid, growth of the O/N culture is performed in SD/-Trp medium and yeast are streaked on SD/-Trp/-Leu plates after transformation. The individual yeast colonies should then be plated on DDO/X/A and QDO/X/A plates, in order to determine protein-protein interactions. Chemical transformation of library (set of plasmids) is also possible and protocol resembles the up-scaled transformation of single prey plasmid.

5. Library or selected prey protein (for confirmation of a defined protein-protein interaction) are clones into pACT2, pGADT7, or any other commercially available or generated vector containing LEU2 nutritional gene and Gal4 DNA AD. Yeast transformed with prey plasmid grows on SD/-Leu plates and constitutively expresses Gal4 AD-fusion protein. Libraries can be purchased as a set of prey plasmids, or already pre-transformed in yeast. Here we describe "Mate & Plate™ libraries" (*Clontech*), which are based on the ability of haploid yeast strains such as Y187 (library) and Y2HGold (bait) to mate with each other and form diploid cell. These libraries are available as plasmids transformed in Y187 yeast strain. Even though in some cases tissue-specific libraries might be preferred, use of universal libraries (obtained from the mixtures of cDNAs from various tissues) is generally recommended.
6. For expression of unacetylated His-tagged SUMO paralogs, cDNAs encoding human SUMO1 (NCBI P63165), SUMO2 (NCBI P61956), and SUMO3 (NCBI-P55854) are cloned in pQE-30 vector (*Qiagen*). Proteins expressed from a pQE-30 plasmid harbor an N-terminal RGS-6xHis-sequence, which can be readily detected with the anti-RGS-His antibody directed against this epitope. Since in in vitro binding experiments outlined here no conjugation of SUMO can occur, we use the mature, processed SUMO forms terminating with the C-terminal GG signature.
7. pAcKRS-3 encodes for an acetyl-lysyl-tRNA synthetase (AcKRS) that is derived from the *M. barkeri* pyrrolysyl-tRNA synthetase. The pCDF PylT-1 encodes the corresponding tRNA that directs the incorporation of acetyl-lysine in response to the amber codon [32]. To this end pCDF PylT-1 additionally carries the ORF for the respective SUMO paralog with an amber codon at the position of desired acetyl-lysine residue. AcKRS and pCDF PylT-1 were kindly provided by Jason W. Chin, MRC Laboratory for Molecular Biology, Cambridge, UK. To facilitate detection of the respective SUMO proteins we also use a 6xHis-tag with the N-terminal RGS sequence extension.
8. For expression of GST fusion proteins, cDNAs encoding SUMO-binding proteins or isolated SIM/phosphoSIM domains are cloned into pGEX vectors (*GE Healthcare*). Phosphomimicking variants are generated through replacement of phosphoserine/phosphothreonine residues by glutamic acid (*see* Figs. 2b and 3b).
9. Quality control is needed to make sure that the full-length proteins were purified to homogeneity without co-purification of bacterial proteins. Detection by Coomassie staining also allows the estimation of protein quantity.

10. While a GST-only control is mandatory in all cases as the minimal negative control, it is preferable to use a binding-deficient control protein with specific amino acid exchanges. For example, when testing canonical SUMO-SIM interactions a SIM variant lacking critical hydrophobic or acidic residues should be used (Fig. 2b) (*see* also **Note 2**).
11. The binding capacity of glutathione sepharose is around 5 mg of GST-fusion protein per ml of packed beads. Forty microliters of the bead suspension from Subheading 3.2.2, **step 7**, should correspond to 20 μl of packed beads or around 10 μg of protein. Accurate quantifications should be made by eluting the purified protein from an aliquot of the beads using glutathione elution buffer. When testing different bait proteins in the same experiment all proteins must be used at equimolar amounts. In case this requires different bead volumes, the final amount of beads is adjusted to 20 μl of packed beads by addition of empty beads.
12. It is important to ensure that the bait proteins are also present in equal amounts after the affinity purification procedure. This controls that during wash steps no loss of bead material has occurred (*see* Figs. 2b and 3b).

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Chapter 7

Real-Time Surface Plasmon Resonance (SPR) for the Analysis of Interactions Between SUMO Traps and Mono- or PolySUMO Moieties

Wendy Xolalpa, Manuel S. Rodriguez, and Patrick England

Abstract

Isolating endogenous SUMOylated proteins is a challenging task due to the high reversibility of this posttranslational modification. We have shown that SUMO traps are useful tools for the enrichment and isolation of proteins modified by SUMO in vitro and in vivo. To characterize the affinity and specificity of different SUMO chains for these traps, that are based on SUMO-interacting motifs, we have used real-time surface plasmon resonance (SPR), which allows a label-free analysis of protein/protein interactions. Here, a protocol to determine the affinities of multivalent SUMO traps for polySUMO chains or mono-SUMO molecules by SPR is presented.

Key words SPR, Protein interactions, SUMO-binding entities, Affinity constants, Avidity, Multivalence

1 Introduction

SUMOylation, the covalent modification of proteins by the Small ubiquitin-like modifier (SUMO), is a posttranslational modification involved in the regulation of several critical cellular processes. The study of SUMOylation (as that of ubiquitylation) is not an easy task due to the labile nature of these modifications [1, 2]. SUMO-binding entities (SUBEs), a.k.a SUMO traps [3], are recombinant proteins that comprise tandem repeats of SUMO-interacting motifs (SIMs) that recognize SUMO molecules on modified proteins [4]. SUMO traps have been shown to be useful affinity purification tools to isolate endogenous SUMOylated proteins in pull-down assays [3, 5]. The characterization of SIM-SUMO interactions has revealed that their affinities are in the range of 2–3 μM [6], and that proteins containing multiple SIMs recognize preferentially polySUMOylated proteins [7, 8]. Surface plasmon resonance (SPR) instruments allow to analyze protein/

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protein interactions in real time without labeling, thus providing a means to fully characterize equilibrium and kinetic parameters [9]. One of the interacting partners (the ligand) is immobilized covalently or non-covalently on the surface of a sensor chip, while the other (the analyte) is routed towards the chip through a microfluidic cartridge thanks to a continuous flow. Here, we describe a protocol to determine by SPR the affinities of SUMO traps (ligands) for poly-SUMO chains or mono-SUMO molecules (analytes). We also show how the ligand density can significantly influence the properties of these interactions. As our SUMO traps comprise a GST moiety, we used an anti-GST antibody as capture molecule [10] to immobilize them non-covalently, thus preserving their native state and exposing the SIMs in an optimal fashion. The general protocol comprises the initial covalent cross-linking of the capture antibody on the surface of the sensor chip, followed by the monitoring of the ligand-analyte interactions themselves, each cycle being composed of three steps: (1) capture of the GST-tagged SUMO traps, (2) injection of SUMO or polySUMO molecules, and (3) regeneration of the anti-GST surface for a new experimental cycle. Individual cycles are performed for each analyte concentration (Fig. 1). The SPR instrument we used (Biacore 2000, GE Healthcare) allows to measure simultaneously the interactions of

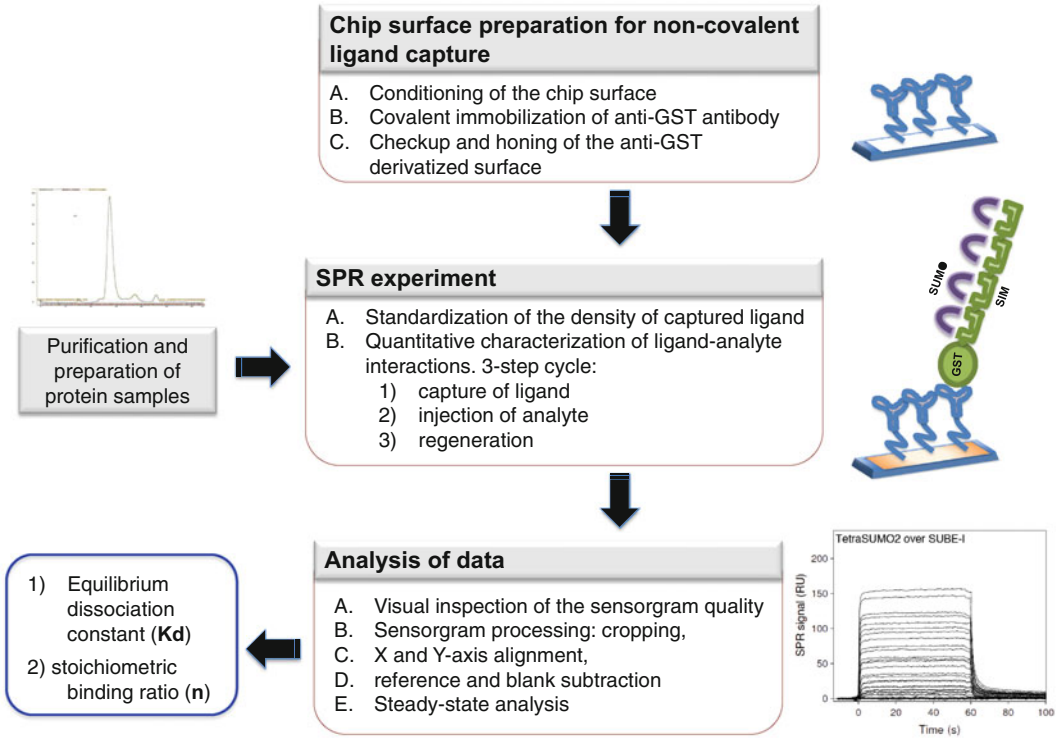

Fig. 1 Schematic diagram of the protocol

an analyte with four ligands: we therefore characterized interactions with an 8-SIM-long SUMO trap (SUBE-l) using two control surfaces, one with a GST-tagged ubiquitin-trap [11] and the other with GST alone.

2 Materials

2.1 Preparation of Ligands and Analytes

1. The SUMO trap SUBE-l and the ubiquitin trap (TUBE-hHR23) used as control were produced as GST-fusion proteins in *E. coli* as described [3, 11] (*see* **Note 1**). SUMO1, SUMO2, and tetraSUMO2 (4×SUMO2), also fused to GST, were kindly provided by R.T. Hay [7]. All proteins were purified using a two-step chromatographic protocol: glutathione affinity chromatography (with glutathione-agarose beads) and size-exclusion chromatography (on a Sephacryl S300 gel filtration column). For SUMO analytes, an additional anion-exchange chromatography step was applied after cleavage of the GST moiety before the final gel filtration (*see* **Note 2**). Proteins were all stored at 4 °C in HBS-EP buffer.
2. Protein concentrations were determined by UV/visible spectrophotometry using the extinction coefficients calculated from each protein sequence (*see* **Note 3**).

2.2 Chip Surface Preparation for Non-covalent Capture of Ligands

1. Sensor Chip CM5 (GE Healthcare).
2. HBS-EP buffer (GE Healthcare, degassed and ready to use) containing 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005 % Tween20.
3. GST Capture Kit (GE Healthcare) including goat anti-GST antibody 0.8 mg/ml, recombinant GST 0.2 mg/ml (in HBS-EP buffer), immobilization buffer (10 mM sodium acetate pH 5.0), and regeneration solution (10 mM glycine-HCl pH 2.2).
4. GST solution (e.g., Sigma).
5. Amine Coupling Kit (GE Healthcare) including 1-ethyl-3-(3 dimethylaminopropyl) carbodimide hydrochloride (EDC) at 0.4 M, N-hydroxysuccinimide (NHS) at 0.1 M, 1.0 M ethanolamine-HCl pH 8.5.
6. 100 mM Glycine-NaOH pH 12.
7. 0.05 % SDS.

2.3 SPR Experiment

1. Biacore 2000 instrument (GE Healthcare).
2. Glass vials (4.0 ml, 16 mm borosilicate screw top).
3. Rounded polypropylene microvials (0.8 ml, 7 mm) with a penetrable cap made of Kraton G.

4. Benchtop microfuge.
5. 10mM Glycine-HCl pH 2.
6. 0.1% SDS.

3 Methods

All procedures were carried out at room temperature unless otherwise specified. A schematic diagram summarizing all the steps of this protocol is shown in Fig. 1.

3.1 Chip Surface Preparation for Non-covalent Ligand Capture

1. Before starting, equilibrate a new CM5 sensor chip (stored at 4 °C) at room temperature for 30 min.
2. Dock the chip in the SPR instrument (set at 25 °C) and prime the system three times with HBS-EP.
3. Operate the Biacore equipment manually. Set flow at 5 μl/min. Open flow cells FC1–FC4. Wait for a stable baseline and monitor resonance units (RU) in real time.
4. *Conditioning of the chip surface*. Prepare a 0.7 mm vial with 100 mM glycine pH 12 and a second vial with 0.05 % SDS, and spin tubes in a microfuge for 10 s (*see* **Note 4**). Inject 5 μl Gly pH 12 followed by 5 μl SDS. Repeat at least twice until a stable baseline is reached (*see* **Note 5**).
5. *Covalent immobilization of anti-GST antibody*. Start a new sensorgram, set flow at 5 μl/min, and open flow cell 4. For surface activation, mix the solutions of EDC and NHS (50 μl + 50 μl) and inject 65 μl over the chip surface (*see* **Note 6**). Prepare the antibody solution (6 μg/ml) in immobilization buffer. After the activation step, immediately inject 100 μl of anti-GST before de-activating the chip with 65 μl of ethanolamine. At the end of this step, record the density of immobilized anti-GST antibody. We routinely attain 11,000–14,000 resonance units (RUs; 1 RU ≈ 1 pg/mm^2). Open flow cell 3, prepare a fresh mix of EDC/NHS, and repeat the anti-GST immobilization protocol as for flow cell 4 (FC4). Follow the same protocol for FC2 and FC1.
6. *Checkup and honing of the anti-GST-derivatized surface*. After immobilizing the antibody in the four channels of the CM5 chip, program a test experiment with commercial GST in order to assess the efficiency of capture by the antibody and of regeneration of the surface. We suggest programming a 20-cycle experiment (overnight) with GST injections followed each time by a regeneration with 5 μl of Gly pH 2 (*see* **Note 7**). Compare the starting baseline with that after regeneration, and check for cycle-to-cycle reproducibility. If regeneration with Gly is not sufficient, add a short (5 μl) injection of 0.1 % SDS.

7. At this point, the surface is ready to start capturing the GST-fused SUMO traps. If not ready on the same day, the system can be left in standby mode for up to a week, without undocking the chip.

3.2 SPR Experiment

1. *Standardization of the density of captured ligand*. Start a new sensorgram, open the four flow cells (FC1–FC4), and briefly rinse with HBS-EP flow at 30 μl/min to stabilize the baseline. Then set the flow rate at 5 μl/min, open each flow cell separately, and adjust the dilutions and injection times of the different GST fusions to reach densities of around 100 RUs (low density), 200–300 RUs (medium density), and 700 RUs (high density). We captured purified GST as a control in FC1, TUBE-HR23 in FC2, and SUBE-1 in FC4. We used successive short 5 μl injections of Gly pH 2 and 0.1 % SDS to regenerate the surfaces.
2. *Quantitative characterization of ligand-analyte interactions*. Once the ligand capture step has been optimized, one can proceed to program multi-cycle SPR methods, each cycle comprising a capture step followed by an injection of analyte and a regeneration step. Each analyte was injected at a minimum of seven concentrations spanning over 2 orders of magnitude. The concentration ranges we used were as follows: for SUMO1, 10–700 μM; for SUMO2, 15–1000 μM; and for tetraSUMO2 0.04–40 μM. In each case, the limiting factor was the amount of analyte available. Each injection was performed in duplicate (*see* **Note 8**).
3. After experiments are finished, perform a few additional regeneration injections. If a new experiment is not foreseen in the next few days, undock the sensor chip and store it dry at 4 °C.

3.3 Analysis of Data

We analyzed the association and dissociation real-time SPR profiles by combining two softwares: Biaevaluation (GE Healthcare) and Scrubber (BioLogic Software).

1. First, sort the experimental curves analyte by analyte using the Biaevaluation software (version 4.1).
2. Check the quality of sensorgrams visually, and ensure that the surfaces were fully regenerated after each cycle.
3. Export sensorgrams to txt format and open them with Scrubber (version 2.0). Crop sensorgrams to select only the analyte injection step, and zero them on the Y- and X-axes (Fig. 2).
4. Double subtract, on the one hand, signals measured on the reference flow cell (with GST alone; *see* **Note 9**) and on the other those from blank injections.

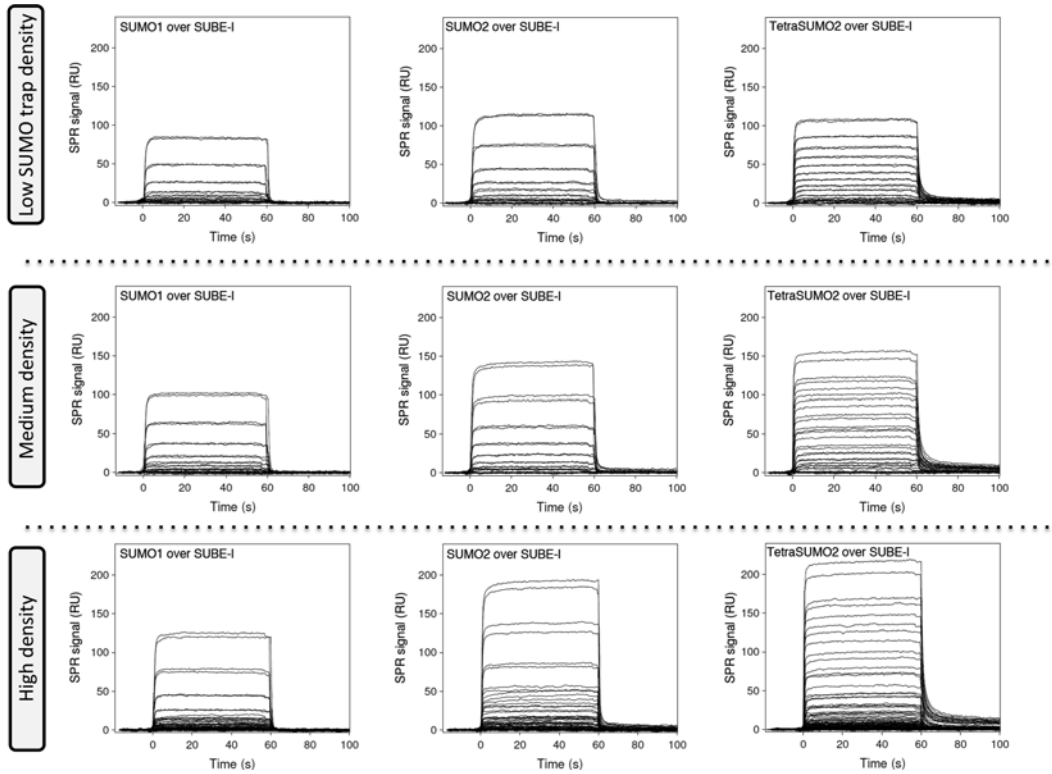

Fig. 2 Representative SPR sensorgram data for the interaction between the SUBE-I multivalent SUMO trap and SUMO1 (*left*), SUMO2 (*middle*), and tetraSUMO2 (*right*) molecules, at different SUMO trap densities (*top*: low; *middle*: medium; *bottom*: high)

5. Save the processed data and export them back to Biaevaluation for further in-depth analysis.
6. Perform a steady-state analysis of the processed data, assuming either one or two binding modes (*see* **Note 10**).

4 Notes

1. GST or GST-fusion proteins were over-expressed in *E. coli* (BL21 or C41) using IPTG induction.
2. Mono- and tetraSUMOs were digested by the thrombin enzyme to eliminate their GST moiety. After affinity chromatography, the analytes were dialyzed against 50 mM Tris-HCl pH 7.5, complemented with 2-mercaptoethanol, and subjected to anion-exchange chromatography using a Q-sepharose resin column. Fractions were analyzed by SDS-PAGE and those corresponding to the expected protein were pooled and dialyzed against 10 mM ammonium bicarbonate before lyoph-

ilization. Before SPR experiments, samples were reconstituted in HBS-EP and submitted to a final round of purification by gel filtration. Fractions containing aggregates or impurities were removed and only those containing highly pure monomeric protein were used. The homogeneity of analytes is crucial to obtain accurate results and good reproducibility in SPR assays.

3. After purification, samples were subjected to centrifugation at 20,000 × *g* during 10 min. Supernatants were saved for spectrophotometric concentration measurement and SPR experiments.
4. Centrifugation allows to eliminate small bubbles remaining in samples, thus avoiding the introduction of air into the Biacore system and spikes in the SPR profiles. Whenever preparing a sample for injection, make sure to centrifuge it for at least 10 s.
5. New sensor chips have to be conditioned when docked for the first time in the Biacore instrument, to get rid of manufacturing residuals remaining on the bioactive dextran layer.
6. Once mixed, the EDC/NHS solution has a shelf life of approximately 30 min, so it is better to prepare it fresh every time. Separately, each of the two reagents is stable at −20 °C, but aliquots should be discarded once thawed.
7. As the antibody is covalently bound to the carboxymethylated dextran matrix of the sensor chip, the anti-GST surface can be regenerated many times and loaded with different GST-containing proteins.
8. Performing replicates, double subtraction or referencing allows to obtain reliable results.
9. The specificity of SUMO moieties for SUMO traps was assessed by using a ubiquitin-trap TUBE as a control ligand, which only displayed a background nonspecific signal at high density (data not shown). The best quality and most reproducible data, according to the overlay of replicates, were obtained for the lowest densities of captured SUMO traps (Fig. 2).
10. The interactions between mono SUMO molecules and SUMO traps could be analyzed as simple 1:1 events (Table 1). On the contrary, as both tetraSUMO2 and SUMO traps are multivalent molecules, their interaction was more complex (as expected), and could be better fitted assuming the existence of two binding modes, each of them most likely involving a different number of SUMO moieties. It is thus possible to distinguish a high-affinity binding mode (Kd1) and a lower affinity one (Kd2) (*see* Table 1). Moreover, we observed that the density of SUMO traps has an influence Suprime both on the equilibrium dissociation constants (Kds), on the stoichiometric binding ratio (SUMO/SUMO trap or tetraSUMO2/

Table 1
Interaction parameters obtained from the analysis of the mono- or tetraSUMO concentration dependence of the SPR response

	SUBE-I (ligand)											
	Low density				Medium density				High density			
Analyte	Kd (μM)		*n*		Kd (μM)		*n*		Kd (μM)		*n*	
SUMO1	1250 ± 50		8		910 ± 40		4.2 ± 0.3		870 ± 40		1.4 ± 0.1	
SUMO2	960 ± 80		8		690 ± 50		4.4 ± 0.3		640 ± 30		1.6 ± 0.1	
	Kd1 (μM)	Kd2 (μM)	n1 (%)	n2 (%)	Kd1 (μM)	Kd2 (μM)	n1 (%)	n2 (%)	Kd1 (μM)	Kd2 (μM)	n1 (%)	n2 (%)
tetraSUMO2	1.3 ± 0.1	115 ± 16	30	70	1.35 ± 0.25	130 ± 40	31	69	1.05 ± 0.20	95 ± 25	33	67
			n = 1				*n* = 0.9				*n* = 0.4	

Kd equilibrium dissociation constant, *Kd1* high-affinity binding constant, *Kd2* low-affinity binding constant, *n* stoichiometric binding ratio, *n1* proportion of molecules binding according to the high-affinity mode, *n2* proportion of molecules binding according to the low-affinity binding mode

SUMO trap), and on the relative proportion of the two binding modes involved in tetraSUMO2/SUMO trap complex formation. This is most likely due to the combination of three phenomena that are exacerbated at high ligand density: (1) steric hindrance upon binding; (2) rebinding upon dissociation; and (3) in the case of tetraSUMO molecules, cooperative binding involving several neighboring SUMO trap molecules. This is why one can observe that the apparent affinity (avidity in the case of tetraSUMO2) increases with ligand density and that the stoichiometric binding ratios on the contrary decrease. One can also note that the relative proportions of the two binding modes observed for the interaction between tetraSUMO2 and the SUMO trap vary in favor of the high-affinity binding mode when ligand density increases (Table 1). This translates into association/dissociation profiles in which the average half-life of the complexes can be seen to increase with ligand density (Fig. 2; *see* also ref. 2).

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Chapter 8

Using Biotinylated SUMO-Traps to Analyze SUMOylated Proteins

Valérie Lang, Elisa Da Silva-Ferrada, Rosa Barrio, James D. Sutherland, and Manuel S. Rodriguez

Abstract

SUMO-interacting motifs (SIMs) recognize SUMOylated proteins with high specificity allowing to connect SUMO-modified proteins. Multiple SIMs fused to distinct tags have been used to increase their affinity and generate more efficient purification tools. Enrichment of SUMOylated proteins using SIMs arranged in tandem (SUMO-traps) facilitates the identification and characterization of protein targets in vitro and in vivo. Here a protocol to produce biotinylated SUMO-traps (bioSUBEs) to capture SUMO chains and typical SUMOylated proteins such as p53 or IkBα is presented. Biotinylated SUMO-traps represent an alternative to reduce the background associated to bigger tags, e.g., during mass spectrometry analysis. Consequently, bioSUBEs are alternative tools to characterize endogenous SUMO targets.

Key words SUMOylation, SIMs, SUBEs, BirA, Biotinylation, Purification, Analysis

1 Introduction

Small ubiquitin modifier (SUMO) is a member of ubiquitin family involved in the regulation of important cellular functions such as protein localization, transcription, DNA repair, cell cycle progression, and pathological processes like cancer, diabetes, or neurological disorders [1, 2]. Three mammalian SUMO isoforms SUMO1, SUMO2, and SUMO3 have been shown to covalently modify several target proteins [2, 3]. SUMO2 and SUMO3 are very similar (97% of sequence identity) and are commonly referred as SUMO2/3. The cellular localization of each isoform is different and associated to different cellular functions. SUMO1 has been linked to, e.g., nuclear import and gene repression [4–6]. SUMO2/3 appear as a large unconjugated reservoir quickly available for conjugation in response to general cellular insults such as heat shock, proteasome inhibitors, and oxidative stress [7, 8].

SUMO molecules also undergo non-covalent interactions with proteins containing short amino acid stretch surrounded by

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hydrophobic residues called SUMO-interacting motifs (SIMs) [9]. The first SIMs were published by Minty and collaborators in 2000 using a two-hybrid approach revealing the existence of an SxS sequence (S = serine, x = any amino acid) flanked by a hydrophobic core and acidic amino acids [10, 11]. More recently, other SIMs with the following sequences were described: SIM-a [PILVM][ILVM]X[ILVM][DSE>]{3}, SIM-b [PILVM][ILVM]DLT, and SIM-r [DSE]{3}[ILVM]X[ILVMF]{2} [12, 13] (*see* **Note 1**).

In order to connect with SUMO proteins, SIMs form a β-strand that interacts in a parallel or antiparallel orientation with the β2-strand of SUMO. SIM sequences bind with low affinity to SUMO with a dissociation constant ranging from 5 to 10 μM [9, 14, 15]. Several SUMO substrates and enzymes that regulate the levels of SUMOylation contain SIMs. Two such examples are the SUMO ligases from PIAS family (protein inhibitor of activated STAT-signal transducer and activator of transcription), and the SUMO target ubiquitin ligase RNF4 (ring finger protein 4) [16–19]. Therefore, the identification of new proteins containing SIMs can be important to further understand the SUMO-dependent regulatory mechanisms.

Multiple SIMs increase the overall affinity for SUMO substrates and this property has been exploited to design efficient purification tools. Based on the artificial repetition of SIMs fused to a glutathione S-transferase (GST) tag, our laboratory developed SUMO-Traps or SUBEs (SUMO binding entities) to purify SUMOylated proteins [20, 21]. This tool facilitates the purification, identification, and characterization of a SUMO target proteins from in vitro and in vivo assays. However, the GST-moiety, due to its relatively large size (~26 kDa), can bind proteins nonspecifically that are co-purified with the SUMO substrates captured by the SUBEs. This can result in a complex protein mixture in which contaminant proteins mask the endogenous SUMO target proteins of very low abundance and therefore complicate their confident identification by mass spectrometry (MS) analysis. Here, a protocol to produce and use biotinylated SUMO-traps to capture SUMO chains and typical SUMOylated proteins is described. Due to the shorter tag length, this strategy should reduce the amount of nonspecific proteins and consequently improve conditions for characterization and identification of endogenous SUMO targets. To optimize and scale up the production of bioSUBEs, we engineered a vector to express SUBEs with incorporated 6xHIS tag (6 Histidines) and an AviTag [22], a short peptide that encodes an optimized substrate for the BirA biotinylation enzyme. BirA was also separately expressed in bacteria to allow in vitro modification. Although some biotinylation occurs in vivo during bioSUBEs expression in bacteria, the in vitro biotinylation reaction after nickel beads purification increases proportion of biotinylated SUBEs and consequently allows a more efficient purification of SUMOylated proteins (*see* **Note 2**).

2 Material

2.1 Cloning

1. pGEX-BirA: The wild-type BirA open reading frame was amplified using high-fidelity PCR from K-12 *E. coli* genomic DNA. The following primers were used: forward: 5′-GATCGGCGGCCGCATGAAGGATAACAC CGTGCC-3′; reverse: 5′-GATCGAATTCTTATTTTTCTGC ACTACGCAGGGATATTTC-3′. The PCR amplicon was digested with NotI and EcoRI restriction enzymes and cloned into a pGEX-6P-1 (with modified MCS) [23] (Fig. 1a).
2. Bio-SUBEs: The SUBE-long coding sequence was amplified from pGEX-SUBE-long using high-fidelity PCR. The amplicon was cloned into a modified version of pMW172, a modified pET vector for T7-based expression in bacteria [24]. The resulting clone encodes the following: 6xHIS-bio-6xHIS-SUBE-l (long) [20] (Fig. 1a). Bio, also known as AviTag, represents the consensus target sequence for biotinylation by BirA. Sequence is available by request.

2.2 Protein Expression and Purification Materials and Reagents

1. Luria Broth (LB) media.
2. 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG).
3. Phosphate-buffered saline (PBS) 1×.
4. 1 M Benzamidine.
5. Triton X-100.
6. Complete protease inhibitor cocktail (EDTA free).
7. Glutathione-agarose beads.
8. Ni-NTA agarose beads (high-density nickel 6BCL-QHNi).
9. High-capacity neutravidin agarose beads.
10. Bradford assay reagent.
11. Dialysis tubing, cutoff 3.5 kDa.
12. Slide-A-Lyzer-7kD.
13. Spectrophotometer.
14. 2× Laemmli buffer.

2.3 Protein Purification Buffers

All solutions should be prepared using ultrapure water (8 MΩ cm at 25 °C), with analytical grade reagents, and stored at 4 °C (unless indicated otherwise). All the waste disposal should be diligently followed according to the specific regulations. In this protocol we did not add sodium azide to the reagents or solutions. The buffers were prepared as follows:

1. Lysis buffer 1: 50 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole, pH 7.5.
2. Binding buffer 1: 50 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole, pH 7.5.

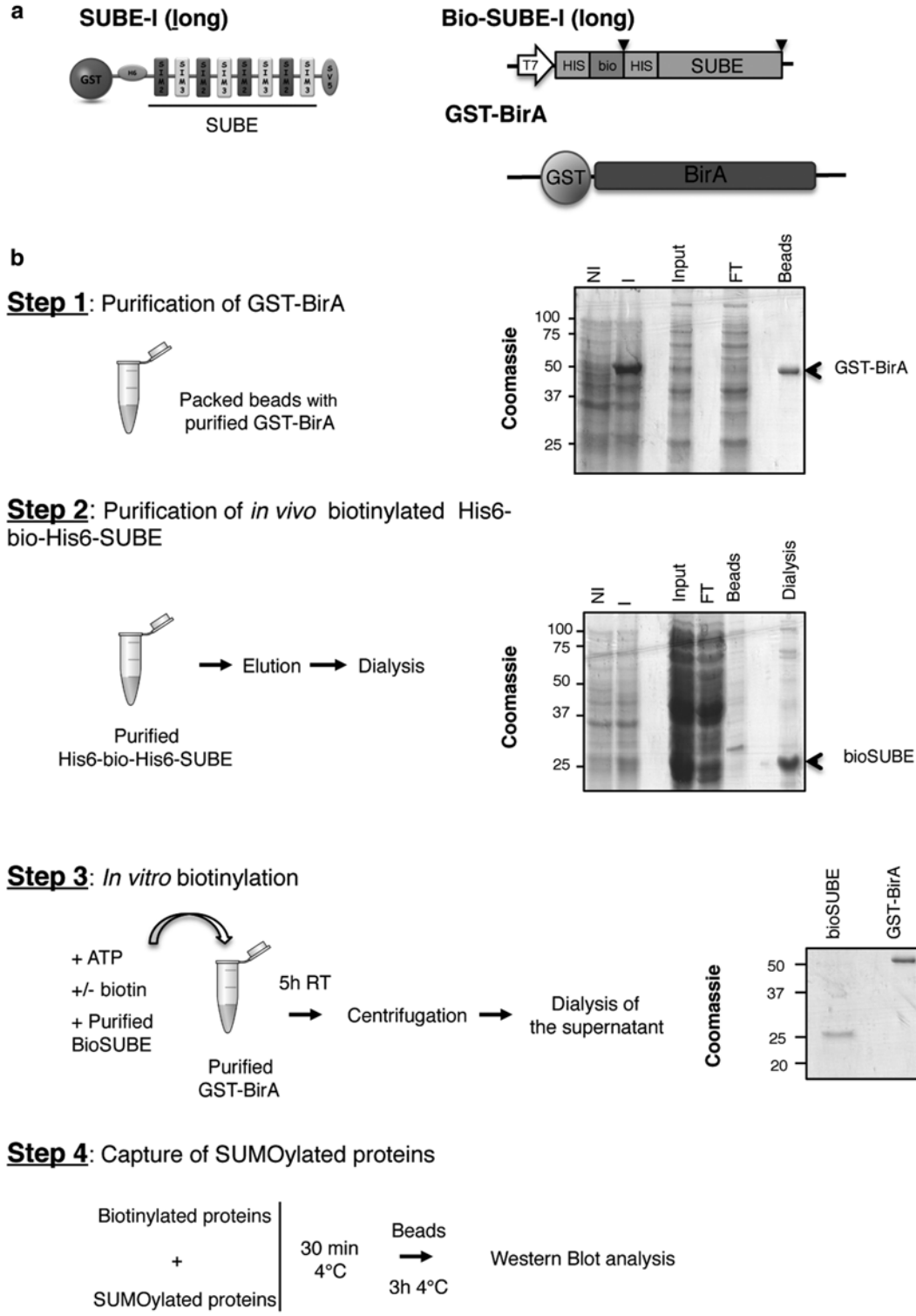

Fig. 1 Integrative diagram of all steps as described in Subheading 3. (**a**) Cartoon illustrating bioSUBE-I (made from SUBE-I (GST tagged)) and GST-BirA constructs used in this manuscript. (**b**) Diagram representing the different steps necessary for the purification of GST-BirA and bioSUBEs proteins, as well as the in vitro biotinylation process and capture of SUMOylated proteins. Coomassie staining detects purified GST-BirA and bioSUBE proteins NI: Non-Induced; I: Induced and FT: Flow-through

3. Washing buffer 1: 50 mM Tris-HCl, 500 mM NaCl, 30 mM imidazole, pH 7.5.
4. Elution buffer 1: 50 mM Tris-HCl, 500 mM NaCl, 150 mM imidazole, 5 mM β-mercaptoethanol, pH 7.5.
5. Dialysis buffer: 50 mM Tris-HCl, 150 mM NaCl, pH 8.0.
6. Lysis buffer 2: 50 mM Tris-HCl pH 8.5, 150 mM NaCl, 5 mM EDTA, 1% Igepal.
7. Washing buffer 2: 50 mM Tris-HCl pH 8.5, 50 mM NaCl, 5 mM EDTA, and 1% Igepal.

2.4 In Vitro Modification Assay and Pull-Down Reagents

1. 1 M Adenosine triphosphate (ATP).
2. 1 mM biotin.
3. Plasmids for the in vitro expression of proteins (IkBα [25] or p53 [26]).
4. TNT-quick-coupled transcription/translation Kit, Promega.
5. Recombinant SUMO2 and SUMO3.
6. Recombinant Ubc9.
7. Recombinant SAE1/2.
8. ATP regenerating system: 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/mL of creatine kinase, and 0.6 U/mL of inorganic pyrophosphatase.
9. Recombinant tetra-SUMO2 fusion protein (4xS2), generously provided by R.T. Hay.
10. Phenylmethylsulfonyl fluoride (PMSF).
11. 1 M Dithiothreitol (DTT).

2.5 Western-Blot Analysis Reagents

1. 12% Poly-acrylamide gels.
2. PVDF membranes.
3. Anti-SUMO2/3 (rabbit polyclonal).
4. Anti-IkBα (mouse).
5. Anti-P53 (DO1, mouse monoclonal).
6. Rabbit anti-mouse HRP-coupled antibody.
7. Goat anti-rabbit HRP-coupled antibody.

3 Methods

All procedures were carried out at 4 °C unless otherwise specified. A diagram integrating all steps of this protocol is illustrated in Fig. 1b.

3.1 Protein Expression and Purification

Glutathione-agarose beads, Ni-NTA agarose beads (high-density nickel 6BCL-QHNi), and high-capacity neutravidin agarose beads were washed three times with ten volumes of 1× phosphate-buffered saline (PBS 1×) by centrifugation at 300 × *g* during 5 min (min) to remove ethanol (preservation solution of agarose beads). At the end, the beads were suspended in PBS 1× to obtain a 50% (v/v) slurry and kept at 4 °C.

3.1.1 Purification of GST-BirA

GST-BirA protein was inducibly expressed in *Escherichia coli* C41 (DE3) using a standard protocol for the production of recombinant proteins as follows:

1. Bacteria culture was grown in 1 L of Luria Broth (LB) media at 37 °C with shaking until to reach an optical density at 600 nm (O.D. 600) of approximately 0.6 units. The expression of GST-BirA was induced by addition of 1 mM of IPTG for 3 h at 37 °C.
2. Bacteria were harvested by centrifugation at 6693 × *g* for 30 min at 4 °C. Then pellet was washed twice with cold (4 °C) PBS 1× and finally resuspended in 10 mL of cold PBS 1× supplemented with 2 mM benzamidine (an inhibitor of proteases) (*see* **Note 3**).
3. Bacteria were lysed on ice by sonication at using 10 μm for a total of 3 min as follows: six pulses of 30 seconds (s) with 30 s of incubation on ice between each pulse (Branson) (*see* **Note 4**).
4. After sonication, lysates were supplemented with Triton X-100 to a final concentration of 1% (v/v) and clarified by ultracentrifugation for 2 h at 48,384 × *g* at 4 °C.
5. The clarified lysate was incubated with 1 mL of glutathione-agarose beads (GST agarose beads) in a 50 mL tube for 2 h at 4 °C.
6. Beads were then loaded into a column and washed five times with one column volume of PBS 1× supplemented with Triton X-100 to a final concentration of 1% (v/v).
7. Finally, GST agarose beads were kept in PBS 1× at 4 °C until used for in vitro biotinylation assay (*see* **Note 5**).

3.1.2 Preparation of Recombinant bioSUBEs

bioSUBEs were inducibly expressed in *Escherichia coli* C41 (DE3) using a standard protocol for the production of recombinant proteins as follows:

1. Bacteria culture was grown in 1 L of Luria Broth (LB) media at 37 °C with shaking until to reach an optical density at 600 nm (O.D. 600) of approximately 0.6 units. The expression of bioSUBEs was induced by addition of 1 mM of IPTG for 4 h at 25 °C.

2. Bacteria were harvested by centrifugation at 6693×*g* for 30 min at 4 °C. Then pellet was washed twice with cold (4 °C) PBS 1× and resuspended in 10 mL of cold lysis buffer 1 supplemented with 2 mM benzamidine and complete protease inhibitor cocktail (EDTA-free).
3. Cells were lysed on ice by sonication at using 10 μm for a total of 3 min as follows: six pulses of 30 s with 30 s of incubation on ice between each pulse (*see* **Note 4**).
4. After sonication, lysates were supplemented with Triton X-100 to a final concentration of 1% (v/v) and clarified by ultracentrifugation for 2 h at 48,384×*g* at 4 °C.
5. The clarified lysate was incubated with 1 mL of Ni-NTA agarose beads (high-density nickel 6BCL-QHNi) in a 50 mL tube for 2 h at 4 °C, pre-equilibrated with binding buffer 1.
6. Beads were loaded into a column and washed five times with binding buffer 1 (~8 column volumes) and washing buffer 1 (~7 column volumes),
7. bioSUBEs were eluted with 1 mL of elution buffer 1. Repeat this step at least five times. Check the protein peak by Bradford assay.
8. Fractions containing bioSUBEs were pooled and dialyzed (dialysis tubing, cutoff 3.5 kDa) overnight at 4 °C against dialysis buffer (*see* **Notes 6** and **7**).
9. Estimate protein concentration by UV absorbance at 280 nm using the coefficient extinction 5750 and M.W. (molecular weight): 1475 kDa.

3.2 In Vitro Biotinylation Assay

Biotinylation reaction was performed in a buffer containing 5 mM ATP (Adenosine triphosphate) (*see* **Note 8**), 0.3 mM biotin (*see* **Note 9**), 30 μL of purified GST-BirA packed beads, and 150 μg of purified 6xHIS-bio-6xHIS-SUBE (+) for 5 hour at room temperature (RT) (*see* **Note 10**). As a negative control the same assay was performed in the absence of biotin (−). Biotinylated proteins and control were dialyzed twice in PBS 1× using Slide-A-Lyzer-7kD (*see* **Note 11**).

3.3 In Vitro SUMOylation Assay

For the SUMOylation assays, in vitro-transcribed/translated IkBα [25] or p53 [26] proteins were incubated in a buffer containing an ATP-regenerating system, 5 μg of SUMO2 and 5 μg of SUMO3, Ubc9 (0.325 μg), and 0.8 μg of purified SAE1/2. For the formation of SUMO2/3 chains, no protein substrate was added to the previously described assay. Reactions were incubated at 30 °C for 2 h before realizing protein pull-down (Beads) or stopped by addition of 2× Laemmli Buffer (input).

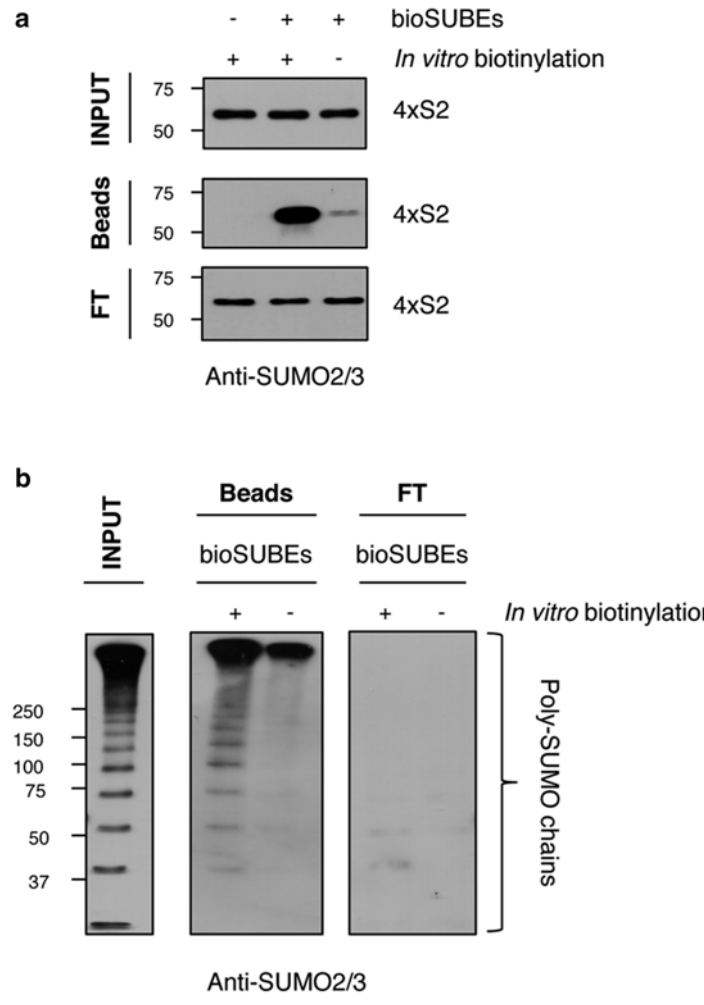

Fig. 2 Capture of poly-SUMO2/3 chains using bioSUBEs. (**a**) SUMO chains containing four SUMO2 molecules (4xS2) or (**b**) SUMO2/3 chains generated in vitro were incubated with biotinylated (+) or non-biotinylated (–) bioSUBEs. Input, flow-through (FT), and bound fractions (beads) were analyzed with anti-SUMO2/3 antibody

3.4 Protein Pull-Down Assays

1. Biotinylated SUMO-traps (and non-biotinylated control; 100 µg each (*see* **Note 12**)) were incubated with the previously reported tetra-SUMO2 fusion protein (4xS2, generously provided by R.T. Hay, Fig. 2a), poly-SUMO2/3 chains (obtained from in vitro SUMOylation assay, Fig. 2b), or in vitro SUMOylated substrates (Fig. 3), for 30 min at 4 °C in lysis buffer 2 supplemented with 1× protease inhibitor cocktail and PMSF.
2. SUMOylated proteins were then purified using high-capacity neutravidin agarose beads according to the manufacturer's instructions in the presence of 0.1 mM DTT for 3 h at 4 °C (*see* **Note 13**).

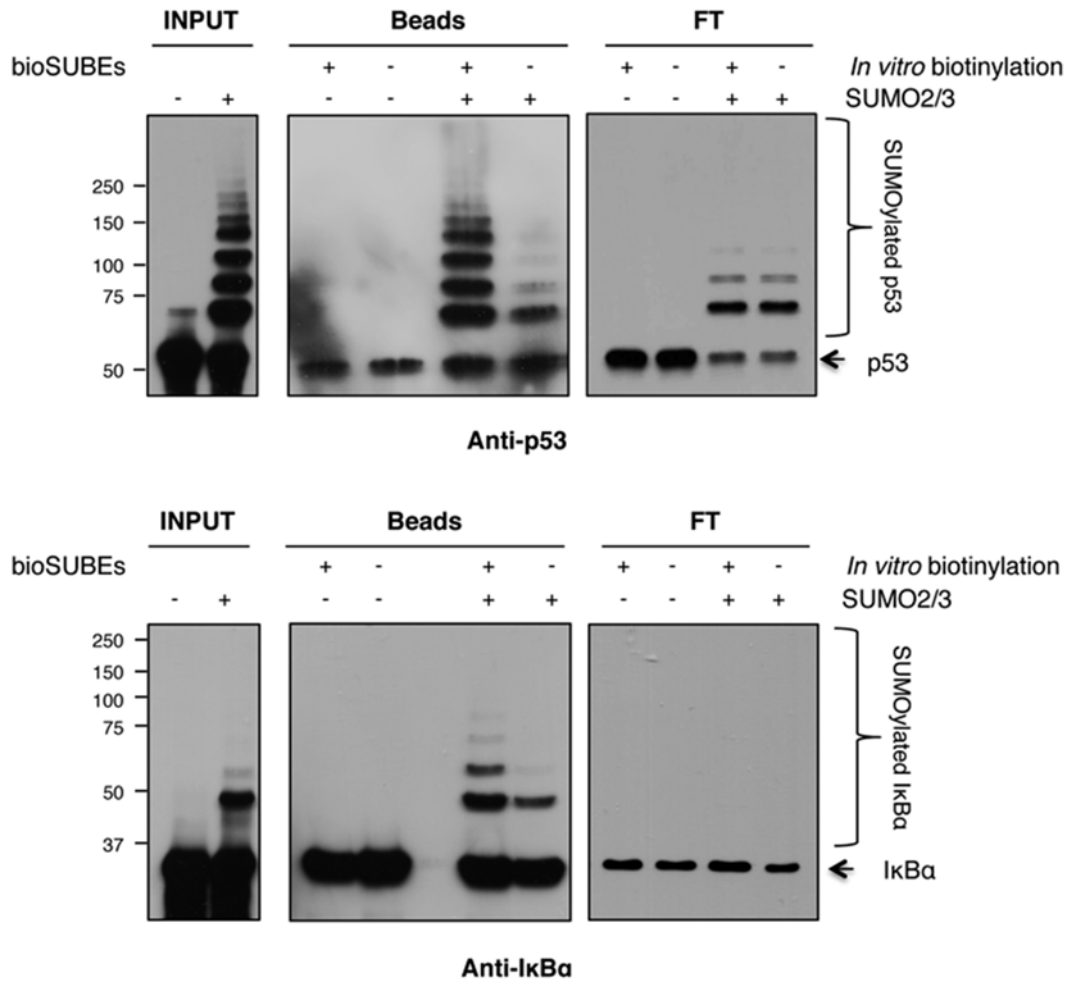

Fig. 3 Capture of SUMOylated p53 and IkBα using biotinylated SUMO traps. p53 (*upper panel*) and IkBα (*lower panel*) were SUMOylated in vitro with SUMO2/3 and incubated with biotinylated (+) or non-biotinylated (–) SUBEs. Control reactions (–) do not contain SUMO-activating enzyme. Input, flow-through (FT), and bound fractions (beads) were analyzed with anti-p53 and IkBα antibodies as indicated. The *arrow* shows unmodified proteins

3. After incubation, beads were pulled down by centrifugation, as previously described, and 1/10th of the unbound fraction (FT) was kept for western blot analysis.
4. Subsequently, the same beads were washed with 30 column volumes of washing buffer 2.
5. Beads were resuspended in one column volume of 2× Laemmli Buffer.

3.5 Western Blot Analysis

For western blot analysis, samples were separated in 12% polyacrylamide gels and membranes were incubated with anti-SUMO2/3 (rabbit polyclonal), anti-IkBα (mouse) and anti-P53 (DO1, mouse monoclonal) antibodies as indicated in the figures.

4 Notes

1. SIM-a: [PILVM][ILVM]X[ILVM][DSE>]{3} = a consensus of seven residues where the first can be either P (proline) and X is any amino acid, I (isoleucine), L (leucine), V (valine), or M (methionine); the second can be either I (isoleucine), L (leucine), V (valine), or M (methionine); the third can be any amino acid; the fourth can be either I (isoleucine), L (leucine), V (valine), or M (methionine); and the last three residues {3} can be either D (Aspartic acid), S (Serine), E (Glutamic acid), or >, the "end of sequence" [13].

   SIM-b: [PILVM][ILVM]DLT = a consensus of three residues, the first can be either P (proline), I (isoleucine), L (leucine), V (valine), or M (methionine); the second can be either I (isoleucine), L (leucine), V (valine), or M (methionine); and the last three residues are a D (aspartic acid), L (leucine), and T (threonine) [13].

   SIM-r: [DSE]{3}[ILVM]X[ILVMF]{2} = a consensus of seven residues, the first three residues {3} can be either D (aspartic acid), S (serine), or E (glutamic acid); the second can be either I (isoleucine), L (leucine), V (valine), or M (methionine); the third can be any amino acid; the fourth can be either I (isoleucine), L (leucine), V (valine), M (methionine), and F (phenylalanine); and the last two residues {2} can be either M (methionine) or F (phenylalanine) [13].

2. Bacterial strains that overexpress BirA (e.g., Lucigen biotin Xcell) may also increase the proportion of biotinylated SUBEs. This method could be preferred over random chemical biotinylation since a single, orientated biotin will be incorporated and SUBEs themselves will not be modified. Excessive biotinylation of SUBEs could result in the inhibition of its capacity to capture SUMOylated proteins.

3. To increase reproducibility of in vitro biotinylation, we work with the same batch of purified GST-BirA. A big batch of IPTG-induced bacterial expression of GST-BirA can be produced. Bacteria can be divided into several aliquots after washing with PBS and pellet kept at −20 °C for short-time storage (<1 month) or −80 °C for long-time storage (<1 year).

4. Sonication conditions such as time or amplitude should be adapted for distinct sonicator models. Set up conditions by analyzing protein recovery in the soluble fraction (Fig. 1b) and function of purified proteins (Fig. 2).

5. In vitro biotinylation can be done with a purified BirA kept on beads or eluted after purification. If you decided to elute the GST-BirA, an additional dialysis after the elution with glutathione and a step of purification on GST agarose beads (1 h at 4 °C) will be

necessary. We did not observe any difference in the final capture of SUMOylated proteins using one or the other procedure.

The protocol used for elution was the following:

(a) Elute GST-BirA with 1 mL of 10 mM reduced glutathione diluted in 50 mM Tris–HCl, pH 9.5. Repeat this step at least five times. Check the protein peak by Bradford assay.

(b) Exchange buffer in PBS by using dialysis tubing with a nominal molecular weight cutoff of 3.5 kDa. Store GST-BirA in PBS at −20 °C.

(c) Estimate protein concentration by UV absorbance at 280 nm.

6. Do not freeze-thaw bioSUBEs more than twice. Prepare small aliquots and store at −80 or −20 °C, for long and short term, respectively. For long-term storage bioSUBEs aliquots should be supplemented with at least 10% of glycerol (v/v).
7. Dialysis buffer must be prepared in advance and stored at 4 °C until use.
8. Prepare small ATP aliquots. ATP is very sensitive to freeze-thaw cycles and should be avoided.
9. D-Biotin is resuspended in H_2O. Its maximum solubility is around 1 mM.
10. Time and temperature of in vitro biotinylation can be adjusted. In our hands 5 h at room temperature (25 °C) gave us the best result. However, there are some in vitro biotinylation protocols performed at 30 °C for 2 h or at 37 °C for 1 h.
11. In some experiments in vivo biotinylation is sufficient and/or gives a better result than an additional in vitro biotinylation. Some tests should be done to appreciate which conditions give better results in the capture of your SUMOylated proteins of interest.
12. The amount of bioSUBEs used for pull-downs depends on the relative abundance of the SUMOylated proteins of interest. It is recommended to set up conditions analyzing by Western blot the input, bound, and unbound material using anti-SUMO2/3 antibodies or against your proteins of interest.
13. Time of incubation with streptavidin beads (containing the bioSUBEs), with, e.g., the cell extracts, can be adjusted to improve the capture of SUMOylated substrates. The addition of SUMO proteases inhibitors might be necessary if the time of incubation is largely increased (e.g., overnight incubations).

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Chapter 9

In Vitro Characterization of Chain Depolymerization Activities of SUMO-Specific Proteases

Julia Eckhoff and R. Jürgen Dohmen

Abstract

SUMO-specific proteases, known as Ulps in baker's yeast and SENPs in humans, have important roles in controlling the dynamics of SUMO-modified proteins. They display distinct modes of action and specificity, in that they may act on the SUMO precursor, mono-sumoylated, and/or polysumoylated proteins, and they might be specific for substrates with certain SUMO paralogs. SUMO chains may be dismantled either by *endo* or *exo* mechanisms. Biochemical characterization of a protease usually requires purification of the protein of interest. Developing a purification protocol, however, can be very difficult, and in some cases, isolation of a protease in its pure form may go along with a substantial loss of activity. To characterize the reaction mechanism of Ulps, we have developed an in vitro assay, which makes use of substrates endowed with artificial poly-SUMO chains of defined lengths, and *S. cerevisiae* Ulp enzymes in crude extract from *E. coli*. This fast and economic approach should be applicable to SUMO-specific proteases from other species as well.

Key words SUMO chain, Ulp/SENP, Protease, In vitro assay, Desumoylation

1 Introduction

Proteomic studies have identified hundreds of cellular proteins that are covalently modified with SUMO [1]. For most of these SUMO substrates, the function of their sumoylation is not known, yet. The SUMO modification of a substrate alters certain parameters such as its localization, interactions with other polypeptides, or DNA binding, to name just a few [2, 3]. Which parameter is affected depends on the respective substrate. Also, like for ubiquitin, both mono- and poly-modifications are possible, and whether a substrate is decorated with only single units or with chains has a different outcome [4, 5]. The precise physiological role of SUMO chains in *Saccharomyces cerevisiae*, even though detected, is largely elusive, since yeast cells expressing a mutant version of SUMO that does not form chains do not exhibit any obvious phenotype except for a meiotic defect [6–8]. One function of SUMO chain formation is to provide a proteolytic control of sumoylated forms of a protein by

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directing them into the ubiquitin/proteasome system [9]. If recognized and targeted by SUMO-targeted ubiquitin ligases (ULS), substrates carrying poly-SUMO chains are further modified by attachment of ubiquitin [5, 10–13]. This can then lead to proteasomal degradation [9]. SUMO molecules are synthesized as inactive precursors, which require processing to expose a diglycine motif at the C-terminus, thereby becoming conjugation competent [14]. Sumoylation is reversible. Deconjugation as well as precursor maturation are carried out by specialized cysteine proteases. Two classes of SUMO-specific cysteine proteases have been identified. The first one is the ubiquitin-like protein-specific protease (Ulp/SENP) group. The second one has only recently been found when the mammalian desumoylating isopeptidase (DeSI-1) protein was identified as a SUMO-specific protease, whose active cysteine residue resides in a papain-like fold that is structurally distinct from the Ulp fold [3, 15]. In *S. cerevisiae*, to date only two SUMO-specific proteases have been identified, namely Ulp1 and Ulp2 [16–18]. In humans, there are six Ulps termed sentrin-specific proteases (SENP-1, -2, -3, -5, -6, -7) catalyzing de-sumoylation [19–24]. One reason for an increased complexity of the mammalian Ulp equipment is the existence of multiple conjugated mammalian SUMO paralogs. SUMO1 shares only ~45% sequence identity with SUMO2 and SUMO3, while the latter two are nearly identical [3]. SUMO2/3 conjugation is induced by various forms of stress, and chains form efficiently. By contrast, SUMO1 modification dominates under non-stressed conditions, and formation of SUMO1 chains is inefficient [25–27]. SENP enzymes display distinct specificities or preferences for the different SUMO paralogs as well as for single SUMO moieties or SUMO chains [19–24].

In general, a biochemical characterization of the activity of a protease requires a purification strategy. Once the hurdle of expressing the protein in a soluble state has been overcome, it is usually separated from the pool of other components present in the expression host, commonly *Escherichia coli*. However, purification is often not only tedious but also, for some proteins, comes along with severe loss of activity. Here we describe an in vitro assay to characterize the chain depolymerization activity of SUMO-specific proteases that works with the enzymes in crude extracts from *E. coli*. The approach can also be used to test whether a certain form of a protein represents a sumoylated form of it. Instead of purified Ulp, this assay only requires the enzyme to be expressed actively in *E. coli*, hence avoiding purification, which can be costly, both in terms of time and resources.

We developed this assay for the yeast ubiquitin-like protein-specific protease 2 (Ulp2) [28]. Ulp2 is a 1034-amino acid protein with an important function in controlling cellular levels of SUMO chains [8, 17, 18]. As other studies showed, Ulp2 does not lend itself well to in vitro analysis, as it is poorly expressed in *E. coli*, coming out with just little activity [17]. As we were interested in the mechanism

by which Ulp2 dismantles SUMO chains, we needed an assay in which the cleavage reaction can be observed until completion. Marginal activity was not enough. In brief, we designed an artificial substrate with a chain consisting of five Smt3 moieties linked to enhanced GFP (eGFP) [28]. The most distal Smt3 moiety is a full-length Smt3, whereas all subsequent units were N-terminally truncated by 17 residues. This design was chosen to closely mimic the linkage pattern of native Smt3 chains, whose units are commonly linked via an isopeptide bond connecting the terminal glycine of one Smt3 molecule to one of several possible lysine residues (in most cases K11, K15, or K19) in a flexible N-terminal extension of the next Smt3 unit. It is nearly impossible to isolate native poly-SUMO chains of defined length from yeast. We verified the integrity of our strategy by performing the assay with a substrate linked to natural lysine-linked poly-SUMO chains generated in a reconstituted sumoylation system in *E. coli* [28, 29]. Therefore, our artificial substrate chains create a suitable model, and also allow for testing protease activity/affinity towards chains of defined length and composition [28].

We have chosen green fluorescent protein (GFP) as a mock substrate because of its stability conferred by the beta-barrel fold [30]. Additionally, its green color conveniently allows tracing the fusion protein throughout the purification process.

Here we describe the approach for Ulp2, but the assay has been successfully employed for analyzing Ulp1, as well [28]. Using this assay, we were able to show that Ulp1 acts on Smt3 chains by an *endo* mechanism, meaning that it stochastically cleaves any of the bonds between an Smt3 moiety and the polypeptide it is linked to, irrespective of whether it is another Smt3 moiety or any other polypeptide. Ulp2, by contrast, acts by an *exo* mechanism [28]. It disassembles Smt3 chains from their distal end by releasing single Smt3 moieties (Fig. 1). It requires a minimum of three Smt3 moieties to bind, and therefore stops when only two SUMO moieties are left on the substrate [28]. Using defined linear substrates, which form the basis for the method described here, and either enzyme dilutions or time courses of their action, allows to readily distinguish between the *endo* and *exo* modes of Ulp enzymes (Fig. 2).

If applied to other SUMO proteases with specificity for distinct SUMO orthologs or paralogs, the substrates should be chosen accordingly. We successfully cloned, expressed, and purified chains of SUMO1 and SUMO2 using the same procedure as described below for poly-Smt3 chains.

2 Materials

Prepare all solutions using deionized ultrapure water. It is not necessary to filter any of the buffers prior to usage. Use sterile (autoclaved) LB medium and glucose solution. Pass additive stocks

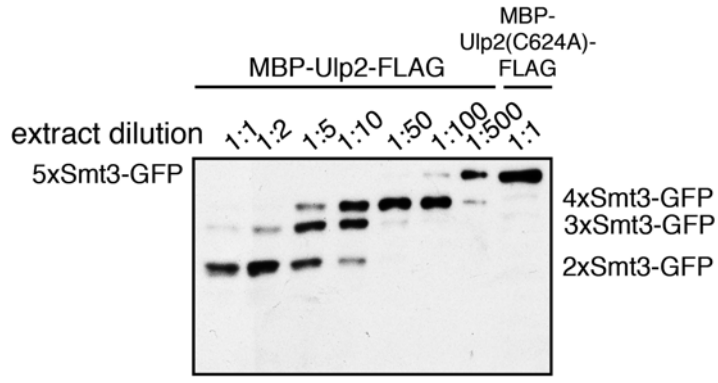

Fig. 1 Example of cleavage assay analysis. 5xSmt3-GFP substrates were incubated with *E. coli* lysates containing Ulp2 diluted in activity test buffer ("1:1" = undiluted lysate) for 2 h at 30 °C. As a control, a lysate was used that contained the inactive (inact.) variant of Ulp2(C624A). Reaction products were then analyzed by SDS-PAGE and anti-HA Western blotting. The full-length substrate is indicated on the *left-hand side* of the blot, and cleavage products are indicated on the *right*. "This research was originally published in the *Journal of Biological Chemistry*. J. Eckhoff and R.J. Dohmen. In vitro studies reveal a sequential mode of chain processing by the yeast SUMO (Small Ubiquitin-related Modifier)-specific protease Ulp2. 2015; 290:12268-12281. © the American Society for Biochemistry and Molecular Biology." [28]

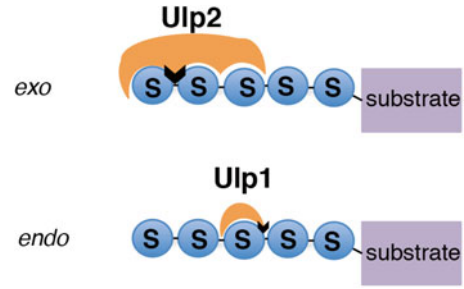

Fig. 2 Schematic representation of *exo* and *endo* cleavage mechanisms exemplified for Ulp2 and Ulp1. Ulp2 binds to three Smt3 units and works by cleaving single Smt3 units off the end of a chain (*exo*). Ulp1 requires only a single Smt3 molecule to bind and can cleave randomly after any Smt3 moiety inside the chain (*endo*). S = Smt3. "This research was originally published in the *Journal of Biological Chemistry*. J. Eckhoff and R.J. Dohmen. In vitro studies reveal a sequential mode of chain processing by the yeast SUMO (Small Ubiquitin-related Modifier)-specific protease Ulp2. 2015; 290:12268-12281. © the American Society for Biochemistry and Molecular Biology." [28]

(except for chloramphenicol) through sterile filter before usage. Make sure to subject any waste that had contact with bacteria to autoclaving before disposal.

2.1 Expression of Ulp2 and Substrate Chains

1. *E. coli* BL21-CodonPlus cells.
2. LB agar plates: 10 g/l Tryptone, 5 g/l yeast extract, 10 g/l NaCl, 2 % agar.
3. 500 ml Erlenmeyer flasks, sterilized.
4. LB medium: 10 g/l Tryptone, 5 g/l yeast extract, 10 g/l NaCl.
5. 50 % Glucose stock solution, sterilized.
6. 30 mg/ml Chloramphenicol stock solution (in EtOH).
7. 100 mg/ml Ampicillin stock solution, sterilized.
8. 1 M Isopropyl-β-d-thiogalactopyranoside (IPTG) stock solution, sterilized.
9. An autoclave.
10. A temperature-controlled shaker/incubator that can accommodate 15-ml glass tubes and 500 ml flasks and can be set to either 20, 30, or 37 °C.
11. A spectrophotometer and cuvette to measure absorbance at 600 nm.
12. A refrigerated centrifuge with rotor fitting 50-ml conical tubes capable of spinning at 2800 × *g*.

2.2 Substrate Chain Purification

1. A refrigerated room set at 4–6 °C.
2. Cell lysis buffer (substrate chains): 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 % glycerol, 1 mM $MgCl_2$, 1× protease inhibitor cocktail (cOmplete, EDTA-free; Roche), 2.5 mg/ml lysozyme, 0.8 mg/ml DNaseI, 1 mM PMSF.
3. 1.5 and 2 ml reaction tubes.
4. Glass beads with a diameter of 0.1–0.11 mm.
5. Vortex.
6. A high-speed centrifuge for 1.5- or 2-ml reaction tubes (e.g., Eppendorf refrigerated centrifuge).
7. Ni purification buffer: 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 10 % glycerol, 1 mM $MgCl_2$.
8. 1 M Imidazole stock solution.
9. Ni Sepharose™ High Performance (GE Healthcare).
10. Disposable drop column.
11. Rotating device (wheel or roller mixer).
12. Buffer exchange system (e.g., PD-10 column (GE Healthcare), spin concentrator, or dialysis equipment).
13. FLAG purification buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 % glycerol, 1 mM $MgCl_2$.
14. Anti-FLAG M2 resin.

15. FLAG peptide.
16. PCR tubes.
17. Liquid nitrogen.

2.3 Preparation of Cell Extract Containing Ulp2

1. A refrigerated room set at 4–6 °C.
2. Extract buffer: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM DTT, 4 mM $MgCl_2$, 1 mM EDTA, 2.5 mg/ml Lysozyme, 1× protease inhibitor cocktail (e.g., complete, EDTA-free; Roche), 0.8 mg/ml DNaseI, 1 mM PMSF.
3. 1.5- and 2-ml reaction tubes.
4. Glass beads with a diameter of 0.1–0.11 mm.
5. Vortex.
6. A high-speed centrifuge for 1.5- or 2-ml reaction tubes (e.g., Eppendorf refrigerated centrifuge).

2.4 Ulp2 Activity Assay

1. Protein LoBind tubes (Eppendorf).
2. Activity test buffer (ATB): 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10 mM DTT, 1 mM EDTA.
3. An incubator set at 30 °C.
4. 6× Laemmli buffer: 380 mM Tris–HCl, pH 6.8, 60% glycerol, 12% SDS, 0.015% bromophenol blue.
5. PCR tubes (preferably strips).
6. A thermocycler.

2.5 Ulp2 Activity Assay

1. SDS-PAGE equipment.
2. Blotting paper.
3. Nitrocellulose membrane.
4. Western blot device.
5. PBS: 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4.
6. Nonfat milk powder.
7. PBS-T: PBS + 0.1% Tween 20.
8. A shaking device to incubate blots on.
9. 3F10 anti-HA antibody.
10. Horseradish peroxidase-coupled goat anti-rat IgG.
11. An ECL detection system.

3 Methods

3.1 Cloning

The gene encoding the enzyme of interest is expressed as a maltose-binding protein (MBP) fusion construct from the "tac" promoter. This is achieved by cloning the target gene into the multiple cloning site of the commercially available pMALc2x vector (NEB) using restriction enzymes. If desired, a TEV protease recognition site (ENLYFQG) can be included in the primer design. Even though pMALc2x contains a factor X cleavage site to remove the MBP-tag, one might want to opt for the more specific TEV protease if considering cleaving off the N-terminal appendix at some point. In addition, a C-terminal tag can be introduced via a suitable reversed primer. In our hands, FLAG tag proved to be a good choice for this purpose. Our expression plasmid for MBP-ULP2 is called pJE12 and will be referred to like that in the following description.

The ORF for the substrate chain is generated in a multistep procedure: First, a fusion of sequences encoding ubiquitin (UBI) and an enhanced version of GFP (EGFP) is prepared via overlap extension PCR, introducing an NsiI restriction site in between the two genes. The resulting fragment, containing the sequencing encoding an EcoRI site followed by a FLAG tag plus a subsequent SacI site at the N-terminus, and an HA tag followed by a KpnI site at the C-terminus, is then ligated into a derivative of pET11a. This vector adds a C-terminal 6xHis tag to the ORF. Since SUMO contains an EcoRI restriction site, it is necessary to first introduce UBI into the construct, and then substitute it with SMT3 via digestion by SacI/NsiI. To this end, generate a fragment encoding SMT3 with a N-terminal SacI site and a C-terminal NsiI site.

To generate a clone encoding a SUMO chain, make use of the NsiI site: generate amplicons encoding N-terminally truncated Smt3 and bearing a 5′ NsiI site as well as a 3′ PstI site. These can now be introduced into the construct one by one, utilizing the compatibility of NsiI- and PstI-generated sticky ends. The final construct is expressed from the T7 promoter. For simplicity, our substrate expression plasmid (pFLAG-Smt3-4xΔ_{17}Smt3-eGFP-HA-6xHis) will be referred to as pJE10 in the protocol.

3.2 Protein Overexpression in E. coli

Apart from the substrate and extract containing Ulp2, a control extract lacking Ulp activity is needed for the desumoylation assay. To this end, transform BL21-CodonPlus cells with the MBP-Ulp2(C624A)-FLAG fusion protein expression vector and proceed as described below for the active variant. Ulp/SENP enzymes are cysteine proteases, and the C624A mutation hits the active cysteine of Ulp2, thereby rendering the enzyme inactive.

1. Transform competent *E. coli* BL21-CodonPlus cells with the MBP-Ulp2-FLAG fusion protein expression vector (pJE12). Do the same with the vector containing the ORF of the substrate chain 5xSmt3-GFP (pJE10). Select transformants on LB

agar plates supplemented with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. Incubate the plates for ~16 h at 37 °C.

2. Pick cells from one colony of the pJE12-transformation, and transfer them to 5 ml LB medium containing 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, and 1% glucose. Pick cells from one colony of the pJE10-transformation, and transfer them to 5 ml LB medium containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. Grow both cultures overnight shaking at 180 rpm and 37 °C.
3. The next morning, inoculate 50 ml of the same media as used for the overnight cultures in 500-ml Erlenmeyer flasks with 0.5 ml of the saturated overnight cultures.
4. Grow the cells at 37 °C with shaking to mid-log phase (OD_{600} ~ 0.5–0.6).
5. Briefly cool down the cultures by placing flasks on ice.
6. Add IPTG:
   (a) For Ulp2 expression to a final concentration of 1 mM.
   (b) For substrate expression to a final concentration of 0.5 mM.
7. Allow expression for:
   (a) ~20 h while shaking at 160–200 rpm at 20 °C.
   (b) 3.5 h while shaking at 160–200 rpm at 30 °C.
8. Measure the OD_{600} of the cultures.
9. Transfer the entire volume of each culture to a 50-ml conical centrifuge tube and pellet the cells by centrifugation (2800 × *g*) for 10 min at 4 °C.
10. Discard the supernatant and resuspend each pellet in 25 ml ice-cold ddH_2O.
11. Pellet cells again (2800 × *g*, 10 min, 4 °C).
12. Discard the supernatant and store pellets at −20 °C for one night or longer (until further processing). This freezing will aid breaking the cells in subsequent cell lysis steps.

3.3 Substrate Chain Purification

Perform all steps at 4 °C. This also applies to centrifugation steps. Ideally, work in a 4 °C room. Unless stated differently, all steps can be regarded to have the addition "at 4 °C." Even though it is possible to pause the procedure by snap-freezing the eluate of the first purification and storing it at −80 °C, the quality of the final product is higher if the purification is done in one go.

1. Thaw a cell pellet from *E. coli* cells expressing FLAG-Smt3-4xΔ_{17}Smt3-eGFP-HA-6xHis (pJD12) on ice.
2. Add 15 μl lysis buffer per 1 OD_{600} of cells.

3. Resuspend cells by gentle manual shaking. Avoid protein degradation by foaming.
4. Incubate cell suspension for 5 min on the bench, and then for another 5 min on ice.
5. To each 1 ml of suspension, add 500 µl glass beads.
6. Subject the lysate to vigorous vortexing for 1 min, followed by 1-min incubation on ice. Repeat three times.
7. Pellet the insoluble cell debris (and proteins) by centrifuging the lysate at 30,000 × *g* for 20 min.
8. Transfer the supernatant to a 15-ml conical tube. Make sure not to transfer any pelleted material.
9. Add NaCl to a final concentration of 500 mM and imidazole to a final concentration of 20 mM.
10. Dilute 1:3 in Ni purification buffer containing 20 mM imidazole.
11. Add ~300 µl Ni sepharose beads.
12. Incubate on a rotating device for 30 min.
13. Transfer resin to drop column.
14. Wash with 15 ml Ni purification buffer containing 20 mM imidazole.
15. Transfer resin to 1.5-ml reaction tube.
16. Add 500 µl Ni purification buffer containing 200 mM imidazole to the resin.
17. Incubate on a wheel for 5 min.
18. Sediment the resin beads by centrifuging at 100 × *g* for 1 min.
19. Carefully transfer the supernatant to a fresh tube. Make sure not to transfer any resin! Rather do not take the entire volume to avoid accidently transferring beads along with the supernatant. It helps to let the resin settle for a few minutes after centrifugation.
20. Repeat **steps 16–19** four times. Pool all eluates.
21. Exchange the buffer to FLAG purification buffer using your favorite procedure. Several methods are possible: PD-10 columns, dialysis, repeated dilution and concentration in spin concentrators. In the latter case, make sure not to reduce the volume of the sample. The protein might precipitate.
22. Add ~50 µl equilibrated anti-FLAG M2 resin to the protein solution.
23. Allow specific binding by incubation on a wheel for 1.5 h.
24. Sediment the beads by centrifugation (100 × *g*, 1 min).
25. Discard the supernatant.

26. Add 1 ml FLAG purification buffer.
27. Sediment the beads by centrifugation (100 × *g*, 1 min).
28. Discard the supernatant.
29. Repeat **steps 26–28** five times. In the process, transfer the resin to a fresh tube twice (e.g., after the second and the fourth washing steps). This helps to get rid of unbound proteins.
30. Add 400 µl FLAG purification buffer containing 150 µg/ml FLAG peptide.
31. Incubate on a wheel for 3 h.
32. Sediment the resin beads by centrifugation (100 × *g*, 1 min).
33. Carefully transfer the supernatant to a fresh tube. Again: Make sure not to transfer any resin.
34. Aliquot the eluate into 200-µl tubes (PCR tubes) (*see* **Notes 1** and **2**).
35. Snap-freeze.
36. Store at −80 °C until usage.

3.4 Preparation of Cell Extract Containing Ulp2

Perform all steps at 4 °C. This also applies to centrifugation steps. Ideally, work in a 4 °C room. Unless stated differently, all steps can be regarded to have the addition "at 4 °C." To obtain control extract, use a pellet of a MBP-Ulp2(C624A)-FLAG expression culture and follow the procedure described below.

1. Thaw a cell pellet of *E. coli* cells expressing MBP-Ulp2-FLAG (pJD12) on ice.
2. Add 15 µl extract buffer per 1 OD_{600} of cells.
3. Resuspend the cells by very gentle manual shaking. Make sure to touch the tube as little as possible to avoid warming. Avoid foaming.
4. Incubate cell suspension for 5 min on the bench, and then for another 5 min on ice.
5. Subject the lysate to vigorous vortexing for 1 min followed by 1-min incubation on ice. Repeat three times.
6. Pellet the insoluble cell debris (and proteins) by centrifuging the lysate at 30,000 × *g* for 20 min.
7. Transfer the supernatant to a fresh reaction tube. Make sure not to carry over any pellet material. You will probably not need much of the extract, so rather take only ~50 % of the total volume than to risk disturbing the pellet.
8. Keep the extract on ice until usage.

3.5 Ulp2 Activity Assay

It is best to prepare the activity assay in a 4 °C room. If no such facility is available, do it on ice.

1. Prepare one protein LoBind tube for each test you want to do. Usually, it is sufficient to test three different concentrations of Ulp2 extract: undiluted extract, a 1:10 dilution, and a 1:100 dilution. Add one control sample containing extract containing inactive Ulp enzyme for each substrate you test.
2. Prepare serial dilutions of Ulp2 extract in activity test buffer.
3. Thaw one aliquot of the substrate preparation.
4. Prepare a suitable dilution of the substrate solution (*see* **Note 3**).
5. Add 12 µl ATB to each tube.
6. Add 2 µl of the substrate dilution to each tube.
7. Add 6 µl of the extract or the appropriate extract dilution to each tube.
8. Mix by pipetting up and down (*see* **Note 4**).
9. Incubate at 30 °C for 2 h.
10. Spin down the reactions at 30,000 × *g* for 5 min at 4 °C.
11. For each reaction, transfer 17 µl to a fresh tube (*see* **Note 5**). Discard the rest.
12. Add 3 µl 6× Laemmli buffer. Mix.
13. Boil for 2–5 min at 100 °C.
14. If not directly subjected to analysis, samples can be stored at −20 °C.

3.6 Assay Analysis

1. Boil the samples briefly.
2. Spin samples down at maximum speed for 1 min.
3. Load the entire volume of each sample on a 10% SDS polyacrylamide gel.
4. Separate the samples by SDS-PAGE.
5. Transfer the proteins to a nitrocellulose membrane using your favorite system (*see* **Note 6**).
6. Block the membrane by incubation in 5% nonfat milk powder in PBS with gentle shaking at room temperature for at least 1 h.
7. Incubate the blot in a 1:5000 dilution of rat anti-HA antibody in PBS-T containing 5% nonfat milk powder with gentle shaking overnight at 4 °C.
8. Wash the blot by incubating it 3× in an excess amount of PBS-T with gentle shaking for 10 min at room temperature.
9. Incubate the blot in a 1:5000 dilution of horseradish peroxidase-coupled goat anti-rat IgG in PBS-T containing 5% nonfat milk powder with gentle shaking for 50 min at room temperature.
10. Repeat **step 8**.
11. Detect the signal by ECL (*see* **Note 7**).

4 Notes

1. It is not necessary to get rid of the FLAG peptide in the eluate. It does not interfere with the assay.
2. We find it convenient to aliquot the substrate solution into PCR tubes. The small volume allows fast thawing (several seconds on ice). The substrate chains are not suitable for storage at 4 °C once they have been thawed.
3. How much substrate you want to apply in each assay depends on the sensitivity of your detection system. You want to have a clear but not too strong signal upon anti-HA Western blotting (see Fig. 1). We recommend to estimate the appropriate dilution by subjecting several dilutions of the final protein solution obtained from FLAG tag purification to SDS-PAGE followed by Western blot detection of HA tag.
4. It is sufficient to pipette up and down 2–3 times after adding the 6 µl of extract. The reaction is very slow when the tubes are kept on ice, so you should work fast but there is no rush.
5. It is most economic to use PCR tubes for this, and do the subsequent boiling step in a thermocycler. Additionally, we find it convenient to first put the Laemmli loading buffer into the tubes and then add the reaction solution once it has been spun down.
6. We routinely apply semidry blotting, but any other blotting system should work, as well.
7. If the signal is too weak, incubate the blot for another night in primary antibody and develop it again the next day. In our hands, this has worked very well on many occasions.

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Part III

Analysis of Protein SUMOylation Using Cell Lines

Chapter 10

Detection of Protein SUMOylation In Situ by Proximity Ligation Assays

Umut Sahin, Florence Jollivet, Caroline Berthier, Hugues de Thé, and Valérie Lallemand-Breitenbach

Abstract

Sumoylation is a posttranslational process essential for life and concerns a growing number of crucial proteins. Understanding the influence of this phenomenon on individual proteins or on cellular pathways in which they function has become an intense area of research. A critical step in studying protein sumoylation is to detect sumoylated forms of a particular protein. This has proven to be a challenging task for a number of reasons, especially in the case of endogenous proteins and in vivo studies or when studying rare cells such as stem cells. Proximity ligation assays that allow detection of closely interacting protein partners can be adapted for initial detection of endogenous sumoylation or ubiquitination in a rapid, ultrasensitive, and cheap manner. In addition, modified forms of a given protein can be detected in situ in various cellular compartments. Finally, the flexibility of this technique may allow rapid screening of drugs and stress signals that may modulate protein sumoylation.

Key words Proximity ligation assay, SUMO, Ubiquitin, SUMO-interacting motif, PML nuclear bodies

1 Introduction

Detection, quantification, and analysis of endogenous SUMO-modified proteins has proven to be a challenging task. Sumoylation is a highly dynamic and transient process, which represents a constant competition between enzymes involved in conjugation and deconjugation [1]. Usually in cells, only a tiny fraction of a given protein is sumoylated and as in the case for other posttranslational modifications (PTM), this may be sufficient to carry out a specific biological function. This minute SUMO-modified version of the substrate may be restricted to particular cell/tissue types or may localize to specific subcellular compartments, such as the chromatin, nuclear pores or bodies [2]. In addition, certain sumoylated proteins may also be polyubiquitinated and thus, insoluble [3, 4]. Critically, the unconjugated SUMO1 peptide is limiting in

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quantity in vivo, pointing to the existence of a vigorous competition among substrates for modification by this peptide [5]. Indeed, most of SUMO1 is conjugated to high affinity targets such as RanGAP1, meaning that endogenous de novo sumoylation by SUMO1 necessitates deconjugation from such targets. Finally, conjugation by SUMO2/3 may occur only in response to a particular stress signal [6]. Taken together, these factors contribute to the difficulty of detecting SUMO-conjugates of a given protein at endogenous levels using conventional methods such as immunoprecipitation followed by immunoblotting. More direct and conclusive techniques such as mass spectrometry often require large amounts of starting material, which may be a challenge while working with primary cells. Similarly in vivo, mass spectrometry may be challenged by cell heterogeneity in tissues. Neither immunoprecipitation nor mass spectrometry are practical techniques to detect and quantify modifications in situ that occur in a specific subcellular compartment or organelle. Finally, sumoylation analysis in overexpression systems may introduce a bias, in particular for paralog specific conjugation [7].

Proximity ligation assay (PLA) bears the potential to address many of the obstacles encountered with the conventional methods and may be a gold standard for future routine needs for screening endogenous sumoylation and ubiquitination. PLA is a versatile technology, initially designed for high-resolution, ultrasensitive, and specific detection of closely interacting protein pairs [8, 9] This approach generally employs a pair of antibodies produced in distinct species, each one specific for a protein in a given complex. These primary antibodies are then targeted by a pair of oligonucleotide-conjugated secondary antibodies. This gives rise to an amplifiable reporter system through ligation and polymerization from the complementary oligonucleotides (Fig. 1). The combination of the requirement for extreme close proximity for ligation to occur (less than 40 nm) and PCR-based amplification reaction results in a highly resolving, specific and sensitive read-out for detection of protein pairs. Efficacy of this technique depends on primary antibody sensitivity and specificity and their concentration needs to be calibrated to avoid false positives.

The PLA technology has been used for studying phosphorylation of some proteins [10, 11] and can easily be adapted to detect other posttranslational modifications such as sumoylation or ubiquitination. In this case, one antibody is directed against the substrate “protein X” while another targets SUMO1, SUMO2/3, or ubiquitin. PLA offers a quick, cheap and ultrasensitive way for initial testing of ubiquitin-like modifications. This can then be confirmed for more robust interpretations using immunoprecipitation or mass spectrometry analyses whenever possible. In principle, PLA could detect “sumoylated protein X” fraction, but also “protein X” interacting with other sumoylated proteins. Indeed,

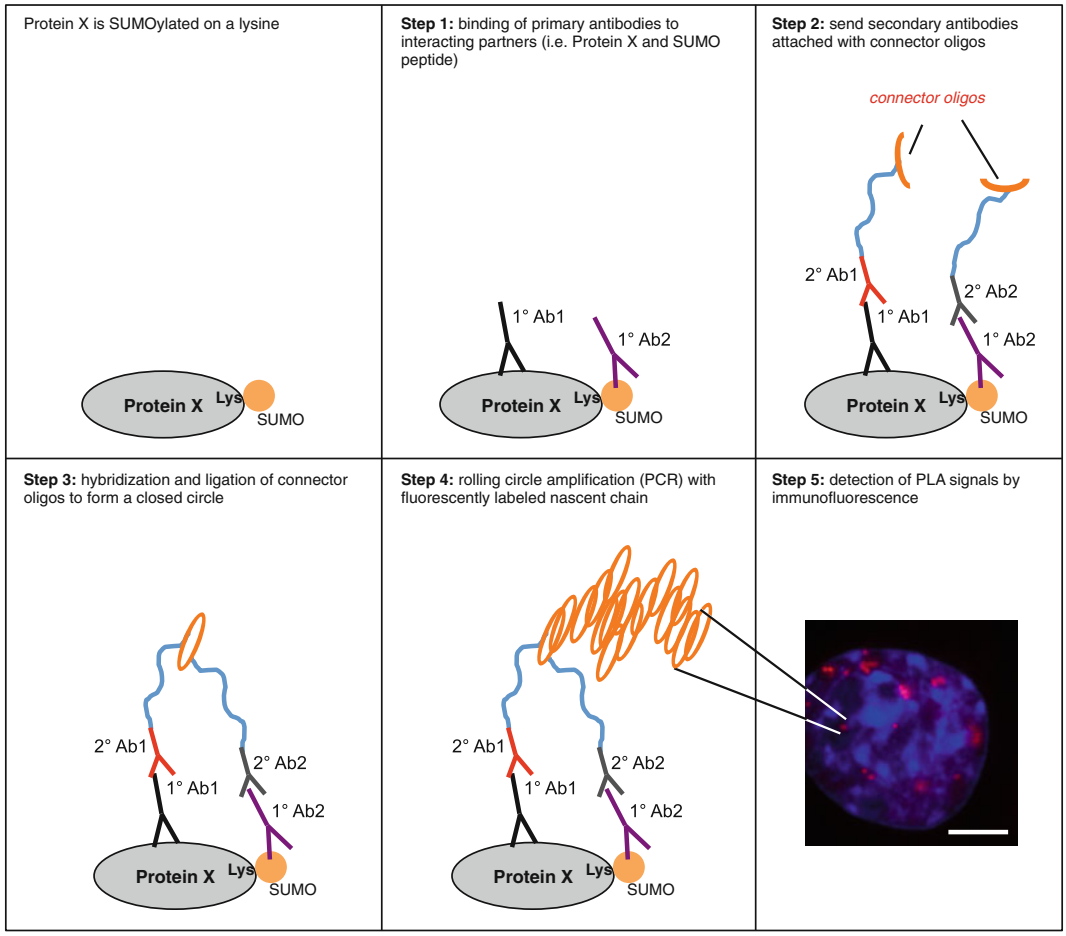

Fig. 1 Schematic representation of the PLA technology. PLA can be adapted to detect SUMO conjugation to a given Protein X (or alternatively, to detect interactions between Protein X and a sumoylated Protein Y, provided that X and Y are in extreme close proximity). Image represents a PML/SUMO2/3 PLA (*red*), DAPI (*blue*). Bar: 5 μm

SUMO peptides may be conjugated covalently to target proteins but can also interact non-covalently with proteins containing SUMO-interacting motifs (SIM). In contrast with other PTMs, sumoylation frequently targets groups of interacting proteins to secure links between partners in a complex (rather than occurring in a sequential cascade on individual proteins). This has been demonstrated for DNA repair proteins that function in a complex, which is stabilized by multiple SUMO/SIM interactions [12]. Thus, PLA may be a useful technique for studying both sumoylation of a given protein and its interactions in a complex of SIM/SUMO proteins. Nevertheless, mutating the SIMs (if any) on "protein X" or SUMO-conjugated lysine(s) on interacting partners could help to discriminate between these two situations.

To illustrate the use of PLA in detecting in situ sumoylation, we studied PML (promyelocytic leukemia), a major SUMO1 and SUMO2/3 target in cells. PML assembles in spherical nuclear subdomains called PML nuclear bodies (NBs) that recruit a large variety of proteins [13]. Recruitment of SIM-containing or sumoylated partner proteins is mediated largely by PML sumoylation [14]. We transduced *pml*$^{-/-}$ Mouse Embryonic Fibroblasts (MEFs) with wild type HA-tagged PML (HA-PML WT) or with a sumoylation-defective PML mutant (HA-PML3K/R). PLA signals (corresponding to PML/SUMO pairs) were obtained only with PML WT and not with the mutant (Fig. 2a), supporting that PLA easily permits to explore both PML sumoylation and its interactions with sumoylated proteins.

PLA is performed on a single-cell scale, allowing researchers to assess the localization of the SUMO- (or ubiquitin-) modified proteins or SUMO/SIM complexes (i.e., cytoplasm, nucleus, nuclear bodies, nuclear pore complex, lysosomes, vesicles, etc.). The assay can be performed simultaneously with immunofluorescence analysis for a third

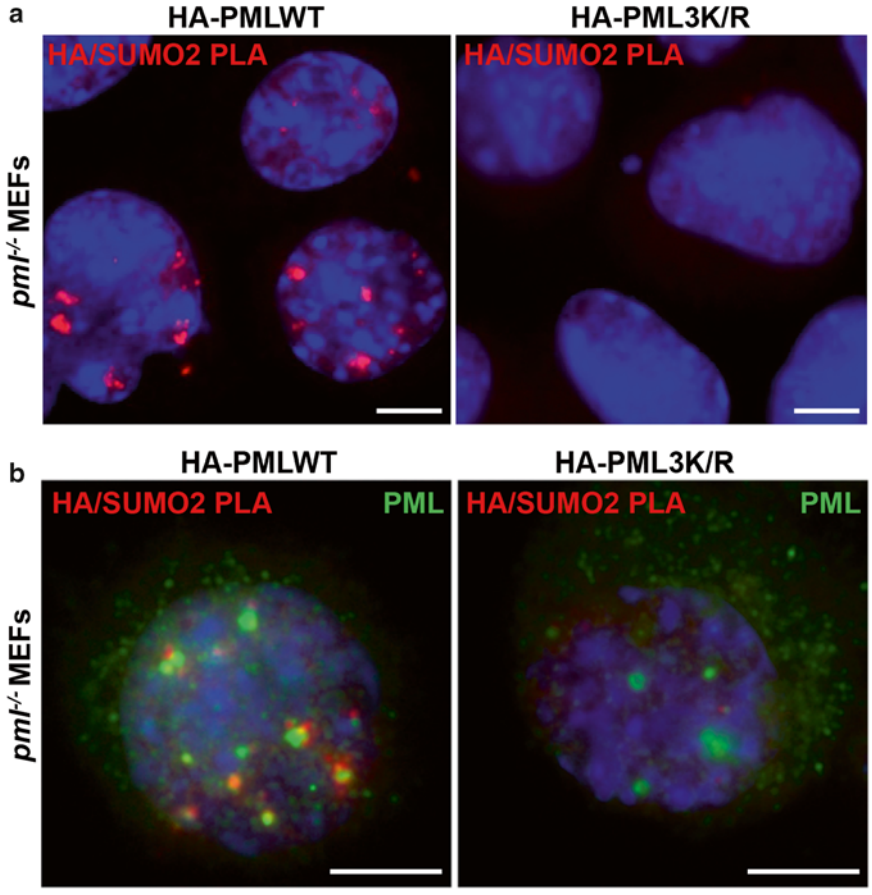

Fig. 2 PML/SUMO interactions detected by PLA. (**a**) *Pml*$^{-/-}$ MEFs were transfected with HA-tagged wild type PML or sumoylation defective PML 3K/R. Absence of PLA signals on right panel is most likely due to PML 3K/R's failure to undergo sumoylation. (**b**) PLA signals (*red*) in (**a**) combined with PML immunostaining (*green*). DAPI (*blue*). Bar: 5 μm

marker. This third antibody could label specific compartments, specific protein complexes or the total pool of the protein of interest. The latter is exemplified in Fig. 2b, where we detected PLA between PML and SUMO2/3 along with the total pool of PML protein.

Studying endogenous sumoylation in different cells (to determine tissue specificity) or assessing the effects of various drugs, treatments and stress signals on sumoylation is easy to manage with PLA. This, thereby, facilitates large scale screens. Importantly, because only a small number of fixed cells are required for PLA, an initial assessment as to whether a protein is sumoylated or involved in SIM/SUMO interactions can be performed on rare cells like stem cells. Depending of the availability of resources and material, researchers may then choose to proceed with complementary techniques such as immunoprecipitation or mass spectrometry.

Recently, using PLA, we have successfully detected SUMO1-conjugated forms of SP100, a PML NB resident protein, in situ in PML NBs (Fig. 3 and see below) [14]. Similarly, we managed to detect SUMO1-, SUMO2/3-, and ubiquitin-conjugated forms of

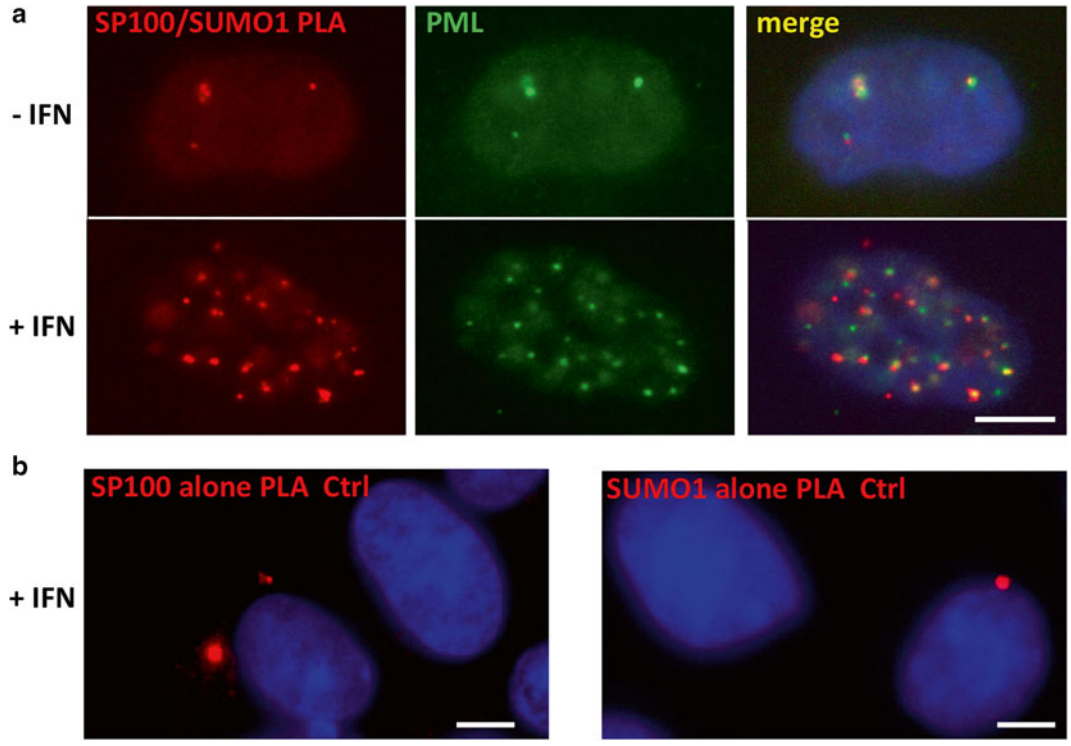

Fig. 3 Interferon-induced SP100 hypersumoylation in PML NBs as detected in situ by PLA. (**a**) HeLa cells were treated with interferon alpha (IFN) to initiate SP100 recruitment in PML NBs and hypersumoylation. SP100/SUMO PLA signals are represented in *red*, PML NBs are in *green*, DAPI in *blue*. Negative controls represent PLA performed with a single antibody (anti-SP100 or anti-SUMO only, rather than anti-SP100 and anti-SUMO in pairs) (IFN treatment: 24 h, 1000 IU/ml). (**b**) PLA controls with anti-SP100 antibody alone or anti-SUMO1 antibody alone. Bar: 5 μm

the HTLV1 Tax oncoprotein in infected T-cells of human patients with or without arsenic–interferon combination therapy [15]. The latter enhances conjugation of Tax by SUMO2/3 and ubiquitin, and tagging it for destruction in the process. We have successfully localized distinct Tax conjugates to specific cellular compartments including the cytosol, nucleus, or PML NBs. In both cases, specificity and robustness of the PLA experiments could be confirmed by immunoprecipitating larger quantities of either SP100 or Tax from the relevant cell systems. Below, we outline in detail the PLA approach to detect endogenous SP100 sumoylation in HeLa cells, in particular upon interferon alpha (IFN) treatment [14]. The technique may easily be applied to other proteins of interest, and adapted to detect conjugation by SUMO1, SUMO2/3, or ubiquitin, either in resting cells or cells undergoing specific treatments.

2 Materials

2.1 Culturing of HeLa cells, Arsenic Treatment, Harvesting, and Fixation

1. HeLa cells growth medium: Dulbecco's modified Eagle's medium (DMEM) + 5% fetal calf serum (FCS) + penicillin–streptomycin.
2. 0.25% trypsin–ethylenediamidetetraacetic acid (EDTA).
3. N-ethylmaleimide (crystalline, >98%) (*see* **Note 1**).
4. Interferon alpha (*see* **Note 2**).
5. Phosphate-buffered saline (PBS) 1×.
6. Phosphate-buffered saline (PBS) 1×, supplemented with 0.1% Tween 20.
7. Phosphate-buffered saline (PBS) 1×, supplemented with 1% bovine serum albumin and 0.05% Triton X-100.
8. Phosphate-buffered saline (PBS) 1×, supplemented with 0.5% Triton X-100.
9. 10% formalin solution, neutral buffered, containing formaldehyde.
10. Cytospin (*see* **Note 3**) (Thermo).

2.2 Proximity Ligation Assays and Detection of PLA Signals

1. PLA kit (Olink Bioscience) Red (*see* **Note 11**).
2. Rabbit anti-SP100 polyclonal primary antibody (homemade, [14]).
3. Mouse anti-SUMO1 monoclonal primary antibody (Invitrogen).
4. Chicken anti-PML polyclonal primary antibody (homemade, [16]).
5. Anti-chicken secondary antibody conjugated with FITC.
6. Bovine serum albumin.
7. 1× PLA final wash buffer: 5.84 g NaCl, 24.24 g Tris in 1 l sterile, nuclease free water. Adjust pH to 7.5.

8. Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI).
9. A fluorescent or laser scanning confocal microscope (Zeiss Axiovert or LSM 510, Zeiss).

3 Methods

A typical PLA involves three major steps:

1. Procurement of cells to be analyzed. Cells may be grown in culture or recovered directly from an animal model. Often an ex vivo or in vivo treatment is involved. Adherent cells may be grown directly on coverslips. Our preference is to fix both adherent and non-adherent cells on microscope slides using Cytospin.
2. In situ antibody incubations followed by PLA enzymatic reactions (ligation and polymerization).
3. Immunofluorescence analysis and quantification.

The protocol below describes detection of SUMO1-conjugated SP100 in adherent arsenic-treated HeLa cells.

3.1 Treatment and Harvesting of Cells

1. 24 h prior to treatment, plate HeLa cells in two 10-cm tissue culture dishes, at a density of 1.8×10^6 cells/plate (*see* **Note 4**).
2. On the next day, treat one plate with IFN at a final concentration of 1000 IU/ml for 24 h (*see* **Note 5**).
3. In order to inhibit SUMO proteases, wash cells once in 5 ml of 1× phosphate buffered saline (PBS) supplemented with 10 mM *N*-ethylmaleimide. This step will disfavor deconjugation of SUMO from its targets, including SP100.
4. Detach cells in 3 ml of 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA). Incubate for 5 min at 37 °C.
5. Stop trypsinization by adding 7 ml of HeLa growth medium. Transfer 10 ml of the cell suspension in a 15 ml Falcon tube. Pipette up and down several times to further dissociate cell clusters.
6. For each condition (untreated and IFN treated) fix 100 μl of cell suspension on microscope slides using Cytospin (*see* **Note 6**) in quadruplicates (*see* **Note 7**).
7. From this point on, all antibody incubations and PLA enzymatic reactions will be performed directly on fixed cells on microscope slides. In order to keep reagents localized on cells during incubations we mark the exterior margin of cells in a circular fashion using a hydrophobic barrier pen (i.e., ImmEdge Hydrophobic Barrier Pen from Vector Laboratories) and let dry (*see* **Note 8**).

8. Add 20–30 µl of 10% formalin solution directly on cells in order to complete fixation. Incubate for 10 min at room temperature.
9. Rinse microscope slides once in 1× PBS. Gently wipe off excess liquid using a paper towel or a Kimwipes.
10. Permeabilize cells with 20–30 µl of 0.5% Triton X-100 in PBS. Incubate for 15 min at room temperature.
11. Rinse once in 1× PBS and gently wipe off excess liquid (*see* **Note 9**).

3.2 Proximity Ligation Assay: Antibody Incubations and Enzymatic Reactions

1. In order to reduce background noise and nonspecific antibody attachment, saturate (block) fixed cells in 20–30 µl of PBS 1×, supplemented with 1% bovine serum albumin and 0.05% Triton X-100 for 30–60 min at room temperature. Rinse once in PBS 1×, supplemented with 0.1% Tween 20.
2. Dilute all primary antibodies 400× in PBS 1×, supplemented with 1% bovine serum albumin and 0.05% Triton X-100. Prepare one set of each of the following primary antibody dilutions: (a) mouse monoclonal anti-SUMO1, (b) rabbit polyclonal anti-SP100, (c) mouse monoclonal anti-SUMO1 + rabbit polyclonal anti-SP100, (d) mouse monoclonal anti-SUMO1 + rabbit polyclonal anti-SP100 + chicken polyclonal anti-PML.
3. Add 20–30 µl of each of the four antibody dilutions on quadriplicate samples. Incubate for 1 h at room temperature.
4. In order to wash off excess primary antibody, incubate slides for 15 min at room temperature in PBS 1×, supplemented with 0.1% Tween 20. Change wash buffer every 5 min.
5. For each condition, dilute oligonucleotide-conjugated secondary antibodies from the PLA kit as indicated : 6 µl mouse secondary antibody (PLUS) + 6 µl rabbit secondary antibody (MINUS) + 28 µl PLA dilution buffer (provided in the kit along with secondary antibodies). Add 40 µl of the secondary antibody mix on cells. Incubate for 1 h at 37 °C.
6. In order to wash off excess secondary antibody, incubate slides for 15 min at room temperature in PBS 1×, supplemented with 0.1% Tween 20. Change wash buffer every 5 min.
7. In order to perform the ligation reaction, prepare the following ligation mix for each condition : 8 µl 5× ligation buffer + 1 µl ligase + 31 µl sterile nuclease free water (all reagents are provided with the kit). Add 40 µl of the ligation mix on cells. Incubate for 30 min at 37 °C.
8. Wash twice in PBS 1×, supplemented with 0.1% Tween 20 for 5 min at room temperature.
9. In order to perform the polymerization reaction, prepare the following polymerization mix for each condition: 8 µl 5×

polymerization buffer + 0.5 μl polymerase + 31.5 μl sterile nuclease free water (all reagents are provided with the kit). Add 40 μl of the polymerization mix on cells. Incubate for 100 min at 37 °C.

10. Wash twice in PBS 1×, supplemented with 0.1 % Tween 20 for 5 min at room temperature.
11. In order to co-label PML NBs, prepare a 1/300 dilution of anti-chicken secondary antibody conjugated with FITC in PBS 1×, supplemented with 1 % bovine serum albumin and 0.05 % Triton X-100. Add 20–30 μl of diluted secondary antibody on cells, incubate for 30 min at room temperature. Wash twice in PBS 1×, supplemented with 0.1 % Tween 20 for 5 min at room temperature (*see* **Notes 10** and **11**).
12. Wash slides twice in 1× PLA final wash buffer, 10 min each at room temperature.
13. Wash slides once in 0.01× PLA final wash buffer for 1 min at room temperature.
14. In order to mount slides, wipe off excess liquid using a tissue paper or a Kimwipes. Add 10 μl of Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) on cells and gently place a coverslip using a pair of fine forceps. Wipe off the excess mounting medium, then let the slides dry for a few minutes at room temperature (*see* **Note 12**).

3.3 Immunofluorescence Analysis and Quantification

PLA signals can be analyzed by a simple fluorescent microscope such as Zeiss Axiovert. Alternatively, for quantification, images should be acquired in high resolution on various consecutive z-stacks by confocal microscopy. To this end, we use either a Zeiss LSM 510 META confocal laser microscope or a Zeiss LSM 710 confocal microscope with a Plan Apochromat 63/1.4 numeric aperture oil-immersion objective using Zen 2009 (Carl Zeiss). For robust statistical analyses, depending on the number of PLA signals per cell, ideally 50–100 cells for each condition are analyzed.

PLA signals indicative of SUMO1-conjugated SP100 (but not of SUMO1 alone or SP100 alone) will appear as distinct red dots under immunofluorescence on samples incubated with both mouse monoclonal anti-SUMO1 and rabbit polyclonal anti-SP100 antibodies (Fig. 3) (*see* **Note 11**). On the other hand, cells incubated with either the mouse monoclonal anti-SUMO1 antibody alone or with the rabbit polyclonal anti-SP100 antibody alone should not display any PLA signals (*see* **Note 7**). Consistent with a role for interferon alpha in promoting SP100 hypersumoylation, SUMO1-SP100 PLA signals are dramatically enhanced in cells that have undergone IFN treatment (Fig. 3). Further labeling of PML NBs in green using a primary chicken polyclonal anti-PML antibody (and an anti-chicken secondary antibody conjugated with FITC)

indicates that SUMO1-SP100 PLA signals greatly co-localize with PML NBs, supporting a role for PML NBs in promoting SP100 hypersumoylation *in situ* (Fig. 3) (*see* **Notes 10** and **11**).

4 Notes

1. We prepare a 0.5 M stock solution of *N*-ethylmaleimide in ethanol, which can be kept at −20 °C for up to a month. Further dilutions must be made at the time of harvesting the cells. *N*-Ethylmaleimide is a highly toxic substance, and therefore, precaution must be observed while handling it.
2. We prepare fresh dilutions of interferon alpha in DMEM prior to each treatment. Keep IFN on ice at all times.
3. PLA may be performed on both adherent (i.e., HeLa, H1299, mouse embryonic fibroblasts, etc.) or non-adherent (i.e., T-cells, etc.) cells. Even though adherent cells may be grown directly on microscopy coverslips, our preference is to grow them in cell culture plates (and perform any required treatment) prior to harvesting and subsequently fixing on microscope slides using Cytospin.
4. Before performing any sort of treatment (i.e., IFN), make sure that the cells have adhered properly at a confluence of 50–70%.
5. Alternatively, arsenic trioxide may also be used, alone or in combination with IFN, to induce hypersumoylation of SP100. To this end, we treat cells with arsenic trioxide (Fluka) at a final concentration of 10^{-6} M for 2 h. Use caution while handling arsenic.
6. While fixing cells on microscope slides using Cytospin, avoid damaging cells by not spinning them at more than 600 rpm.
7. PLA signals arise from ligation and polymerization of complementary oligonucleotides attached to two distinct antibodies raised against a specific protein in a complex of two closely interacting proteins (i.e., SUMO1 and SP100 in the context of SUMO1-conjugated SP100). In order to ensure the specificity of PLA signals and assess any possible background noise, PLA must also be performed using each of the two primary antibodies alone, one at a time. These negative controls should not give rise to any PLA signals.
8. Alternatively, nail polish may be used to mark the exterior margin of cells fixed on microscope slides. Make sure that the nail polish has completely dried before proceeding with formalin fixation.
9. Fixed and permeabilized cells (on microscope slides) may be stored for several weeks at 4 °C in PBS 1×, supplemented with 0.1% Tween 20. Take necessary precautions to avoid bacterial or fungal contamination while storing the slides in this buffer. We normally do not add sodium azide at this stage.

10. Note that co-labeling is an optional step in order to localize PLA signals to specific cellular structures or compartments. In this case, SP100-SUMO1 PLA signals were co-labeled with PML NBs. One may perform PLA analyses either singly, or in combination with other immunofluorescence markers.
11. In order to distinguish between SP100-SUMO1 PLA signals and PML NB co-labeling, PLA was performed using a PLA Red kit which gives rise to red PLA signals. On the other hand, PML NBs were stained in green (using FITC conjugated secondary antibodies). Alternatively, depending on the kit, PLA signals may vary in color.
12. Mounted and dried microscope slides may be stored at 4 °C in an appropriate slide box, protected from light.

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Chapter 11

In Situ SUMOylation and DeSUMOylation Assays: Fluorescent Methods to Visualize SUMOylation and DeSUMOylation in Permeabilized Cells

Eri Yuasa and Hisato Saitoh

Abstract

This chapter deals with the fluorescence detection of SUMOylation and deSUMOylation in semi-intact cultured human cells, the so-called "in situ SUMOylation assay" and the "in situ deSUMOylation assay," respectively. In the in situ SUMOylation assay, the recombinant green-fluorescence protein fused to the SUMO1 (GFP-SUMO1) protein is used to visualize the nuclear rim, nucleolus, and nuclear bodies. These GFP signals represent cellular regions where SUMOylation efficiently takes place. If the recombinant SUMO-specific protease SENP1-catalytic domain is added after in situ SUMOylation, GFP signals can be erased. Therefore, the in situ SUMOylation assay can be used to assess deSUMOylation enzymatic activity.

Key words SUMOylation, DeSUMOylation, Fluorescence detection, Cell-permeabilization, Cell-based assay

1 Introduction

Using recombinant SUMO1 fused to yellow-fluorescent protein (YFP-SUMO1), Pichler et al. first developed a procedure to visualize the location where SUMOylation efficiently takes place in detergent-permeabilized, semi-intact cultured human cells [1]. We also observed a similar phenomenon using recombinant SUMO1 protein fused to green-fluorescent protein (GFP-SUMO1) and elucidated its rationale, leading to modification of the procedure of Picher et al. and designating our method as "in situ SUMOylation assay" [2–4].

In Fig. 1, an outline of the procedure of the in situ SUMOylation assay is illustrated. Typically, adherent-type cells are cultured, such as human cervical carcinoma HeLa or Ca Ski cells, and these cells are grown on a coverslip sunk in culture medium. The coverslip is then removed from the culture medium and the cells on the coverslip are permeabilized in situ by detergent, such as digitonin or Triton X-100. Because the in vivo cellular environment is expected

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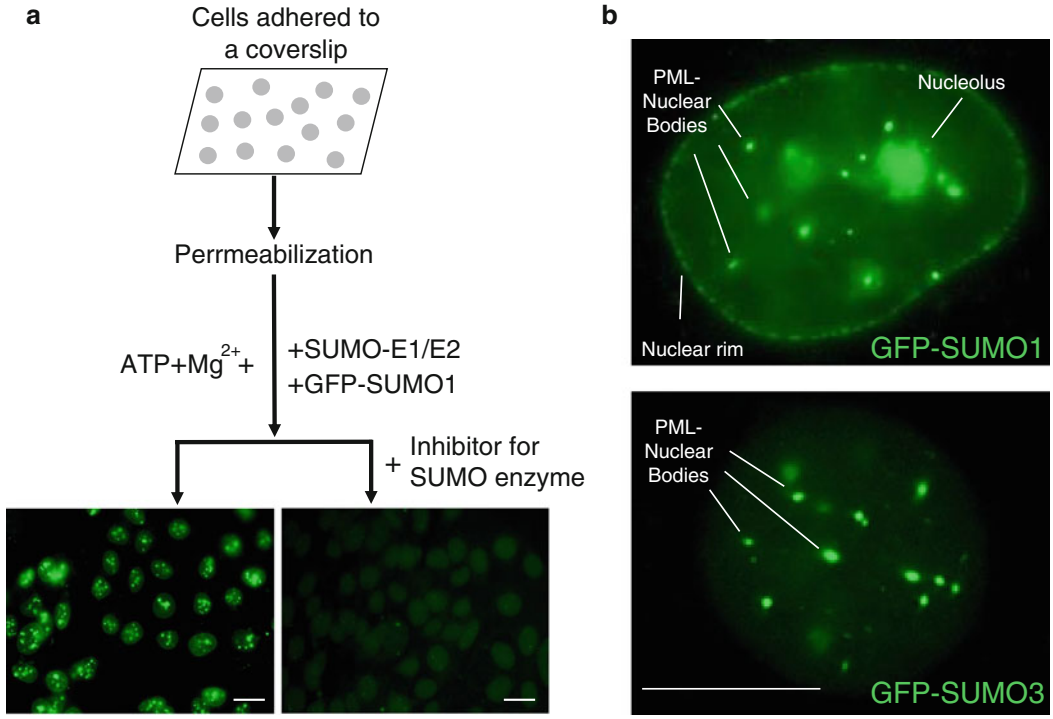

Fig. 1 Visualization of SUMOylation sites in the in situ SUMOylation assay using permeabilized Ca Ski cells. (**a**) Basic procedure and expected results of the in situ SUMOylation assay are shown. Bar represents 20 μm. (**b**) Larger view of the representative results of the in situ SUMOylation assay using GFP-SUMO1 or GFP-SUMO3 as a probe is shown. Typically, GFP-SUMO1 detects the nuclear rim, the nucleolus, and multiple PML-bodies (*upper* panel), while GFP-SUMO3 visualizes PML-bodies and barely detects the nuclear rim and the nucleolus (*lower* panel). Bar represents 10 μm

to be largely maintained in permeabilized semi-intact cells, exogenously added recombinant SUMOylation enzymes and fluorescence-labeled SUMOs (e.g., GFP-SUMO1 and -SUMO2/3) should access target sites and recognize target proteins in situ.

As shown in the bottom picture of Fig. 1, using GFP-SUMO1 we routinely detect fluorescence signals at the nuclear rim, nucleolus, and multiple nuclear bodies, which may represent promyelocytic leukemia (PML) bodies [2]. The GFP signals represent covalent attachment of the SUMO-moiety to cellular components via its C-terminal di-glycine motif (GG), and not non-covalent SUMO interactions with cellular components, because no signal is detected in the absence of adenosine 5′-triphosphate (ATP) or if the SUMOylation deficient SUMO1-delta G mutant fused to GFP (GFP-SUMO1ΔG) is added to the reaction [2–4]. Although the exact numbers and repertoire of proteins being SUMOylated in semi-intact cells remain to be clarified, the reproducibility and high signal-to-noise ratio of this assay enable the researcher to elucidate the location where SUMOylation reactions efficiently occur in semi-intact cells by microscopy.

In this chapter, we describe our detailed conditions of the in situ SUMOylation assay using GFP-SUMO1 as a probe. Additionally, this chapter includes our newly developed "in situ deSUMOylation assay," in which the activity of a deSUMOylation enzyme can be monitored by a decrease in the intensity of the fluorescent signals detected in the in situ SUMOylation assay [5].

2 Materials

Use analytical grade reagents and deionized distilled water to prepare all solutions in the protocol.

2.1 Cell Culture and Permeabilization

1. Ca Ski cells [e.g., American Type Culture Collection (ATCC) cat. no. CRL-1550] (*see* **Note 1**).
2. DMEM with l-glutamine and phenol red medium supplemented with 5% fetal bovine serum (FBS) and 100 U/mL penicillin G and 100 μg/mL streptomycin sulfate.
3. Culture dish, 10 × 35 mm.
4. Circular coverslips, 12 mm in diameter (e.g., Fisher Scientific, cat. no. 12-545-80) (*see* **Note 2**).
5. Phosphate-buffered saline (PBS): Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of distilled H_2O. Adjust the pH to 7.4 with HCl. Add H_2O to 1 L. Dispense the solution into aliquots and sterilize by autoclaving. Store this solution at room temperature.
6. 0.1% Triton X-100 solution: 0.1% Triton X-100 [polyoxyethylene (10) octylphenyl ether] in PBS. The solution is store at 4 °C.
7. Forceps.

2.2 In Situ SUMOylation Reaction

1. Recombinant E1 enzyme (*see* **Note 3**).
2. Recombinant E2 enzyme (*see* **Note 4**).
3. Recombinant GFP-SUMO1 (*see* **Note 5**).
4. 10× Reaction buffer: 500 mM Tris–HCl, pH 7.9, 1 M NaCl, 100 mM $MgCl_2$, 10 mM dithiothreitol, supplemented with 10 mM ATP.
5. Phosphate-buffered saline (PBS; *see* **item 5** in Subheading 2.1).
6. Paraffin film.
7. Humidified box: a box containing a piece of water-wetted Kimwipes, which prevents the specimen from desiccation.

2.3 In Situ deSUMOylation Reaction

1. Recombinant E1 enzyme (*see* **Note 3**).
2. Recombinant E2 enzyme (*see* **Note 4**).
3. Recombinant GFP-SUMO1 (*see* **Note 5**).

4. Recombinant *Oryzias latipes* SENP1-catalytic domain (*Ol*SENP1-CD) protein (*see* **Note 6**).
5. 10× Reaction buffer (*see* **item 4** in Subheading 2.2).
6. *N*-Ethylmaleimide (NEM) solution: 10 mM solution in ethanol. Stored at −20 °C until use.
7. Phosphate-buffered saline (PBS; *see* **item 5** in Subheading 2.1).
8. Paraffin film.
9. Humidified box: a box containing a piece of water-wetted Kimwipes, which prevents the specimen from desiccation.

2.4 Microscope Observations

1. 4% paraformaldehyde–PBS solution: Perform the following steps in the hood.

   In 10 mL H_2O, add 0.8 g paraformaldehyde. Bring to 80 °C in a 50-mL conical tube in a beaker of distilled water. Stop heating when the solution begins to clear. Add drops of 1 N NaOH until completely clear. Add 10 mL 2× PBS. Allow to cool before fixing cells. Aliquot and store at −20 °C or up to 1 month at 4 °C in a dark box.
2. 4′,6-Diamidino-2-phenylindole (DAPI) solution: Add 5 μL of 1 mg/mL DAPI. Add PBS solution to a final volume of 1 mL. Store up to 1 week at 4 °C in a dark box.
3. 1,4-Diazabicyclo[2.2.2]octane (DABCO) solution: Add 2.5 g DABCO (e.g., Wako Pure Chemical Industries, cat. no. 649-25712). Add 80 mL glycerol. Add 20 mL PBS. Aliquot into small tubes and store at −20 °C in a dark box.
4. Phosphate-buffered saline (PBS; *see* **item 5** in Subheading 2.1).
5. Nail oil.
6. Kimwipes.
7. Slide glass.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Cell Culture and Cell Permeabilization

1. Prepare a culture dish (10 × 35 mm) in which a coverslip is located (*see* **Note 7**).
2. Culture Ca Ski cells in the dish until the cell density reaches approximately 1.0×10^7 cells/mL in 2 mL of DMEM supplemented with 5% FBS, penicillin, and streptomycin in a humidified atmosphere with 5% (v/v) CO_2 at 37 °C.
3. Aspirate the medium and add 1 mL of 0.1% Triton X-100 solution. Be sure to not allow the specimen to dry completely.

4. Incubate for 20 min at room temperature to permeabilize cells on the coverslip.
5. Remove the 0.1% Triton X-100 solution and add 1 mL PBS gently, followed by incubation for 3 min.
6. Remove the PBS and add 1 mL of fresh PBS gently, followed by incubation for 3 min without allowing the specimen to dry completely (*see* **Note 8**).

3.2 In Situ SUMOylation Reaction

1. Place 50 μL of 1× Reaction buffer supplemented with 0.5 μg of purified recombinant E1, E2, and GFP-SUMO1 proteins at the center of a piece of paraffin film (5 × 5 cm). Using forceps, place the coverslip from **step 6** in the previous section face down on the solution (*see* **Note 9**).
2. Incubate for 20 min at room temperature in a humidified box without allowing the specimen to dry completely (*see* **Note 10**).
3. Take the coverslip using forceps and put it face up in a new 10 × 35 mm culture dish (*see* **Note 11**).
4. Wash three times with 1 mL PBS without allowing the coverslip to dry.
5. Take the coverslip using forceps and put it face up in a new 10 × 35 mm culture dish (*see* **Note 11**).
6. Wash three times with 1 mL PBS without allowing the coverslip to dry.

3.3 In Situ deSUMOylation Reaction

1. Perform **steps 1–4** in the previous section (*see* Subheading 3.2).
2. Place 50 μL PBS supplemented with no reagent, 0.5 μg of purified recombinant *Ol*SENP1-CD or *Ol*SENP1-CD plus 1 mM NEM at the center of a piece of paraffin film (5 × 5 cm), then using forceps place the coverslip from **step 4** in the previous section face down on the solution (*see* **Note 12**).
3. Incubate for 10 min at room temperature in a humidified box without allowing the specimen to dry (*see* **Note 13**).
4. Take the coverslip using forceps and place it face up in a new 10 × 35 mm culture dish (*see* **Note 14**).
5. Wash three times with 1 mL PBS. The coverslip should not be allowed to dry.

3.4 Microscope Observations

1. Aspirate PBS from the sample of **step 8** in the previous section and add 1 mL of the 4% paraformaldehyde–PBS solution without allowing the specimen to dry.
2. Incubate for 15 min at room temperature to fix the specimen to the coverslip.
3. Remove the 4% paraformaldehyde–PBS solution and wash three times with PBS (*see* **Note 15**).

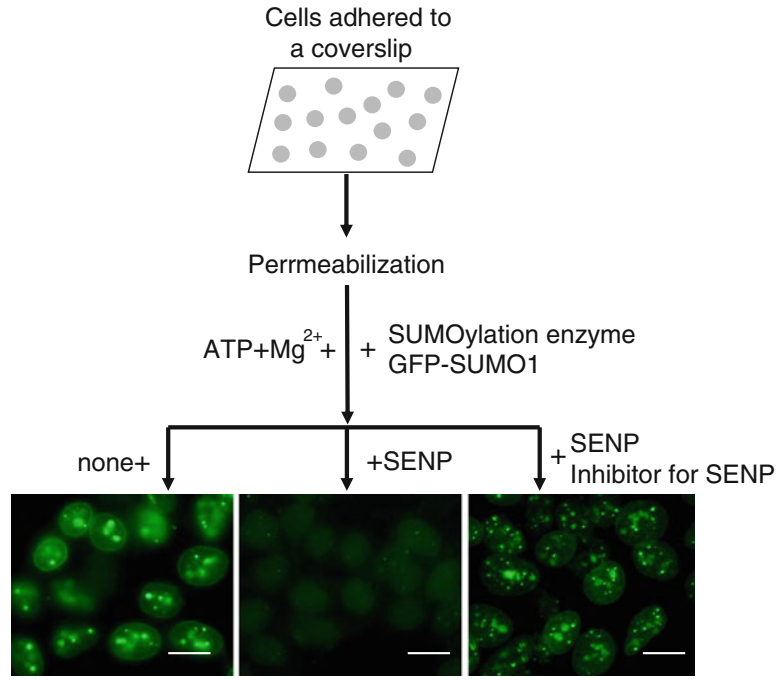

Fig. 2 Visualization of SUMOylation and deSUMOylation activities using permeabilized Ca Ski cells. Basic procedure of the experiment and results are shown. The in situ SUMOylation assay using GFP-SUMO1 as a probe is presented in the *left* panel. After in situ SUMOylation, *O*/SENP1-CD is incubated in the absence (*middle* panel) or in the presence of the SENP-inhibitor NEM (*right* panel). Bar represents 20 μm

4. Place a small drop of the anti-fade reagent DABCO solution on a glass slide and then take the coverslip from the dish and put it face down on the drop.
5. Push the coverslip gently using a finger and attach to the glass slide.
6. Remove excess liquid outside the coverslip using a piece of Kimwipes paper.
7. Seal the edges of the coverslip by nail oil and let the oil dry.
8. The slide is now ready to view under a fluorescent microscope (Fig. 2).

4 Notes

1. Other cell lines, beside Ca Ski, may be suitable to use with this assay. For example, we obtained reasonable signals when HeLa cells [American Type Culture Collection (ATCC) cat. no. CCL-2] were used in the assay [2–5]. However, from our experience, we recommend Ca Ski cells for the assay, because this cell line appears to give better signals with respect to nuclear dots, which may represent PML bodies, than HeLa cells [2].

2. Although we recommend the use of circular coverslips (12 mm in diameter), other sizes and shapes can be used.
3. We generally use the recombinant mAos1-Uba2 fusion protein expressed in a baculovirus-insect cell system as the SUMO-E1 source [4]. Bacterially expressed recombinant Aos1 and the Uba2 heterodimer are also functional in this assay [2].
4. We generally use the bacterially expressed recombinant *Xenopus laevis* Ubc9 protein as a SUMO-E2 source [2–5]. As Ubc9 is highly conserved among eukaryotes, the Ubc9 protein derived from other species is expected to be functional in this assay [1].
5. It should be also noted that researchers must use the mature/processed form of SUMO, and not the full-length/unprocessed form of SUMO. The C-terminus of the SUMO moiety in the GFP-fusion protein should contain the di-glycine motif (GG). Instead of recombinant GFP-SUMO1, recombinant GFP-SUMO2/3 can be used. However, GFP signals are different among the SUMO-subtypes; GFP-SUMO1 efficiently accumulates at the nuclear rim, nucleolus, and PML bodies, whereas GFP-SUMO3 signals are located at PML bodies and are barely observed at the nuclear rim and nucleolus (Fig. 1b). Similar results have been published [2] and indirect-immunofluorescence patterns of anti-SUMO1 and anti-SUMO2/3 antibodies are also consistent with the pattern of in situ SUMOylation, arguing for heterogeneity between SUMO1 versus SUMO2/3 subtypes [6, 7].
6. Although we use the recombinant SENP1-catalytic domain protein derived from Medaka fish (*Oryzias latipes*) [5], SENP proteins derived from other species and/or subfamilies are expected to be suitable for use.
7. All solutions and equipment coming into contact with living cells must be sterile. Before setting the coverslip in the culture dish, it should be cleaned with ethanol followed by flaming for sterilization. Two coverslips ($\varphi = 12$ mm) can be placed in a single culture dish (10×35 mm), if required for multiple reactions.
8. We recommend that, at this step, researchers should observe the specimen under a phase-contrast microscope and ensure that a reasonable number of cells have adhered onto the coverslip. We sometimes observe that very few cells are present on the coverslip after permeabilization. In such events, reduce the concentration of Triton X-100 to 0.05 or 0.02%. Alternatively, use digitonin (e.g., Sigma-Aldrich, cat. no. D-141) or saponin (e.g., Sigma-Aldrich, cat. no. 47036) as alternatives to Triton X-100 for cell permeabilization [1–5].
9. You will be able to add a chemical compound of interest into the reaction mixture, and then assess the effect of the added chemical. Several types of chemical compounds that inhibit SUMOylation activities have been identified using this assay [8–10].

10. Note which side of the coverslip the cells are adhered to and make sure the cell-adherent surface faces the reaction solution such that the cells can interact with the SUMOylation enzymes and GFP-SUMO1. Make sure the coverslip attaches to the solution and no air bubbles exist between the coverslip and the surface of the solution.
11. Make sure you know which side of the coverslip faces to cells. The cell-adherent surface of the coverslip should be facing up such that nonspecifically bound GFP-SUMO1 can be washed away efficiently.
12. You will be able to add the chemical compound of interest into the reaction mixture and monitor subsequently the effect of the added chemical. Indeed, the well-known SENP inhibitor NEM inhibits deSUMOylation activities in the in situ deSUMOylation assay (right panel in Fig. 2), suggesting that the assay is suitable for searching for chemical compounds, besides NEM, that inhibit deSUMOylation activities.
13. You have to notice which side of the coverslip has the adhered cells and make sure the cell-adherent surface faces the reaction solution such that the cells can interact with the deSUMOylation enzymes and/or drugs of interest. Make sure that the coverslip attaches to the solution and no air bubbles exist between the coverslip and the surface of the solution.
14. Make sure you know which side of the coverslip faces to cells. The cell-adherent surface of the coverslip should face up such that free GFP-SUMO1 detached from the target proteins by *Ol*SENP1-CD can be washed away efficiently.
15. After fixation, indirect-immunofluorescence analysis using specific antibodies and/or specific dye staining can be performed [1–5].

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Chapter 12

Analysis of SUMOylated Proteins in Cells and In Vivo Using the bioSUMO Strategy

Lucia Pirone, Wendy Xolalpa, Ugo Mayor, Rosa Barrio, and James D. Sutherland

Abstract

Posttranslational regulation of proteins by conjugation of ubiquitin- and ubiquitin-like molecules is a common theme in almost every known biological pathway. SUMO (small ubiquitin-related modifier) is dynamically added and deleted from many cellular substrates to control activity, localization, and recruitment of other SUMO-recognizing protein complexes. The dynamic nature of this modification and its low abundance in resting cells make it challenging to study, with susceptibility to deSUMOylases further complicating its analysis. Here we describe bioSUMO, a general method to isolate and analyze SUMOylated proteins from cultured cells, using *Drosophila* as a highlighted example. The method also has been validated in transgenic flies, as well as human cells. SUMOylated substrates are labeled by in vivo biotinylation, which facilitates their subsequent purification using streptavidin-based affinity chromatography under stringent conditions and with very low background. The bioSUMO approach can be used to validate whether a specific protein is modified, or used to analyze an entire SUMO subproteome. If coupled to quantitative proteomics methods, it may reveal how the SUMO landscape changes with different stimuli, or in diverse cell or tissue types. This technique offers a complementary approach to study SUMO biology and we expect that the strategy can be extended to other ubiquitin-like proteins.

Key words SUMOylation, Biotin, BirA, Mass spectrometry, *Drosophila*

1 Introduction

SUMO, a 90-amino acid ubiquitin-like protein, is highly conserved among eukaryotes and is one of the most studied Ubiquitin-like proteins (UbLs). Like ubiquitination, the process of SUMOylation consists of the covalent attachment of SUMO to a target substrate, involving the activation, conjugation, and ligation of the SUMO moiety by E1, E2, and E3 enzymes, respectively [1]. SUMOylation is required for various cellular and developmental processes, especially in the modification of factors involved in the regulation of gene expression and DNA damage responses, among others. There are four SUMO homologues in vertebrates (SUMO1-4) and a

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single homologue in *Drosophila* (Smt3). In this model organism, SUMOylation is essential during development; Smt3 mutants die as late embryos and early larvae, depending on allele strength and maternal contribution [2–4].

Several studies in vitro and in vivo have been made to identify SUMO-conjugates. The most common approach is based on the expression of tagged SUMO versions, allowing isolation of SUMOylated proteins by affinity chromatography and identification by mass spectrometry [4–14]. Compared to ubiquitination, the SUMO subproteome is much less abundant. The SUMOylated fraction of a given protein is usually very low, making its identification more challenging. SUMOylation is stimulated under stress conditions, so many of the published protocols use heat-shock treatments to increase the proportion of SUMOylated proteins to facilitate their detection.

Here, we describe a new technique developed for the identification of SUMOylated proteins in *Drosophila* cells and in vivo. Briefly, the method is based on the in vivo biotinylation of SUMO and its subsequent conjugation to substrate proteins, followed by purification of substrates by streptavidin-based chromatography. The bioSUMO conjugates can then be analyzed by western blot or mass spectrometry [15]. The method has been adapted from the bioUb approach, which has been successfully used in cells and in vivo to identify ubiquitinated proteins [16, 17]. In this strategy, we fuse a short peptipe (*bio*; also called AviTag) that serves as a biotinylation target for *E. coli* biotin ligase (BirA) to the N-terminus of *Drosophila* Smt3. Specifically, we use a version of Smt3 that is degenerate at the nucleotide level, which renders it insensitive to RNAi-based silencing, but encodes wild-type Smt3 protein. When coupled to RNAi of endogenous Smt3, the resulting bioSmt3 molecule is the most abundant source of cellular SUMO [18]. To facilitate the expression of both bioSmt3 and BirA in cells, we use multicistronic expression vectors (Ac5-STABLEs). Open reading frames are separated by short "2A-like" sequences, also called CHYSEL (cis-acting hydrolase element) peptides, which are derived from viral proteins that direct "ribosome-skipping" and allow stoichiometrically balanced expression of multiple proteins from the same vector [19]. When bioSmt3 and BirA are expressed in cells, the bioSUMOylated substrates accumulate and can later be purified using streptavidin-based affinity chromatography (Fig. 1). The high affinity between biotin and streptavidin allows lysis and pulldown analysis to be performed using stringent conditions, which both inactivates potential deSUMOylases and generates high yields of SUMOylated proteins with almost no background from non-covalent SUMO-interacting proteins and nonspecific contaminants.

Beyond the bioSUMO strategy in *Drosophila* cell culture described here, we mention two additional applications in progress. The bioSmt3 strategy has been adapted for use in transgenic

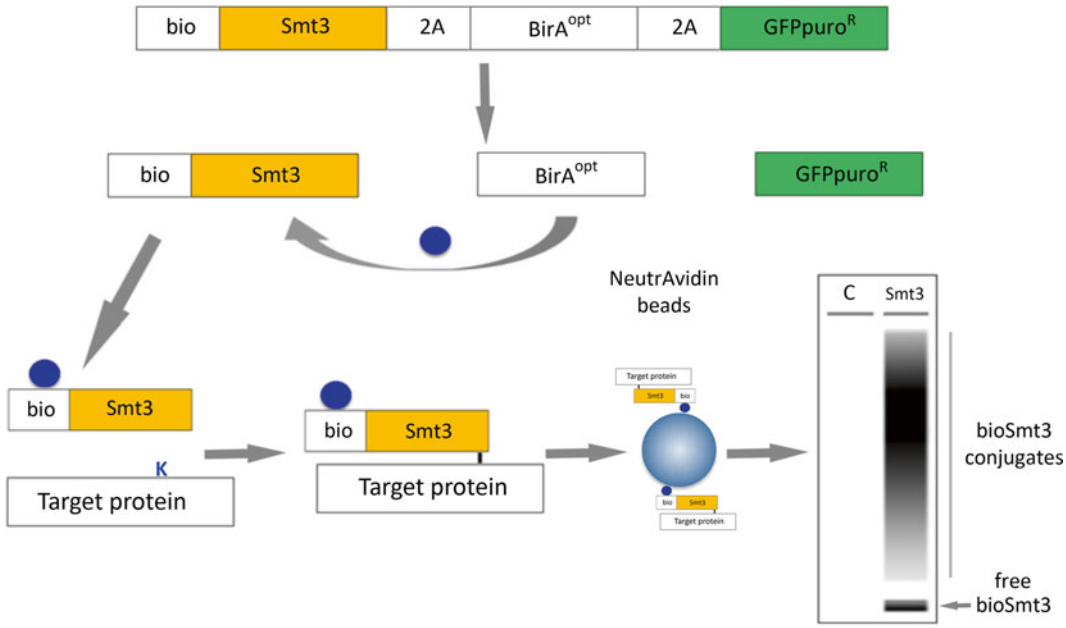

Fig. 1 Schematic diagram outlining the bioSUMO strategy. The bioSmt3 and BirA are expressed in cells using multicistronic, 2A-based vectors. In the case of *Drosophila* cells, the constitutive Actin-5C promoter is used. After 2A-processing, bioSmt3 is biotinylated, activated, and conjugated to substrates using BirA and the cellular E1 and E2 enzymes, respectively. Modified substrates are then released from cells in denaturing lysis conditions and captured by NeutrAvidin-based affinity chromatography. After stringent washes, eluted proteins can be separated by gel electrophoresis and analyzed by western blot or mass spectrometry. Using anti-biotin, a stylized depiction is shown of free, unconjugated bioSmt3 and a smear of higher molecular weight conjugates

flies using the classical UAS-GAL4 system [20], allowing characterization of SUMOylated proteins from particular tissues using the same purification protocol. Furthermore, by adapting the vectors to be used in mammalian cells, the same strategy has been validated using human SUMO homologues to explore the SUMO proteome in various human cell lines (L. Pirone, J. Sutherland, R. Barrio, unpublished).

In summary, the bioSUMO strategy can be used in *Drosophila* cells, as well as in transgenic flies and mammalian cells (with adaptations to the expression system). It can be utilized to test the SUMOylation of a protein of interest, using specific antibodies and western blotting. Alternatively, it can be used to generate SUMO subproteomes when coupled to mass spectrometry analysis. The isolation/identification of the SUMOylated substrates can be achieved with low expression of bioSmt3, and further optimized by simultaneous RNAi-silencing of endogenous Smt3. Notably, we have been able to identify many SUMOylated proteins in the absence of heat-shock, suggesting a high sensitivity of the system. We expect that the technique can be applied to other ubiquitin-like proteins and model organisms, to allow a better understanding of UbL modifications in a range of physiological contexts.

2 Materials

1. Cell lines (*see* **Note 1**) or *Drosophila* larvae expressing the *bioSmt3-T2A-BirA* constructs, or *BirA control*, together with GAL4 driver of choice.
2. Biotin solution: 1 mM; dissolve 12 mg of biotin in 50 ml of water or cell culture media. Biotin has low solubility in water; vortex until fully dissolved. Sterilize using 0.22 μm syringe filter and store at 4 °C for up 1 month or at −20 °C for longer periods.
3. Buffers: Prepare all solutions using ultrapure water (*see* **Note 2**). Volumes to prepare can be adjusted according to experimental needs. Once dissolved, filter the solutions with a 0.22 μM membrane filter. Store at room temperature for no more than 1 month. Urea-containing solutions are unstable and for mass spectrometry applications, fresh solutions are recommended.
4. Lysis buffer: 8 M urea, 1% sodium dodecyl sulfate (SDS), 60 mM *N*-ethylmaleimide (NEM), Protease inhibitor cocktail (Complete mini; EDTA-free). For 10 ml of Lysis buffer, add 70 mg of NEM crystals and 1 tablet of protease inhibitors to 1 ml of filtered washing buffer (WB) 5, agitate in vortex until dissolved, and adjust volume to 10 ml using WB5. Prepare fresh daily; do not re-filter. Store WB5 at room temperature (urea/SDS will precipitate at 4 °C).
5. Binding buffer: 3 M urea, 1 M NaCl, and 0.25% SDS in 1× phosphate buffered saline (PBS). Add 180.18 g of urea, 58.44 g of NaCl, 12.5 ml of 20% SDS solution, and 100 ml of 10× PBS to ultrapure water up to 1 l (*see* **Notes 2**–**4**).
6. WB1: 8 M urea, 0.25% SDS in 1× PBS. Add 480.48 g of urea, 12.5 ml of 20% SDS solution, and 100 ml of 10× PBS to ultrapure water up to 1 l (*see* **Notes 2** and **4**).
7. WB2: 6 M guanidine hydrochloride in 1× PBS. Add 573.18 g of guanidine hydrochloride and 100 ml of 10× PBS to ultrapure water up to 1 l.
8. WB3: 6.4 M urea, 1 M NaCl, and 0.2% SDS in 1× PBS. Add 384.384 g of urea, 58.44 g of NaCl, 10 ml of 20% SDS, and 100 ml of 10× PBS to ultrapure water up to 1 l (*see* **Notes 2**–**4**).
9. WB4: 4 M urea, 1 M NaCl, 10% isopropanol, 10% ethanol, and 0.2% SDS in 1× PBS. Add 240.24 g of urea, 58.44 g of NaCl, 10 ml of 20% SDS, 100 ml of isopropanol, 100 ml of ethanol, and 100 ml of 10× PBS to ultrapure water up to 1 l.
10. WB5: 8 M urea and 1% SDS in 1× PBS. Add 480.48 g of urea, 50 ml of 20% SDS, and 100 ml of 10× PBS to ultrapure water up to 1 l.
11. WB6: 2% SDS in PBS. Add 100 ml of 20% SDS and 100 ml of 10× PBS to ultrapure water up to 1 l.

12. 5× SDS sample buffer: 5% 2-mercaptoethanol, 0.02% bromophenol blue, 30% glycerol, 10% SDS, 250 mM Tris-Cl pH 6.8. For 50 ml: 2.5 ml of 2-mercaptoethanol, 10 mg of bromophenol blue, 5 g of SDS (powder; use mask when weighing), 25 ml Tris–HCl (0.5 M, pH 6.8). Mix well and bring up to 50 ml using glycerol (87% or anhydrous) to obtain 30% and ultrapure water. Aliquot and store at −20 °C.
13. Elution buffer: 4× SDS sample buffer, 100 mM DTT. For 1 ml, use 800 µl of 5× SDS sample buffer, 100 µl 1 M DTT, 100 µl ultrapure H2O. Prepare fresh daily.
14. Equilibrated high-capacity NeutrAvidin-agarose resin: After resuspension, remove required amount of resin and equilibrate with 10 bed volumes of 1× PBS (once) and binding buffer (twice; RT).

3 Methods

3.1 Cell Transfection and Biotin Supplementation

Different cell types, transfection protocol and efficiency, and the desired type of assay will influence the amount of starting material necessary. For example, western validation of SUMOylation for endogenous substrates will require more cells than an exogenous substrate expressed by cotransfection. If end goal is mass spectrometry of SUMO subproteome, then even more cells will be required. Experimental optimization is necessary. As a rule of thumb, SUMO conjugates should be detectable by western blot (using Streptavidin-HRP or anti-biotin-HRP) using $2–3 \times 10^7$ transfected *Drosophila* S2 cells. This can be achieved by plating 8×10^6 cells in 10 ml of S2 media into a 10 cm dish, transfecting with 3–5 µg of DNA using calcium phosphate method and collecting cells after 3–4 days. Other transfection protocols (e.g., Effectene, which uses less DNA) can be used. If the bioSUMO plasmid has GFPpuro marker, it can be used to follow expression using fluorescent microscopy. Otherwise, GFP co-transfection can be used to monitor transfection efficiency. Although untested in cells, we expect that co-transfection of double-stranded RNA to induce RNAi of the endogenous Smt3 gene will lead to a higher efficiency of bioSmt3 incorporation. This effect has been validated in transgenic fly experiments (unpublished). Even without, recovery of bioSmt3 conjugates from cells is very efficient.

To ensure efficient biotinylation, we supply exogenous biotin (50 µM, from a 1 mM stock) by adding directly to the media and mixing, at least 24 h prior to harvest. Biotin can also be added to the fly food in case of the in vivo experiments.

3.2 Collection of Samples

1. Collect cells after 72–96 h (*Drosophila*) or 24–48 h (mammalian) after transfection.

2. Wash the cells one or two times in cold 1× PBS. Collect cells by scraping in 1 ml cold 1× PBS and transfer into 2 ml eppendorf (*see* **Note 5**). Washing removes serum proteins and exogenous biotin from the cells.
3. Centrifuge (500 × *g*, 4 °C, 5 min), discard the supernatant, keep pellet on ice (*see* **Note 6**).
4. In case of in vivo experiments, collect the tissue of interest (larvae or dissected tissues) in cold 1× PBS, wash as necessary, and proceed with Subheading 3.3.

3.3 Lysis and Binding

From this point, all the steps should be performed at room temperature (urea-containing solutions can precipitate on ice).

1. Add 1 ml of lysis buffer (to pellet of approximately $2–3 \times 10^7$ S2 cells, or $0.5–1 \times 10^7$ human cells). Resuspend gently by inversion since lysate will be viscous.
2. Sonicate the sample to reduce viscosity (Sanyo Soniprep 150 or similar, with microprobe; 3 × 15 s, 10 μm setting). Ice is not necessary, but let the sample cool between sonication steps. Do not overheat sample or cause frothing.
3. Centrifuge for 20 min, at 18,000 × *g* to remove insoluble material.
4. Transfer the cleared supernatant into a 5 ml tube. We recommend polypropylene with rounded bottom to ensure even mixing.
5. Add 3 volumes of binding buffer (e.g., 3 ml for 1 ml of lysis buffer; binding buffer may need to be prewarmed; *see* **Note 4**). Additional NEM and protease inhibitors can be added if problems with degradation or recovery efficiency are observed.
6. Mix well and remove an 80 μl aliquot (amount for endogenous targets; for exogenous, co-expressed targets, less volume can be taken). Add 20 μl of 5× SDS sample buffer, mix and store at −20 °C. This will serve as Input sample.
7. Incubate the sample with equilibrated NeutrAvidin beads previously prepared. For 1 ml of initial lysate use 50 μl of beads (bed volume). If screw- or snap-top tubes are not used, then seal tubes well with Parafilm.
8. Incubate overnight at room temperature (12–18 h), with gentle agitation by placing the tubes on a rotating wheel or roller mixer.

3.4 Washes

1. Centrifuge the samples in a tabletop centrifuge (100 × *g*, 5 min, RT). Pelleted resin may be difficult to see.
2. Separate carefully the supernatant from the beads and save as the flow-through fraction (FT). Take an 80 μl aliquot, mix with 20 μl of 5× SDS sample buffer and store at −20 °C (or equivalent volume as in Subheading 3.3, **step 6**). Remaining FT can be saved at RT until end of experiment.

3. Resuspend beads in 500 µl of WB1 and transfer the beads into a 1.5 ml microfuge tube (*see* **Note 7**).
4. Use 500 µl of buffer per wash, inverting the tube 3–4 times each time to mix beads. Perform the washes as follow: 2× WB1, 3× WB2, 1× WB3, 3× WB4, 1× WB1, 1× WB5, and 3× WB6. To collect beads, centrifuge at low speed in microfuge (1000 rpm, 3–5 min). Discard the supernatants (*see* **Note 8**).

3.5 Elution

1. After final wash, add 50 µl of elution buffer to pelleted beads. Mix well by vortexing.
2. Boil for 5 min at 99 °C in a thermoblock. Vortex vigorously, spin briefly, and boil for a second time, 5 min, 99 °C (*see* **Note 9**).
3. Centrifuge in microfuge (5 min, maximum speed >14,000 rpm) to separate eluate from the beads. Carefully separate the eluate (supernatant) from the beads and transfer to a fresh tube. Alternatively, eluate can be recovered using a spin filter (*see* **Note 9**).
4. Input and FT samples should be boiled 5 min as well. Input, FT, and elution samples can be stored at 4 °C for 1 week or −20 °C for longer storage. Boil briefly, vortex, and spin before loading on PAGE.
5. Check your Input, FT, and elution samples by western blot or process eluates for mass spectrometry analysis (*see* **Note 10**).

4 Notes

1. *Drosophila* S2, S2R+, and Kc167 have been tested and validated. For human cells, 293FT, U2OS, and MDA-MB-231 cell lines have been tested and validated.
2. Use deionized ultrapure H_2O (18 MΩ-cm resistivity, 25 °C) for all buffers.
3. The buffer takes more than 30 min to dissolve—heating is not recommended. Adjust volume with water from the beginning. Use magnetic stirrer until completely dissolved.
4. Binding buffer and WB3 precipitate at room temperature. Pre-warm the buffers at 37 °C for 30 min before use. Diluting the sample with binding buffer serves to reduce the urea concentration and encourage better biotin–streptavidin interaction. We have seen that this dilution improves recovery in *Drosophila* samples. With human cell lysates, we routinely perform binding directly in lysis buffer without dilution, and maintain high recovery rates. Optimization is recommended if problems are encountered.

5. Most *Drosophila* cell lines are semi-adherent, so scraping is not absolutely necessary; pipetting is enough to detach cells from the plate. Washes can be done gently on the plate, or by transferring the cells to 15 ml falcon tubes.
6. The cell pellets can be processed immediately or frozen at –20 or –80 °C.
7. Washes are easier in 1.5 ml microfuge tubes. Transfer the beads using clipped large-bore tip to avoid damage. Pipette carefully and transfer all beads into the 1.5 ml tube.
8. 500 µl is the minimal recommended wash volume (i.e., 10 bed volumes); more can be used to further reduce nonspecific background. Elimination of the supernatant can be performed using a vacuum aspirator. Using a long thin tip (e.g., gel loading tips) allows better control of the aspirator flow. Avoid touching the pelleted beads. Also note that WB3 sometimes forms precipitate around the pellet. The tube can be warmed to avoid this, or simply leave the precipitate along with the pellet. It will dissolve completely in the next WB4 step.
9. The biotin–NeutrAvidin interaction is very strong. High heat, high SDS, and mechanical mixing are used to maximize the recovery of biotinylated proteins. We find that removal of the eluate from the resin soon after boiling also enhances recovery. Quick separation can be done using spin filters (e.g., Sartorius Vivaclear Mini 0.8 µm PES or equivalent). After boiling, use a cut yellow tip to transfer the sample (beads and liquid) to the spin filter. Spin (5 min, maximum speed) to recover the eluate. Save filter/beads until end of experiment.
10. Loading amounts depends on desired targets. If analysis by mass spectrometry is planned, take all necessary precautions to avoid contamination (gloves, sterile tips and low protein-binding plasticware if available). It is recommended to consult with your planned mass spectrometry partner to decide on the strategy for sample processing, i.e., gel conditions, direct digest of proteins from resin, and other alternatives.

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Chapter 13

Label-Free Identification and Quantification of SUMO Target Proteins

Ivo A. Hendriks and Alfred C.O. Vertegaal

Abstract

Mass spectrometry-based approaches are utilized with increasing frequency to facilitate identification of novel SUMO target proteins and to elucidate the dynamics of SUMOylation in response to cellular stresses. Here, we describe a robust method for the identification of SUMO target proteins, and the relative quantification of SUMOylation dynamics, using a label-free approach. The method relies on a decahistidine (His_{10})-tagged SUMO, which is expressed at a low level in a mammalian cell line or model organism. The His_{10}-tag allows for a single-step, high-yield, and high-purity enrichment of SUMOylated proteins, which are then digested and analyzed by high-resolution mass spectrometry. Matching between runs and label-free quantification integrated in the freely available MaxQuant software allow for a high rate and accuracy of quantification, providing a strong alternative to laborious sample or cell labeling techniques. The method described here allows for identification of >1000 SUMO target proteins, and characterization of their SUMOylation dynamics, without requiring sample fractionation. The purification procedure, starting from total lysate, can be performed in ~4 days.

Key words SUMO, SUMO target purification, Mass spectrometry, Proteomics, Label-free quantification, His_{10} pulldown

1 Introduction

SUMO proteomics approaches have become increasingly prolific over the last decade, with a wide range of studies reporting hundreds of SUMO target proteins, often in a setting where the dynamic nature of SUMOylation is revealed [1–6]. More recently, technical hurdles associated with the identification of SUMO sites have been largely overcome, allowing the identification of hundreds [7–12] to thousands [13] of SUMOylated lysines. The proteomic study of SUMO is generally challenging due to SUMO-specific proteases which remain active under all but the harshest lysis conditions, SUMO being a low-stoichiometry modifier, and the transient nature of SUMO modification. Therefore, the large majority of these approaches rely on the exogenous expression of an epitope-tagged

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SUMO, as epitope tags facilitate efficient and cost-effective purification at high yield. Whereas endogenous approaches for the purification of SUMO exist [4, 14, 15], they are technically challenging and require large amounts of starting material.

In the large majority of published SUMO proteomics studies, stable isotope labeling by amino acids in cell culture (SILAC) [16] is used in order to gain quantitative insight into SUMOylation dynamics. SILAC boasts several unique advantages, such as mixing several samples at an early stage in the purification procedure to minimize technical variance, and high-accuracy quantification of peptide pairs. However, the SILAC method also has various drawbacks. Biologically, we have observed that most cell lines do not grow optimally on SILAC medium, often due to the dialyzed serum that has to be used. This in turn can have negative effects on SUMO conjugation dynamics, in addition to altered biological behavior of the cells. Technically, SILAC labeling is laborious with cells often needing at least 2 weeks of growth on SILAC medium to sufficiently integrate the SILAC labels. SILAC analysis is also limited to three labels, and whereas multiplexed analysis can still be performed by sharing one common label for one common condition while alternating the other two labels and conditions [5], this complicates experimental design and normalization of data. Finally, using duplex or triplex SILAC labeling linearly increases sample complexity through an increased amount of peptides in the sample, which in turn will decrease the overall depth of analysis.

We have recently adapted label-free quantification (LFQ) [17] as a standardized approach in our lab, and have noted excellent quantitative accuracy and performance when applied to the identification of SUMO targets [10], while simultaneously being more cost-effective and simplifying experimental design. Performance of LFQ has steadily increased over the last years with increasingly high-throughput mass spectrometers, and moreover through optimized software solutions, notably the "matching between runs" which is integrated in the freely available MaxQuant software [18, 19]. This pivotal feature allows matching of MS/MS-identified peaks from one sample to unidentified peaks with identical chromatography gradient time and m/z characteristics from other samples, thereby greatly increasing depth of analysis and quantification accuracy as more samples and replicates are added to the analysis. Essentially, this substitutes the role of co-eluting SILAC peptide pairs. Technically, whereas LFQ displays slightly larger variance than SILAC, it is more reproducible and allows for more proteins to be detected and quantified [20]. Furthermore, LFQ is not limited in experimental design, and any amount of samples may be combined. LFQ is also applicable in model organisms where SILAC labeling is impossible, such as in most plants, which synthesize their own amino acids.

In this chapter, we describe a method for label-free identification and relative quantification of target proteins

modified by decahistidine-tagged (His_{10}-tagged) SUMO (Fig. 1). From our experience involving several epitope tags such as His_6, HA, and FLAG, as well as endogenous purification methods, the His_{10}-tagged strategy performed the best in terms of overall yield, purity and robustness of the method. At the same time, the tag is small and does not overly increase the size of the protein. Our His_{10}-SUMO2 construct includes a non-fused GFP, and can be efficiently transduced into any mammalian cell system using a third generation lentiviral approach. A stable cell line expressing low and homogenous levels of His_{10}-SUMO2 can then be acquired through fluorescence-aided cell sorting (FACS). Following cell lysis, a single step purification procedure, a concentration step, in-solution digestion, and peptide desalting are required to generate samples ready to be analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). To facilitate label-free quantification, at least three biological replicates of each sample have to be prepared, in addition to at least one set of control samples originating from the parental cell line not expressing His_{10}-SUMO2 (Fig. 1). A similar strategy may be used for the study of SUMO1, ubiquitin, and ubiquitin-likes. Overall, the sample preparation takes ~4 days.

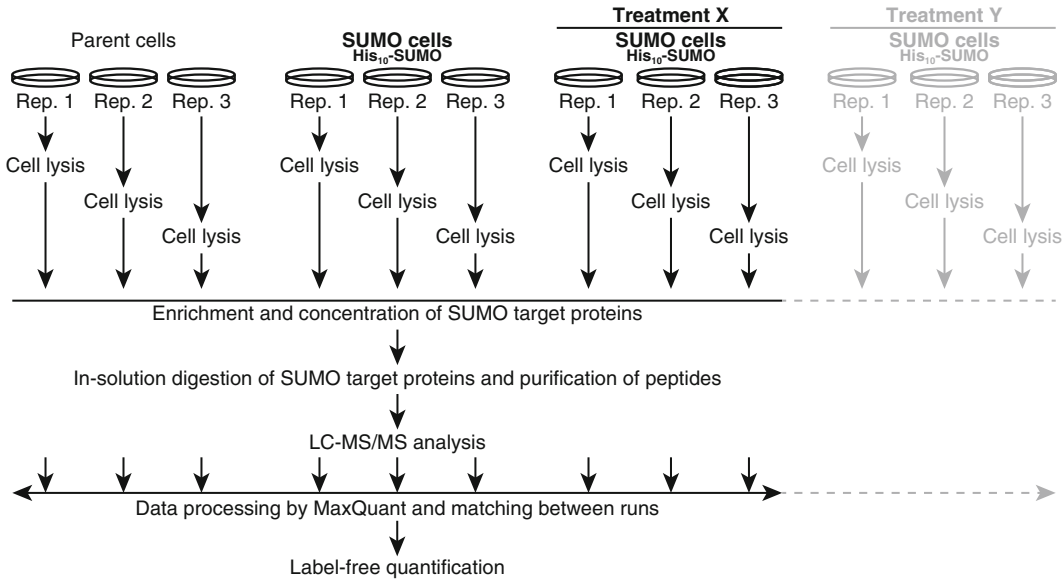

Fig. 1 Schematic representation of the label-free quantification approach when identifying SUMO target proteins. At minimum, a set of samples derived from parental cells are needed to facilitate comparison to a set of samples derived from cells expressing His_{10}-SUMO2. An additional set of His_{10}-SUMO2 cells treated in a certain way allows for quantification of SUMOylation dynamics, and further sets may be added as desired. For all sets, at least three biological replicates are required, which may be prepared and lysed at different points in time. Purification of SUMOylated proteins, and further technical handling, should occur simultaneously for all samples. After LC-MS/MS analysis, matching between runs by MaxQuant greatly extends coverage and overlap of peptide and protein identification between samples

Studying SUMO acceptor lysines has become increasingly facilitated over the last years, since it directly identifies SUMO target proteins and greatly aids in functional study of selected target proteins by revealing the modified lysines. Regardless of these advantages, the study of SUMOylation dynamics can confidently be performed at the protein-level, as site-level SUMO dynamics often do not significantly differ from protein-level SUMO dynamics [11]. Furthermore, there are considerably more peptides available for quantification of any single protein when performing a protein-level study. Nonetheless, the His_{10}-SUMO2 method described here can be adapted to facilitate identification of sites, either using K0-Q87R His_{10}-SUMO2 with the K0 method [13], or using wild-type His_{10}-SUMO2 with the PRISM strategy [11].

The method described here, in combination with a high-resolution and modern Orbitrap mass spectrometer such as a Q-Exactive, can routinely and accurately identify and quantify a considerable portion of the human SUMO proteome without requiring sample fractionation [10]. Typical numbers include relative quantification of >500 SUMO targets under standard growth conditions, and >1000 SUMO targets under stress conditions. Dynamic changes of a factor >3 between samples can be readily identified from three biological replicates, with a possibility to increase quantitative accuracy and sensitivity by increasing the number of biological replicates.

2 Materials

2.1 Cell Growth and Lysis

1. A stable cell line expressing His_{10}-SUMO2 [10, 12], or any other SUMO family member, ubiquitin, or ubiquitin-like with a His_{10}-tag. A lentiviral construct carrying His_{10}-[SUMO/Ub/Ubl]-IRES-GFP behind a CMV promoter is ideal for transduction and subsequent FACS-sorting of cells expressing the construct at a low and homogenous level. Our CMV-[His_{10}-SUMO2-IRES-GFP] lentiviral construct is available on request.
2. Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum and 100 U/ml penicillin–streptomycin (Gibco).
3. Any reagents or infrastructure required for studying SUMO dynamics, e.g., MG-132 for proteasome inhibition, or a separate incubator at 43 °C for heat shock.
4. Phosphate Buffered Saline (PBS).
5. Cell scrapers.
6. Guanidine Lysis Buffer: 6 M guanidine-HCl, 93.2 mM Na_2HPO_4, 6.8 mM NaH_2PO_4, 10 mM Tris–HCl, pH 8.0.
7. Any denaturing buffer for generation of total lysates.

2.2 Enrichment of SUMO Target Proteins

1. Microtip sonicator.
2. BCA Protein Assay Kit.
3. Ni-NTA agarose (Qiagen).
4. 1 M Tris buffers, pH 6.3, 7.0, and 8.0. Set the Tris buffers to the required pH with HCl.
5. 5 M imidazole solutions, pH 7.0, and 8.0. Set the imidazole solutions to the required pH with concentrated HCl. Note that to reach pH 7.0 for 5 M imidazole, the imidazole must be dissolved entirely in 6 M HCl. This process is very exothermic and should be performed in clean glassware, very slowly, while mixing, and on ice.
6. Wash Buffer 1: 6 M guanidine-HCl, 93.2 mM Na_2HPO_4, 6.8 mM NaH_2PO_4, 10 mM Tris–HCl, 0.1% Triton X-100, 10 mM imidazole, 5 mM 2-mercaptoethanol, pH 8.0.
7. Wash Buffer 2: 8 M urea, 93.2 mM Na_2HPO_4, 6.8 mM NaH_2PO_4, 10 mM Tris–HCl, 0.1% Triton X-100, 10 mM imidazole, 5 mM 2-mercaptoethanol, pH 8.0.
8. Wash Buffer 3: 8 M urea, 21.6 mM Na_2HPO_4, 78.4 mM NaH_2PO_4, 10 mM Tris–HCl, 10 mM imidazole, 5 mM 2-mercaptoethanol, pH 6.3. Use 5 M imidazole pH 7.0 for making this buffer.
9. Wash Buffer 4: 8 M urea, 21.6 mM Na_2HPO_4, 78.4 mM NaH_2PO_4, 10 mM Tris–HCl, 5 mM 2-mercaptoethanol, pH 6.3.
10. Elution Buffer: 7 M urea, 58 mM Na_2HPO_4, 42 mM NaH_2PO_4, 10 mM Tris–HCl, 500 mM imidazole, pH 7.0.
11. Urea Buffer: 8 M urea, 93.2 mM Na_2HPO_4, 6.8 mM NaH_2PO_4, 10 mM Tris–HCl, pH 8.0.
12. LoBind microcentrifuge tubes, 1.5 ml (Eppendorf).
13. Microcentrifuge tubes with spin filter, 0.45 μm (Millipore).

2.3 Concentration and In-Solution Digestion of SUMO Target Proteins

1. Microcentrifuge concentrators, 100K MWCO (Sartorius Stedim).
2. Temperature-controlled microcentrifuge.
3. Bradford Protein Assay.
4. 1 M ammonium bicarbonate in MQ water.
5. 250 mM chloroacetamide (CAA) in MQ water, prepare just before use.
6. 1 M dithiothreitol (DTT) in MQ water, keep at 4 °C and store at −20 °C. Diluted 50 mM DTT stock should be prepared just before use.
7. Lysyl Endopeptidase, mass spectrometry grade (Lys-C; Wako).
8. Modified Trypsin, sequencing grade (Promega).

9. TFA solution: 50% trifluoroacetic acid (TFA), HPLC grade, in MQ water.
10. Activation Buffer: 100% methanol, HPLC grade.
11. StageTip Buffer A: 0.1% formic acid, HPLC grade.
12. StageTip Buffer B: 80% acetonitrile in 0.1% formic acid, HPLC grade. Prepare freshly on the same day.
13. C18 disks (Empore).
14. Needle: Kel-F hub (KF), point style 3, gauge 16 (Hamilton).
15. Plunger: Assembly N, RN, LT, LTN for model 1702 (Hamilton).

2.4 LC-MS/MS Analysis of SUMO Target Proteins

1. A liquid chromatography system, e.g., an EASY-nLC 1000 (Thermo).
2. A nano-electrospray ionization (ESI) source.
3. A C18-packed analytical column.
4. A high resolution Orbitrap mass spectrometer, e.g., a Q-Exactive (Thermo), and any other relevant equipment that was not listed above. Refer to the expertise of the local mass spectrometry operator(s).
5. Relevant chromatography buffers for an elution gradient; e.g., 0.1% FA and 95% ACN in 0.1% FA.
6. Freely available MaxQuant software [18] for initial processing and quantification of the raw proteomics data.
7. Freely available Perseus software [21] for further processing of the data, performing two-sample testing on LFQ values, and other statistical analyses.

3 Methods

The protocol for identification and quantification of SUMO target proteins described herein can essentially be subdivided into four parts: cell culture to generate batches of His_{10}-SUMO2 cells and subsequent lysis, purification of SUMO conjugates through nickel-affinity chromatography and concentration, two-step in-solution digestion of SUMO conjugates followed by desalting and concentration of peptides by StageTip, and analysis of the peptide mixture by LC-MS/MS with subsequent label-free quantification (Fig. 2).

For cell culturing, it is important to acquire a stable cell line expressing His_{10}-SUMO2 at a low level. Cells expressing a high level of His_{10}-SUMO2 result in a large presence of unconjugated SUMO, which complicates purification of SUMO conjugates. For any experiment, at least one parental control should be included, and all biological conditions should be prepared in at least experimental triplicate. For this purpose, different batches of cells may be grown,

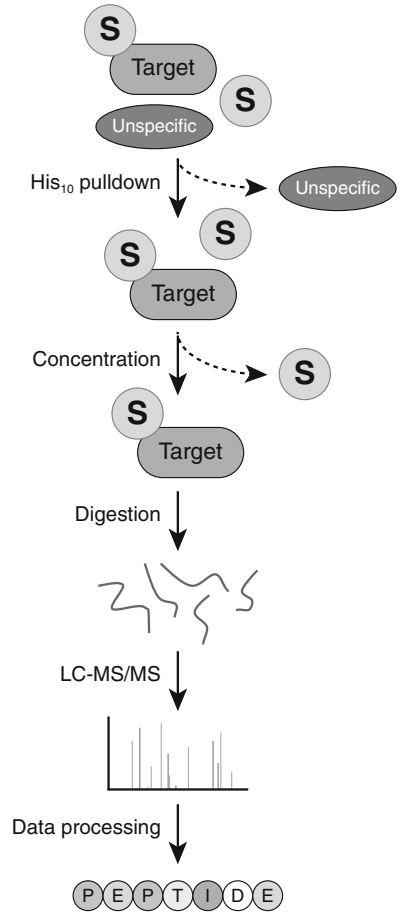

Fig. 2 Schematic overview of the His_{10}-SUMO2 purification procedure. Guanidine lysates are prepared, and a single stringent His_{10}-pulldown facilitates removal of the large majority of all background proteins. Concentration under denaturing conditions on 100K MWCO filters separates unconjugated SUMO from SUMO target proteins. Concentrated SUMO target proteins are digestion in-solution, the resulting peptides are desalted, and analyzed by LC-MS/MS. MaxQuant is used to process raw data and perform label-free quantification

treated and lysed on different days, as long as the purification procedure is performed simultaneously on all samples. In general, one nearly confluent 15-cm dish (20 million cells) can yield 1–5 μg SUMO conjugates, and provides sufficient material for several mass spectrometry runs. However, scaling up to 3–5 dishes per experiment generally results in a higher sample purity and a greater reproducibility. Cells are lysed in a highly denaturing guanidine buffer, which completely inhibits any SUMO protease activity.

In order to purify SUMO target proteins, a fairly standard nickel-affinity chromatography procedure is performed, albeit with a high concentration of competing imidazole, facilitated by the His_{10} tag. A set of stringent washes follows an overnight pulldown,

and multiple elutions are performed in order to maximize yield. A critical step in the protocol is the subsequent high level of concentration which is performed in denaturing conditions on a 100K MWCO filter, resulting in the loss of all free SUMO but retaining all SUMO conjugates [12, 13].

Purified and concentrated SUMO conjugates are subjected to in-solution digestion in order to generate tryptic peptides, which may be analyzed by mass spectrometry. It should be mentioned that the concentrated SUMO target proteins are also ideally suited to immunoblot analysis, and virtually any SUMO target may be visualized in this manner. A double digestion of Lys-C and trypsin is performed, to yield the highest achievable cleavage efficiency. Peptides are desalted according to standard StageTip protocol [22], eluted, vacuum dried, and resuspended in a small volume of buffer compatible with liquid chromatography.

Finally, the peptides are analyzed through nanoflow liquid chromatography coupled to tandem mass spectrometry. We generally use elution gradients of 120 min in length, and measure all biological samples as two technical replicates, but it is up to the experimenter to decide what would be the most feasible. The generated data is analyzed through MaxQuant, which will identify peptides and match identified peaks between different samples in order to increase quantitative coverage in all samples. Ultimately, Perseus software is used in order to handle the output from MaxQuant, and perform statistical procedures on the data such as imputation and two-sample testing, in order to identify SUMO target proteins and quantify SUMOylation dynamics.

3.1 Cell Growth and Lysis

1. Per biological replicate, grow one batch of His_{10}-SUMO2 cells per experimental condition you wish to evaluate, and at least one control batch using the parental cell line. In total, at least three biological replicates of all samples and controls must be acquired, although culture, treatment and lysis of different batches may take place on different days (*see* **Note 1**).
2. Treat the cells as required for your experiment. Take into account that the time required for harvesting and lysing of the cells is typically 30 min per batch of five 15-cm dishes; plan accordingly.
3. Remove the medium from cells ready to be harvested, and place the dishes on ice.
4. Wash the cells twice with ice-cold PBS, taking care to remove all PBS after the second wash.
5. Add 2 ml ice-cold PBS per 15-cm dish, and use a cell scraper to gently release all the cells from the dish.
6. Collect the cells in a sufficiently large tube, and centrifuge them down briefly. A tube of 15 ml is typically sufficient for 5× 15-cm dishes or less, if pellet size exceeds 500 µl multiple 15-ml tubes or a 50-ml tube may be preferable (*see* **Note 2**).

7. Resuspend the cells in 2 ml ice-cold PBS per 15-cm dish, and take a 100 µl aliquot.
8. Centrifuge down the small aliquots and lyse them in any denaturing total lysate buffer, to serve as an input control.
9. Centrifuge down the main batch of cells, and remove all PBS.
10. Vigorously lyse the cells by adding 10 pellet volumes of room temperature Guanidine Lysis Buffer directly onto the cells while vortexing. After 5 s, close the tube and shake the tube vigorously for 5 s.
11. Alternate vigorous vortexing and shaking for 5 s each, for 3–6 cycles, over 30–60 s total, ensuring that no visible cellular debris remain (*see* **Note 3**).
12. Snap freeze the lysate using liquid nitrogen. Frozen lysates can be stored at −80 °C for several months, and form an ideal pause point.

3.2 Enrichment of SUMO Target Proteins

1. Gently thaw out the lysate, on a roller at room temperature. Avoid sudden temperature differences or water baths during thawing (*see* **Note 4**).
2. Sonicate the lysate to homogenize it, using a microtip centrifuge, and 2–4 bursts of 5 s sonication at ~30 W. Ideally, the lysate becomes pipettable with a microtip (*see* **Note 5**).
3. If any visible debris remains in the lysate, centrifuge the lysate until it is clear again.
4. Determine protein levels in all lysates using the BCA assay, according to the manufacturer's instructions. Equalize total protein levels across all samples if differences >10 % are observed.
5. Prepare 20 µl Ni-NTA agarose beads (dry volume, i.e., 40 µl from a 50 % slurry) per 1 ml of lysate. Equilibrate the beads by washing them four times in Guanidine Lysis Buffer supplemented with 50 mM imidazole and 5 mM 2-mercaptoethanol (*see* **Note 6**).
6. Add imidazole to your lysates to a concentration of 50 mM, 2-mercaptoethanol to a concentration of 5 mM.
7. Add the equilibrated Ni-NTA beads to the lysates, and incubate the suspension overnight at 4 °C in a rotator-mixer. Sample mixing should be extensive, but not so much as to cause a large amount of foaming.
8. After overnight incubation, pellet the beads by centrifugation. Remove all supernatant, but do not discard it in case the purification was not optimal (*see* **Note 7**).
9. Transfer the beads to a clean microcentrifuge tube using 5–10 buffer volumes of Wash Buffer 1 (*see* **Note 8**).
10. Wash the beads for 10 min by incubating in a rotator-mixer at room temperature. Centrifuge the beads and remove all wash buffer.

11. Add 5–10 buffer volumes of Wash Buffer 2, and incubate for 10 min in a rotator-mixer at room temperature. Centrifuge the beads and remove all wash buffer.
12. Add 5–10 buffer volumes of Wash Buffer 3, transfer the beads to a clean LoBind microcentrifuge tube, and incubate for 15 min in a rotator-mixer at room temperature. Centrifuge the beads and remove all wash buffer (*see* **Note 9**).
13. Add 5–10 buffer volumes of Wash Buffer 4, and incubate for 15 min in a rotator-mixer at room temperature. Centrifuge the beads and remove all wash buffer.
14. Add 5–10 buffer volumes of Wash Buffer 4, transfer the beads to a clean LoBind microcentrifuge tube, and incubate for 15 min in a rotator-mixer at room temperature. Centrifuge the beads and remove all wash buffer.
15. Elute the beads by adding one bead volume of Elution Buffer, and shaking the beads for 20 min at 1250 RPM at room temperature.
16. Equilibrate 0.45 μm spin filter columns by washing them once with Elution Buffer.
17. Briefly spin down the beads, clear the elution by passing it through the 0.45 μm filter, and transfer it to a clean LoBind tube.
18. Repeat the elution of the beads twice more, clearing them through the same 0.45 μm filter units, and pool subsequent elutions from the same replicate with the first (*see* **Note 10**).
19. The cleared elutions may be frozen and stored at −80 °C for several months, and can be readily investigated through immunoblotting.

3.3 Concentration and In-Solution Digestion of SUMO Target Proteins

1. Thaw out the elution samples at room temperature while shaking at 750 RPM.
2. Equilibrate one 100K MWCO filter per sample, by washing them with 250 μl Urea Buffer. The Urea Buffer should be prepared freshly and pre-filtered through 0.45 μm filter units in order to remove any potential debris and prevent blocking the 100K MWCO filter units.
3. Concentrate the sample over the 100K MWCO filter, applying at most 400 μl sample to the filter unit, and centrifuging at 8000 × *g* for 10–15 min at 25 °C in a temperature-controlled centrifuge. Concentration rate is approximately 50 μl/min. In case the sample exceeds 400 μl, add another 400 μl after each concentration round (*see* **Note 11**).
4. Flowthrough may be discarded, although a small aliquot should be kept in order to ensure that only free SUMO is passing through the filter.
5. Continue concentration until 5–10 μl of sample remains per 15-cm dish used for the sample. Avoid overconcentration to <1 μl of sample.

6. Wash the sample with 250 µl Urea Buffer, and re-concentrate.
7. Repeat the wash step once.
8. Remove the concentrated SUMO target proteins from the filter by centrifuging the filter upside down for 1 min at 1000×*g* in the microcentrifuge tubes supplied with the filter units (*see* **Note 12**).
9. Transfer the sample to a clean LoBind tube.
10. Perform a Bradford assay to determine the concentration of the SUMO target proteins in your sample (*see* **Note 13**).
11. Increase the volume of your sample to a convenient volume using Urea Buffer, e.g., 25 or 50 µl. Concentrated SUMO target proteins may be investigated with immunoblotting, and are ideal for visualizing low-abundance SUMO targets.
12. Add ammonium bicarbonate (ABC) to a concentration of 50 mM (*see* **Note 14**).
13. Add DTT to a concentration of 1 mM, and incubate at room temperature for 30 min (*see* **Note 15**).
14. Add CAA to a concentration of 5 mM, and incubate at room temperature for 30 min (*see* **Note 16**).
15. Add 5 mM DTT, to a total concentration of 6 mM, and incubate at room temperature for 15 min.
16. Add Lys-C to the sample, in a 1:50 enzyme-to-protein ratio (*see* **Note 17**).
17. Incubate for 3–5 h at room temperature, still, and in the dark.
18. Add three volumes of 50 mM ABC to the sample, diluting the urea to <2 M.
19. Add trypsin to the sample, in a 1:50 enzyme-to-protein ratio.
20. Incubate overnight at room temperature, still, and in the dark.
21. Acidify the sample by adding TFA to a concentration of 2% (*see* **Note 18**).
22. Samples may be frozen and stored at −80 °C for several months.
23. In case samples were frozen, thaw them at room temperature while shaking at 750 RPM.
24. Prepare StageTips for desalting and concentrating the peptides, essentially according to published protocol [22]. When making StageTips, stack three C18 disks on top of each other to maximize peptide retrieval.
25. Activate the StageTips by passing 100 µl methanol over them by centrifuging at 1000×*g*. It is vital that the C18 column material remains wetted during the entire procedure, until the peptides are ready to be eluted (*see* **Note 19**).
26. Condition the StageTips with 100 µl Buffer B.
27. Equilibrate the StageTips with 100 µl Buffer A.

28. Load your sample on the StageTip. If your sample exceeds 100 µl in volume, only load 100 µl per loading cycle.
29. Wash the StageTips twice with 100 µl Buffer A. After the second wash, it is safe to completely dry the C18 column material. Peptide-loaded StageTips may be stored at 4 °C or −20 °C for several weeks.
30. Prepare LoBind microcentrifuge tubes with a hole punctured in the lid, allowing a StageTip to be placed in the tube lid with the tip reaching approximately until the 0.5 ml mark.
31. Place the StageTip in the LoBind tube, and elute the peptides by passing 25 µl Buffer B over the tip at 1000 × *g* for 3–5 min.
32. Repeat the elution once, leaving the StageTip in the same tube so the elutions are pooled.
33. Transfer the elution to a clean LoBind tube.
34. Vacuum dry the peptides using a SpeedVac. The peptides may be dried entirely (*see* **Note 20**).
35. Resuspend the peptides in 10 µl Buffer A, and gently tap the tube to assist in dissolution of the peptides (*see* **Note 21**).
36. Sonicate the peptides in a water bath for 2 min.
37. Briefly centrifuge the peptides, and store them at −20 °C or −80 °C until they are ready to be analyzed by LC-MS/MS.

3.4 LC-MS/MS Analysis of SUMO Target Proteins

1. In general, it is advisable to consult with local mass spectrometry experts when determining how to analyze samples. Regardless, for purposes of label-free quantification, it is of high importance that all samples are analyzed on the same machine, the same column, using the same chromatography gradient and buffers, and preferably back-to-back (*see* **Note 22**).
2. Analyze a small amount of sample, e.g., 0.1–0.2 µl from the total sample, using a short diagnostic 30 min gradient.
3. Determine the overall concentration of the sample. Several of the major peaks should generally correspond to internal SUMO peptides, the most prominent SUMO target proteins, and possibly trypsin or Lys-C.
4. Based on sample concentration, load 1–4 µl of sample, using a longer chromatography gradient, e.g., 120 min. Perform an elution gradient starting at 0.1% FA, and gradually increasing to 30% ACN in 0.1% FA over the majority of the gradient. Towards the end of the gradient, increase to 90% ACN in 0.1% FA (*see* **Note 23**).
5. Depending on the amount of samples in the experimental design, and the amount of available mass spectrometry time, it may be advantageous to measure all biological samples in technical duplicate. In general, the SUMO proteome is not sufficiently complex to warrant fractionating of the peptides prior

to analysis, provided a modern mass spectrometer with high peptide identification speed (>10/s) is used.

6. Analyze the raw data using MaxQuant software version 1.5.3.8 (or newer). To this end, the default settings of MaxQuant are largely sufficient. Critical settings that must be changed to facilitate label-free quantification:
   (a) Label-free quantification → Set to "LFQ" → Disable "Fast LFQ".
   (b) Sequences → Fasta files → Add a copy of the human proteome derived from Uniprot, named "HUMAN.fasta". If using a different organism, use the appropriate proteome.
   (c) Adv. identification → Enable "Match between runs" → Match time window [min] → Set to "1".

   Other optional changes that optimize processing speed, accuracy, and coverage:
   (d) Modifications → Max. number of modifications per peptide → Set to "3".
   (e) Instrument → Main search peptide tolerance → Set to "6".
   (f) Instrument → Max. charge → Set to "6".
   (g) Sequences → Min. peptide length → Set to "6".
   (h) Sequences → Max. peptide mass [Da] → Set to "6000".
   (i) Protein quantification → Disable "Discard unmodified counterpart peptides".
7. Set up the experimental design for the data files in MaxQuant. Give different samples and individual biological replicates a unique experiment name. Technical replicates from the same biological replicate should be given the same experiment name. Do not assign different fraction numbers to the data files; this is only intended for samples, which were fractionated prior to LC-MS/MS and where matching between runs should exclude certain match-ups (*see* **Note 24**).
8. MaxQuant will provide several text tables as output. The proteinGroups.txt file contains virtually all relevant protein information, including quantification. In addition, the summary.txt file will give insight into the identification rate of all MS/MS spectra. In case less than 10% of the MS/MS spectra are being identified as peptides, it may be advantageous to optimize the chromatography and mass spectrometry method, or load more sample.
9. Analyze the proteinGroups.txt output using Perseus software. Statistical analysis and validation of data is not a simple process that can be described in a few steps, and therefore we refer to the online MaxQuant and Perseus documentation and tutorials, and the expertise of the experimenter's local

bioinformaticians. We will provide a few straight-forward steps that are part of a typical data analysis pipeline, but these are by no means exhaustive (*see* **Note 25**).

10. Import the data from the proteinGroups.txt, setting the LFQ intensity values as the main expression columns. Other information, such as non-normalized intensity values, peptide counts, and MS/MS counts, may be imported as numerical data to provide additional insight.
11. Perform a categorical annotation of rows, i.e., setting up an experimental grouping with Perseus. Here, multiple biological replicates corresponding to the same condition should be given the same experiment name. Name this grouping "Experiment".
12. Perform a secondary categorical annotation, and indicate which samples correspond to the cell line expressing His_{10}-SUMO and which samples do not. Name this grouping "His_{10}-SUMO".
13. Filter the data by excluding reversed database hits and "Only identified by site" hits. We do not recommend a priori excluding potential contaminants, as some of these may be SUMO targets.
14. Perform a $\log_2$ transformation on the LFQ intensity values.
15. Perform a filtering based on valid values, demanding that at least three valid values are found in at least one experimental grouping. For this purpose, use the "Experiment" grouping (*see* **Note 26**).
16. Perform imputation to derive any missing values. Set the mode for imputation to "Total matrix", with the default numerical settings.
17. Perform a two-sample test to uncover SUMO target proteins. For this purpose, perform a two-sample test on the "His_{10}-SUMO" experimental grouping, setting "Yes" as the first group and "No" as the second group. Set the S0 value to 0.5, and leave the FDR settings at 0.05. Perseus will add several columns indicating the ratio difference between the His_{10}-SUMO cells and the parental control, the *p*-value of the observed difference, the maximum *q*-value for the comparison, and whether the change is significant within the constraints of the FDR.
18. Perform a two-sample test to uncover any differences between experimental conditions. For this purpose, perform a two-sample test on the "Experiment" grouping, setting a "treated" or "positive" condition as the first group, and a "control" condition as the second group. Set the S0 value to 0.5. Leave the permutation-based FDR at 0.05, or alternatively change the testing to a direct *p*-value assessment in case multiple hypothesis testing is not preferred (*see* **Note 27**).
19. Several other statistical functions of Perseus may prove useful, such as scatter plot analysis, principle component analysis, heatmap analysis, or term enrichment analysis.

20. Export the processed data as a text file, which may be readily investigated with programs such as Excel (*see* **Note 28**).

4 Notes

1. For proper statistical evaluation of data, and thus for accurate label-free quantification (LFQ), at least three biological replicates are required. In general, LFQ can reliably quantify differences of a factor >3 under any conditions, but may be able to quantify smaller differences provided the analysis is carried out accurately and consistently. Increasing biological replicates to 4 or 5 can increase the overall sensitivity of the protocol, and is recommended when only subtle changes in the SUMO proteome are anticipated. Different biological replicates may be prepared over the course of several weeks, i.e., growing, treating, and lysing the cells. However, all technical handling of the samples should be performed simultaneously. In general, it is preferable to have more biological replicates and less technical replicates, e.g., five biological replicates measured once would be preferable over three biological replicates measured twice.

   For large systematic differences, such as comparing SUMO target proteins in a His_{10}-SUMO2 line versus the contaminants in a parental line, the overall experimental consistency is not as important. A similar scenario would exist when causing large differences in the SUMO proteome, by performing heat shock or proteasome inhibition. Contrarily, when subtle differences are expected, e.g., affecting <50 targets by <2-fold in a population of >500 target proteins, it is absolutely critical to minimize technical variation, and it may even be necessary to minimize biological variation by culturing all biological replicates simultaneously. Often overlooked variables in SUMO dynamics are factors such as age of the DMEM, passage number of the cells, cellular confluence, and how often the incubator is opened and closed during the experiment.

2. In our hands, a confluent 15-cm dish of HeLa cells will result in 75–150 μl of cell pellet. This equals in the range of 15–30 million cells, or 3–6 mg of total protein. Depending on treatment and efficiency of purification, 1–5 μg of SUMOylated protein can be purified from this amount of cells expressing His_{10}-SUMO2 at a level that is between 1 and 5 times that of endogenous SUMO2. Cell lines overexpressing SUMO2 will have an overrepresentation of non-conjugated SUMO2, which interferes with purification and SUMOylation dynamics.

3. A swift and complete lysis of the cells is critical to preserve SUMOylation and allow for efficient purification of SUMO target proteins. Some dexterity is required in adding lysis buffer directly onto the cell pellet while vortexing, and practicing

this action beforehand may be beneficial, e.g., by pipetting water into an open tube while vortexing. Not long after adding the lysis buffer onto the cell pellet and briefly lysing, the tube should be quickly and firmly closed, and vigorously shaken in order to disturb the large clump of chromatin. Vortexing alone is not sufficient for this purpose. When cells are treated harshly, or are otherwise unhealthy in culture, lysis may prove to be more difficult. Ensure that the tube is sufficiently large for the lysis, with at least 1/2 and preferably at least 2/3 of the tube remaining empty. Regardless, after 30–60 s of lysing most proteins will be dissolved and the sample should be snap frozen. Homogenization by sonication, and centrifugation to remove any remaining debris, will complete preparation of the lysate.

4. Snap freezing of the viscous lysate after vigorous lysing may trap a significant amount of air in the lysate. Furthermore, small amounts of liquid nitrogen may leach into the tube if it is not closed properly prior to snap freezing. Therefore, avoid rapid thawing out of the lysates, e.g., by using a water bath. Sudden temperature and pressure differences may cause the tubes to rupture or even explode. Always gently thaw the lysates by air, at room temperature, on a roller-mixer. Furthermore, after lysis and just before snap freezing, it is advantageous to briefly open and then firmly reseal the tube.
5. Aqueous guanidine is stable, and heating of the lysate during sonication is not harmful. However, we still recommend that lysate temperatures do not exceed 40 °C, and letting the samples cool to room temperature between sonication cycles is generally a good idea.
6. The amount of Ni-NTA resin used in this protocol roughly corresponds to 20 μg of beads per 1 μg of SUMOylated protein. The reported Ni-NTA binding capacity of 1 μg beads is 8 μg of target protein. We thus use ~160 times more beads than would be theoretically necessary. However, while the reported binding capacity may hold true under ideal and non-denaturing conditions, or when re-purifying an already pure target protein, we have observed the binding capacity to be much lower in guanidine buffer and when purifying from complex mixtures of proteins. The amount of beads necessary may be titrated for individual cell lines, but keep in mind that guanidine lysates cannot be directly analyzed through SDS-PAGE because SDS and guanidine are incompatible in solution at high concentrations.
7. There are several reasons why SUMOylated proteins are not or poorly recovered from the lysate. Firstly, the amount of imidazole in the lysate could either be too low, allowing competitors to bind, or too high, preventing binding of any proteins. Secondly, the pH of the lysis buffer could be in excess of 8.0. Buffers in the range of 8.2–8.4 or even higher lead to very high background

binding of many proteins regardless of imidazole concentration. Setting the lysis buffer to a pH of 7.5–7.7 will lead to a cleaner pulldown, but may reduce overall yield. Thirdly, the amount of beads could be insufficient, or the amount of free SUMO could be too high. While this would mostly be due to expressing level of SUMO, cellular treatment, or inefficient lysis, increasing the amount of Ni-NTA beads can be a work-around.

8. Urea is unstable in aqueous solutions, especially at alkaline pH and elevated temperatures, and should always be dissolved freshly on the same day. For the urea-containing buffers in this protocol, we recommend preparing 50 ml of 9 M urea stock by dissolving 27.03 g of urea in MQ water, and filling to 50 ml. Dissolving of urea is endothermic and may be accelerated by incubating the urea solution in a 37 °C water bath while frequently mixing. Remove the 9 M urea stock to room temperature once all urea is dissolved, and discard any leftover 9 M urea stock at the end of the day.
9. After changing the buffer from guanidine to urea, and especially after the Triton X-100 is no longer present in the buffer, it becomes increasingly difficult to pellet all beads. Beads may also start sticking to the side of the microcentrifuge tube upon centrifugation. To optimize recovery of beads, we recommend the use of a swing-out centrifuge, or performing a two-step centrifugation in a fixed-angle rotor where the tube is rotated 180° between the steps. Ideally, a centrifuge with a slow deceleration is used, as sudden deceleration can lead to dislodging of some beads. To prevent beads sticking the tube wall, LoBind tubes generally perform quite well. Otherwise, just prior to centrifugation, add more wash buffer to almost entirely fill the tube.
10. Filtering of the elutions through a 0.45 μm filter is critical, as any beads and other large particles present in the sample will compromise concentration over 100K MWCO filter units. During the third and final elution, the entire bead suspension may also be passed through the 0.45 μm filter to optimize sample recovery. The first and second elutions tend to have a similar yield in SUMO target proteins, whereas the third elution will have markedly less but still sufficient to warrant pooling with the first two elutions.
11. Whereas one may think that smaller SUMO target proteins are able to pass through the 100K filter and are lost as a result, we have proven that under denaturing 7 M urea conditions, only free SUMO passes through the filter [12, 13]. Concentration speed may depend on the yield of the sample and any contaminants remaining in the buffer. Note that Triton X-100 forms micelles that exceed 100 kDa in size and will not pass through the filter unit, therefore it is absolutely vital that all Triton X-100 is removed from the sample in the last few washing steps. Initially, it may be beneficial to monitor the concentration

speed of the samples every few minutes, to avoid overconcentration. Although it is hard to estimate the exact volume, a small amount of liquid should remain visible when looking through the side of the filter unit. Prior to starting the wash steps, the sample should be concentrated to <50 µl. Concentrating to a smaller volume after the final wash step is okay. Note that even if the column appears completely dry, there will still be some liquid left in the dead volume. If the total recovered volume of concentrated proteins from the filter is <10 µl, it is advantageous to add ~10 µl of Urea Buffer to the filter unit, gently tap the filter unit, and incubate for a few minutes at room temperature. Recover the Urea Buffer and pool it with the rest of the concentrated proteins.

12. While it is also possible to remove the concentrated sample directly from the filter unit into a LoBind tube, the filter unit does not fit perfectly into the LoBind tube, and as a result the assembly will not fit in most microcentrifuges. Furthermore, if not carefully placed, the filter unit may dislodge and get launched during centrifugation, which is both highly undesirable and dangerous.
13. A mini-Bradford essay is typically sufficient. Adding 0.5 µl of concentrated material to 10–50 µl Bradford should cause a visible coloration to blue, whereas control Urea Buffer should not. The parental control should cause much less or no visible blue coloration. Use a NanoDrop instrument to determine the exact concentration of SUMOylated proteins. We have observed that 1 µg of BSA in 50 µl of Bradford corresponds to about 0.070 absorbance (1 mm path at 595 nm) on a NanoDrop. Values exceeding 0.100 for a 1 mm path length are no longer linear; dilute the samples if necessary.
14. We recommend adding ABC in a 1:20 ratio from a 1 M stock.
15. DTT is not stable in solution. Working solutions should be kept on ice, and stored at −20 °C for longer term.
16. Chloroacetamide is highly unstable in aqueous solutions, and should be dissolved freshly before use. We recommend making a 250 mM stock in water. Iodoacetamide may also be used.
17. Lys-C digestion is not strictly necessary, and while beneficial, adding too much Lys-C will cause extensive contamination of the sample with Lys-C peptides, which may reduce chromatographic performance. It is therefore important to accurately determine the SUMOylated protein concentration. Otherwise, assume a concentration of 1 µg of SUMOylated protein per 15-cm plate, thus adding 20 ng of Lys-C per 15-cm plate of material processed. Alternatively, the Lys-C digestion may be skipped at the experimenter's behest.
18. Concentrated TFA is a highly corrosive and toxic substance, handle with care, in a fume hood, and wear protective equipment. It may be advantageous to prepare a 50% TFA solution

in water, which does not fume and is safer to handle at the bench, and is easier to accurately pipet.

19. The dryness of the column material can directly be observed by eye. Dry column material is pristine white, whereas wetted column material is dullish grey. Drying out of the column will happen from top to bottom, and happens much faster when volatile buffers were last passed over the column, i.e., acetonitrile or methanol. If the column significantly dries out during the equilibration procedure prior to loading the sample, repeat the whole equilibration. Leave a small bit of liquid (~1–2 mm) on the column at all time to minimize drying. After loading the sample and washing twice, it is safe to let the tips dry completely.
20. We have not observed significant differences between peptides that were dried to completion, or peptides that were dried until ~10–20% liquid remained, as long as all acetonitrile has evaporated. Regardless, avoid heating of the samples during vacuum drying. The temperature should not exceed room temperature when high concentrations of acetonitrile are still present in the sample, and should never exceed 40 °C even after most acetonitrile has evaporated.
21. While we perform chromatography with 0.1% formic acid, the experimenter's preference, or the recommendation from the mass spectrometry facility, may be different. 0.5% formic acid, 0.1% trifluoroacetic acid, or 0.5% acetic acid are commonly used in the field and should work perfectly fine, although we have no experience with using these buffers for analyzing the SUMO proteome.
22. For matching between runs, MaxQuant matches identified features by m/z and chromatography elution time. There is a certain threshold for technical variation, i.e., a 20-min window for aligning the entire gradient, in addition to a 1 min window for aligning separate peaks. These windows may be adjusted by the experimenter if required. Therefore, samples measured on similar columns and separated with similar gradients should align properly, even if not all samples can be measured in one session. We still recommend running samples with a similar expected composition back-to-back.
23. The expected chromatography pattern will be one of medium complexity (Fig. 3), i.e., in between the "hill" pattern usually observed for a total lysate digest, and the "spiky" pattern usually observed for a digest of a limited number of proteins. A high degree of "spikiness" is observed when insufficient sample is loaded, and overrepresented peaks from SUMO itself, trypsin, Lys-C, and other common contaminants are the only prevalent peaks. A sufficiently high load will cause several of the major peaks to take multiple minutes to elute, but the "plateau" in between the peaks should reach sufficiently high, in

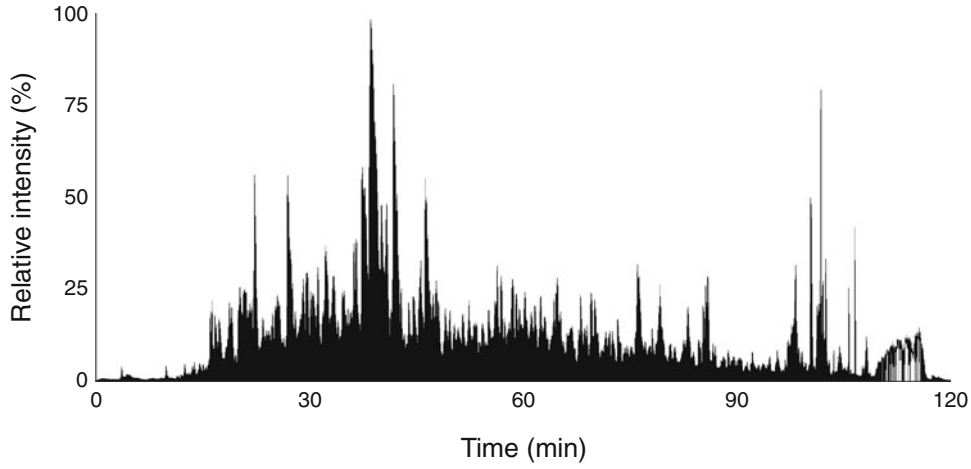

Fig. 3 A representative chromatographic profile when analyzing a suitable amount of His_{10}-SUMO2 target peptide sample using a 120 min elution gradient. The majority of the peaks should exceed 5–10 % of the maximum relative intensity. The amount of material required per run may vary depending on cell line and purification yield, and is generally in the range of 2–20 million cells, or 0.5–5 mg of total protein, or 0.5–5 μg of SUMOylated protein, or 10–100 % of a nearly confluent 15-cm dish, when using HeLa or U2OS cells

the range of 10–20 %, to allow for identification of less abundant SUMO target protein peptides (Fig. 3).

24. In case the experimenter decides to fractionate the samples prior to analysis, for example by size-separation of SUMOylated proteins on SDS-PAGE prior to pursuing an in-gel digestion strategy, or by ion-exchange chromatography of the final purified peptide mixture, fraction numbers have to be assigned in the experimental design. Here, all fractions that are expected to yield comparable groups of proteins, i.e., all of the top slices from SDS-PAGE, or all of the lowest-salt elutions from ion-exchange chromatography, should be given the same fraction number. Neighboring fractions, i.e., adjacent gel slices in terms of size, or subsequent elution fractions from ion-exchange chromatography, should be given neighboring and valid numbers. Matching between runs will compare peaks between samples with the same fraction numbers, and additionally to peaks in samples with an adjacent fraction number, e.g., a fraction 2 sample will be matched to all fraction 2 samples, and secondarily to fraction 1 and 3 samples.

25. Documentation, tutorials, and advice for using MaxQuant may be found at: http://coxdocs.org/doku.php?id=maxquant:start, http://groups.google.com/group/maxquant-list

    Documentation, tutorials, and advice for using Perseus may be found at: http://coxdocs.org/doku.php?id=perseus:start, http://groups.google.com/group/perseus-list

26. Depending on experimental design, the experimenter's choice for filtering the data may differ. For example, when there are eight samples representing a similar condition, e.g., four biological replicates using two different knockdown viruses targeting the same protein, it may be advantageous to group these together, and demand valid values in at least 6 out of 8. This would ensure a triplicate participation of both viruses, or quadruplicate of one and duplicate of the other. A more stringent filtering for 8 out of 8 valid values could be enforced, which is entirely feasible as long as matching between runs performs adequately. Note that even with a less stringent filtering, the missing values will be imputed and are unlikely to correlate with the real values, therefore increasing standard deviation and increasing the *p*-value of the two-sample test, rendering the observed difference insignificant.
27. Permutation-based FDR is a highly stringent method of filtering for significant differences, and will generally report no differences between moderately dynamic SUMO proteomes. Comparing SUMO-enriched samples to the parental control will readily report hundreds of significant targets, as will comparing SUMO proteomes resulting from cellular treatments causing extensive SUMOylation dynamics, such as heat shock or proteasome inhibition. Overall, increasing the amount of biological replicates, optimizing the intended dynamic effect of the cellular treatment or procedure, or expanding the purification procedure to minimize the presence of non-dynamic SUMO target proteins, will lead to greater perceived differences. When assessing smaller changes in the SUMO proteome, such as those observed during the DNA damage response, or when knocking down one protein, it is often better to assess differences by *p*-value alone. However, we recommend great caution in accepting any change of less than a factor 2, and any *p*-value greater than 0.05. In general, changes in excess of a factor 3 and *p*-values less than 0.01 are reliable, provided that sufficient peptides identify the protein (3+) and sufficient MS/MS scans were identified (equal to or greater than the total amount of biological samples). Additionally, differences observed outside of permutation-based FDR should be double-checked and revalidated by performing manual normalization and quantification of the non-LFQ intensity values. For proteins detected by a small amount of peptides or MS/MS scans, LFQ may sometimes behave erratically and generate aberrant ratios.
28. Perseus is an excellent tool for rapid filtering and statistical processing of the data, and adding columns reporting ratios of change, *p*-values, and *q*-values. Several other statistical analyses can also be rapidly performed. However, the program is somewhat cumbersome and not suited for generating a table with a

comprehensive and intuitive layout, and does not facilitate easy browsing of the data for proteins of interest. Therefore, once statistical processing is complete, it is advantageous to export the data, and use Excel for further formatting and evaluation of the data.

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Chapter 14

The Use of Multimeric Protein Scaffolds for Identifying Multi-SUMO Binding Proteins

Elisa Aguilar-Martínez and Andrew D. Sharrocks

Abstract

The use of in vitro assays, such as glutathione S-transferase (GST) pull-downs, enables the study of complex cellular processes in a simplified form. Pull-down assays facilitate the discovery and detailed study of protein–protein interactions, which can then be extrapolated to the cellular environment. Here, we describe the expression, purification and use of a multi-SUMO platform to identify SUMO-interacting proteins. This SUMO-platform can be easily expressed and purified from bacterial cells for use as baits in pull-down assays. This methodology facilitates the discovery of novel SUMO-binding proteins or further characterization of SUMO with known binding partners.

Key words Multi-SUMO, Poly-SUMO, Protein–protein interaction, Protein purification, Pull-down

1 Introduction

SUMOylation is a dynamic posttranslational modification that can permit or disrupt protein–protein interactions (Reviewed in ref. [1]). Proteins have been shown to interact with SUMO through a consensus sequence, the SUMO-Interacting Motif (SIM). The consensus motif consists of hydrophobic amino acids followed or preceded by acidic amino acids [2, 3]. Multiple SIM motifs can be found in a single protein. For example, ZMYM2 and Arkadia/RNF11 contain three functional SIMs whereas RNF4 contains four SIMs [4–6]. SUMO is conjugated to lysine residues that are usually preceded by a bulky hydrophobic amino acid and followed by a nonspecific amino acid spacer and then an acidic residue (ψ-K-X-E/D) [7]. SUMO2/3 can be incorporated into its substrate as a monomer on multiple lysine residues, to create a multi-SUMO platform, or on the same lysine residue as SUMO chains, to create poly-SUMO (Fig. 1). In contrast, SUMO1 is not thought to form chains but has the potential to form a multi-SUMO platform through multi-site conjugation.

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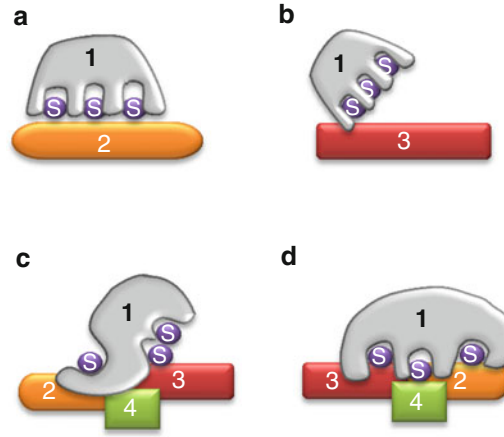

Fig. 1 Models for the presentation of multi-SUMO and interaction modalities with different multi-SUMO platform configurations. Putative multi-SIM containing proteins (1), SUMOylated interaction partners (2-4) and SUMO (purple circles) are depicted (**a**, **b**) SUMO can be conjugated to several lysines on the same protein forming a multi-SUMO scaffold (**a**) or on the same lysine forming poly-SUMO chains (**b**). (**c**, **d**) Multi-SUMOylated scaffolds can be potentially formed by the interaction of a SIM-containing protein with different SUMOylated proteins in a complex

A recent mass spectrometry study identified around four thousand SUMOylation sites in over one thousand proteins. Among these proteins are ZNF451 which contains 40 SUMOylation sites, MIS18BP1 with 25 and hnRNPUL with 6 [8] and hence these proteins have the potential to form complex multi-SUMO platforms. The formation of multi-SUMO platforms could therefore occur due to the formation of chains or multi-site SUMOylation on a single protein but equally might also arise by presenting SUMO on several different proteins involved in the same cellular process. One process in which this scenario appears likely is DNA repair, where multiple different proteins were found to be SUMO modified and the individual modifications contribute collectively to the efficiency of the DNA repair process [9]. Functionally, multivalent SUMO–SIM interactions likely strengthen the SIM–SUMO interaction, and/or allow the interaction of different SIM-containing proteins with a multi-SUMOylated substrate (Reviewed in ref. [10]). In this context, the role of poly-SUMO chains as a platform for additional protein interactions has been widely studied (Reviewed in ref. [11]). Moreover, in vitro poly-SUMO chains, consisting of head to tail fused SUMOs have been previously used to study the interaction of SIM-containing proteins [6]. However, these chains represent only one way in which multiple SUMO moieties can be presented. Alternative in vitro platforms to study different multi-

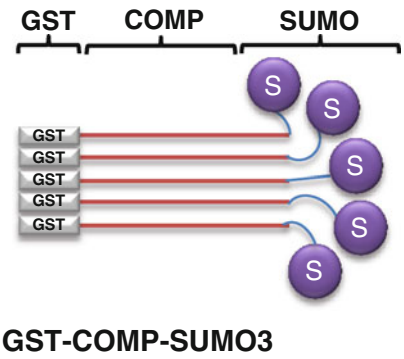

Fig. 2 Schematic diagram of the multi-SUMO platform GST-COMP-SUMO3. This multi-SUMO scaffold consists of GST (*gray boxes*) fused to the coiled-coil pentamerization domain of COMP (*red lines*; amino acids 29–74), a influenza virus hemagglutinin peptide-derived linker (*blue lines*; GSGSGS), and SUMO3 (*purple circles*)

SUMO-SIM configurations were previously not available. Therefore, in order to identify and study new multi-SIM containing proteins we designed a novel multi-SUMO platform (Fig. 2) [4]. This platform mimics multi-SUMOylation, can be expressed and purified from bacteria and was developed by taking advantage of the coiled-coil domain of cartilage oligomeric matrix protein (COMP). The coiled-coil domain of COMP consists of seven heptad repeats that form a five-stranded domain [12], and therefore can be used to create a pentameric scaffold on which five SUMO moieties are presented. Using the influenza virus hemagglutinin peptide (GSGSGS) as an artificial linker [13], SUMO3 was fused to the C-terminus of the coiled-coil domain of COMP (amino acids 29–74). To facilitate affinity purification of the fused protein a GST tag was added to the N-terminus of COMP (amino acids 29–74) to create the construct GST-COMP-SUMO3. This construct resembles a potential multi-SUMOylated protein that can be used to identify multi-SIM containing proteins independently of the substrate to which SUMO is attached. When GST-COMP-SUMO3 is expressed, the coiled-coil domain of COMP forms a pentameric structure without the need to add any reagent or substrate. One important feature is the glycine-serine linker between COMP and SUMO which provides potential flexibility to SUMO positioning. Incorporation of the GST tag not only facilitates the purification of the protein but allows its use for pull-down assays.

While the system we describe is specifically designed to isolate multi-SUMO binding proteins, in principle, the same approach could be used to study interactions with any ubiquitin-like modifier when presented as a multimerized form.

2 Materials

All the solutions should be prepared using ultra-pure water. Unless stated differently buffers should be kept at 4 °C.

2.1 Bacterial Growth

1. High salt Luria Broth (LB) media, 1% tryptone, 0.5% yeast extract, 1% NaCl. Sterilize by autoclaving.
2. Antibiotics stock solutions, 200 mg/ml ampicillin in water, 34 mg/ml chloramphenicol in absolute ethanol. Sterilize by filtration using a 0.22 μm pore filter. Store at −20 °C.
3. 50% glycerol in water. Sterilize by autoclaving. Store at room temperature.
4. One molar stock solution of Isopropyl β-d-1-thiogalactopyranoside (IPTG) is made in water and sterilized by filtration using a 0.22 μm pore filter. Store at −20 °C.

2.2 Protein Purification

1. 5× phosphate buffered saline (PBS), 685 mM NaCl, 13.5 mM KCl, 50 mM Na_2HPO_4, 10 mM KH_2PO_4. Sterilize by autoclaving. Store at room temperature.
2. GST lysis buffer, 1× PBS, 0.5% Triton X-100.
3. Protease Inhibitors Cocktail, Complete tablets (Roche). Store at −20 °C.
4. GST-wash buffer I, 1× PBS, 400 mM NaCl.
5. GST-wash buffer II, 1× PBS, 400 mM NaCl, 0.5% Triton X-100.
6. Glutathione (GSH)-agarose beads (Sigma).

2.3 Pull-Down

1. SUMO-binding buffer, 50 mM Tris base pH 7.5, 250 mM NaCl, 0.1% Igepal, 5% glycerol.
2. SUMO-wash buffer, 50 mM Tris base pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 5% glycerol.
3. 2× SDS loading buffer, 0.25 M Tris–HCl pH 6.8, 20% glycerol, 0.2% bromophenol blue, 0.4% SDS, 0.4% β-mercaptoethanol. Store at room temperature.

3 Methods

3.1 Protein Expression in Bacteria

1. Transform *Escherichia coli* BL21-CodonPlus(DE3)RIL (*see* **Note 1**) with 100 ng of each plasmid encoding a GST-fusion protein. Plate the transformants in high-salt LB agar plates with 200 μg/ml ampicillin and 34 μg/ml chloramphenicol.
2. Next day, inoculate 5 ml of LB media in a culture tube (*see* **Note 2**) with a single colony from the plates. Add ampicillin

and chloramphenicol to a final concentration of 200 μg/ml and 34 μg/ml respectively. Grow overnight at 37 °C with shaking at 200 rpm (*see* **Note 3**).

3. The following day add ampicillin (200 μg/ml final concentration) to 50 ml of LB in a 250 ml conical flask (*see* **Note 4**). Inoculate the LB with 1 ml of the overnight culture (giving an OD_{600nm} of approximately 0.05–0.1). Grow the culture at 37 °C, with shaking at 200 rpm until it reaches an OD_{600nm} of 0.5–0.6, (approximately 2–2.5 h) (*see* **Note 5**).
4. Induce protein expression by adding IPTG to a final concentration of 200 μM (*see* **Note 6**).
5. Continue to grow the cells for an additional 4 h with shaking at 200 rpm, at 25 °C (*see* **Note 7**).
6. Spin down the cells at 2230 × *g*, 4 °C for 15 min (*see* **Note 8**).
7. Remove supernatant and either proceed to affinity purification (Subheading 3.2) or freeze the pelleted cells at −20 °C.

3.2 GST-Fusion Protein Purification from Escherichia coli

GST-tagged proteins are purified from bacteria lysates by affinity binding to solid bead supports.

3.2.1 Preparation of the Beads

1. Add 400 μl glutathione agarose beads to a 2 ml tube (*see* **Note 9**).
2. Wash the beads, by adding 1.5 ml of GST lysis buffer, mix the beads and spin down for 1 min at room temperature at 100 × *g* and discard the supernatant (*see* **Note 10**).
3. Repeat **step 2** once.
4. Add 1.5 ml of GST lysis buffer. Leave the beads to equilibrate at room temperature while preparing the cell lysate.

3.2.2 GST-Fusion Protein Binding and Washing

1. Add 0.04 g bacteria pellet (Subheading 3.1, **step 7**) (approximately one quarter of a 50 ml bacterial culture) to a 1.5 ml tube.
2. Add 800 μl of GST lysis buffer.
3. Add protease inhibitors (Complete) to a final dilution of 1:50.
4. Briefly vortex to resuspend the pellet.
5. Sonicate the cells for 10 cycles of 30 s on, 30 s off (*see* **Note 11**).
6. Spin down the cells at 15,700 × *g*, 4 °C for 10 min and retain the supernatant.
7. Transfer 20 μl of the supernatant to a new tube and keep it on ice for analysis of total protein available for binding (Input).
8. Spin down the beads (Subheading 3.2.1, **step 4**) for 1 min at room temperature at 100 × *g*. Remove and discard the buffer.

9. Add the supernatant (**step 6**) to the beads.
10. Incubate for 1 h at 4 °C with gentle agitation.
11. Spin down the beads for 1 min at room temperature at 100 × *g*, take 20 μl and keep it for analysis of unbound protein (Flow Through, FT).
12. Discard the rest of the supernatant.
13. Wash the beads once as in **step 2** (Subheading 3.2.1) using GST-lysis buffer and discard supernatant.
14. Wash the beads by adding 1.5 ml of GST-wash buffer I. Incubate for 5 min at 4 °C with gentle agitation. Spin down for 1 min at room temperature at 100 × *g*. Remove and discard the supernatant.
15. Repeat **step 14** twice.
16. Wash the beads once with GST-wash buffer II. Incubate for 5 min at 4 °C with gentle agitation. Spin down for 1 min at room temperature at 100 × *g*. Remove and discard the supernatant.
17. Wash the beads once with 1.5 ml 1× PBS. Spin down for 1 min at room temperature at 100 × *g*. Carefully remove and discard the supernatant (*see* **Note 12**).
18. Wash the beads once with 1.5 ml SUMO-binding buffer. Spin down for 1 min at room temperature at 100 × *g*. Carefully remove and discard the supernatant.
19. Add 200 μl SUMO-binding buffer to the pelleted beads and add protease inhibitors (dilution 1:50).
20. Take 10 μl of the resuspended beads for analysis (purified protein).
21. Keep the remaining beads at 4 °C.
22. Verify the expression and purification of the protein by SDS-PAGE by running the three fractions, ie input (**step 7**), flow through (**step 11**) and purified protein (**step 20**) (*see* Fig. 3 for an example).

3.3 GST Pull-Down Assay

1. In a 1.5 ml microcentrifuge tube, add the necessary amount of beads (Subheading 3.2.2, **step 21**) to have 1 μg of GST-fusion protein. If the volume of the beads is smaller than 30 μl top it up using GSH-agarose beads without protein (*see* **Note 13**).
2. Incubate, either a complex protein mixture or a purified putative interacting protein (*see* **Note 14**) to the beads-bound GST-fusion protein in a total volume of 250 μl of SUMO binding buffer in the presence of protease inhibitors (dilution 1:50), and incubate at room temperature for 2 h with gentle agitation.

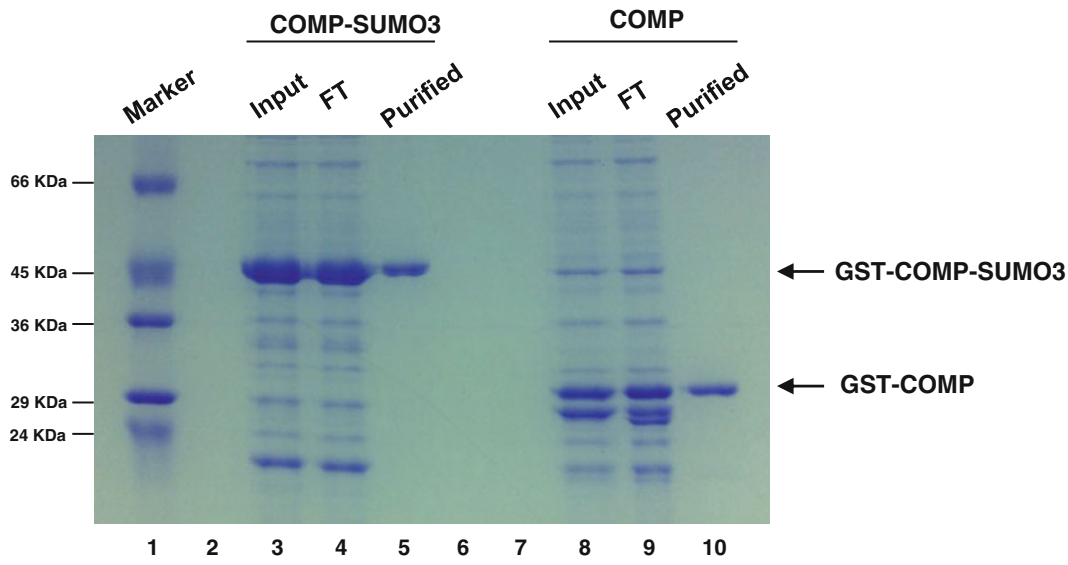

Fig. 3 SDS-PAGE analysis of the protein purification steps. Bacteria expressing either GST-COMP-SUMO3 (multi-SUMO platform) or GST-COMP (control) were lysed to release total cellular proteins (Input), and incubated with GSH-agarose beads. Proteins that did not bind to the beads (FT, flow through) and the final purified protein attached to the GSH-agarose beads are shown. The positions of the bands corresponding to GST-COMP-SUMO3 and GST-COMP are indicated. *Lanes 5* and *10* show successful purification of GST fusion proteins although a substantial portion of the protein remains unbound in the FT fractions (*lanes 4* and *9*)

3. Spin down for 1 min at room temperature at 100 × *g*. Remove and discard the supernatant.
4. Wash the beads by adding 1 ml of SUMO-binding buffer. Spin down for 1 min at room temperature at 100 × *g*. Remove and discard the supernatant.
5. Wash the beads by adding 1 ml of SUMO-binding buffer. Incubate the beads for 5 min at room temperature with gentle agitation.
6. Spin down for 1 min at room temperature at 100 × *g*. Remove and discard the supernatant.
7. Wash the beads by adding 1 ml of SUMO-wash buffer. Incubate the beads for 5 min at room temperature with gentle agitation.
8. Spin down for 1 min at room temperature at 100 × *g*. Remove and discard the supernatant.
9. Repeat **steps 7** and **8** once more.
10. Spin down the beads once more, 1 min at room temperature at 100 × *g*.
11. Carefully remove the remaining supernatant (*see* **Note 12**).

12. Elute the proteins from the beads by adding 25 μl of 2× SDS-loading buffer.
13. Identify the interacting proteins by western blotting or mass spectrometry (*see* **Note 15**).

4 Notes

1. BL21-CodonPlus(DE3)RIL are used as they are codon-optimized for mammalian protein expression. However, other bacterial strains can be used as long as they retain an intact Lac regulatory system (yields may be lower with other strains).
2. For bacterial culture do not fill up tubes or conical flasks with LB. Media is better oxygenated when at least 75 % of the tube/flask is empty resulting in better bacterial growth.
3. Overnight growth of bacteria transformed with plasmids conferring ampicillin resistance and encoding toxic proteins is not recommended since it could lead to plasmid loss and therefore lack of expression of the desired protein. However, this is not the case for GST-COMP or GST-COMP-SUMO.
4. Since chloramphenicol inhibits translation, do not add it when growing bacteria to express protein.
5. A glycerol stock can be made using the overnight culture. Take 600 μl of the overnight culture, spin it down in a microcentrifuge for 3 min at 3300 × *g*. Remove the supernatant. Resuspend the pellet in 600 μl of fresh LB. Add 600 μl of sterile 50 % glycerol and mix gently. Store at −80 °C. This stock can be used in the future instead of having to re-transform bacteria.
6. Allow the cultures to cool down to room temperature before adding IPTG.
7. GST-COMP fusions are relatively insoluble proteins and a better yield of soluble proteins is achieved if cells are grown at 25 or 28 °C after the addition of IPTG.
8. Pelleted cells can be kept frozen at −20 °C for at least a year.
9. For protein purification either GSH-agarose or GSH-magnetic beads can be used. The nature of the beads does not affect the results. The use of magnetic beads reduces the time of the experimental procedures. If using lyophilized beads, incubate them over night in ultrapure water. Wash the beads three times in 1× PBS. Store the beads in 1× PBS, 20 % ethanol at 4 °C. To wash the beads, spin them down at 100 × *g* for 1 min. To avoid losing beads allow a small amount of buffer to remain on top of the pelleted beads. If using magnetic beads do not leave the tubes for long periods of time on the magnetic rack to avoid aggregation of the beads.

10. Spin is done a low speed, $100 \times g$ to prevent compacting the beads, which could potentially trap unbound proteins.
11. Sonication can be performed either using a water bath or an immersion probe. To avoid protein degradation, set the water bath temperature to 4 °C or if using an immersion probe place the tube containing the resuspended cells in ice.
12. To avoid losing beads when removing the remaining supernatant, squeeze the end of the pipette tip with forceps.
13. GST proteins are highly expressed resulting in a high amount of protein bound to the beads after purification. Therefore, only a small amount (typically 5–15 μl of a 50:50 suspension) of beads is needed for the pull-down assay. However, the use of small volumes of beads could lead to the loss of significant amounts of the beads during the assay. To prevent this, GSH-agarose beads, previously washed three times and equilibrated in an equal volume of SUMO binding buffer, should be added to the protein-bound beads to have a final volume of 30 μl beads (50:50 suspension).
14. The protein to be tested as multi-SUMO interacting protein can be obtained from different sources. Total or nuclear cell extracts of transfected or un-transfected cells represent complex protein mixtures, whereas in vitro transcribed-translated protein or purified protein allows analysis of binary interactions. The amount of protein needed for the assay varies depending on the methods used to express the protein or to detect the interaction and needs to be standardized for each protein depending on abundance or expression levels. If the protein of interest is easily overexpressed in for example, 293T cells, 4×10^6 cells are sufficient to see the interaction by western blotting. A third of a standard 25 μl in vitro transcription-translation reaction or 1 μg of purified protein from bacteria are a good starting point for the assay.
15. Since GST or the coiled-coiled region of COMP could interact with the tested proteins, GST-COMP lacking the SUMO moiety, should be included in parallel assays to facilitate the identification of false positive interactions. When identifying the interacting protein by western blot it is recommended to verify the integrity of the bait proteins. This can be done by staining the nitrocellulose membrane with Ponceau red after the transfer. A photograph of the stained membrane should be taken for future reference. If mass spectrometry is the chosen method, run the proteins in a SDS-PAGE, stain the gel with Coomassie G-250 and proteins should be visible. If de-staining is required, use ultrapure water. A photograph of the gel should be taken for future reference. Cut the bands of the proteins of interest and perform mass spectrometry analysis.

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Chapter 15

Isolation of In Vivo SUMOylated Chromatin-Bound Proteins

Tasneem Bawa-Khalfe

Abstract

SUMO posttranslational modification directs gene transcription and epigenetic programming to support normal cell function. The dynamic nature of SUMO-modification makes it difficult to identify endogenous protein substrates. Isolation of chromatin-bound SUMO targets is exceptionally challenging, as conventional immunoprecipitation assays are inefficient at concentrating this protein population. This chapter describes a protocol that effectively precipitates chromatin-associated fractions of SUMOylated heterochromatin protein 1α in cultured cells. Techniques to enrich endogenous SUMO substrates at the chromatin are also demonstrated and discussed. This approach could be adapted to evaluate chromatin-bound SUMO targets in additional in vivo systems.

Key words SUMO, Chromatin-bound, HP1α, SENP7

1 Introduction

Chromatin-bound proteins are subject to posttranslational modification (PTM) via *s*mall *u*biquitin-like *mo*difiers (SUMO). SUMO-PTM (or SUMOylation) alters the function, cellular distribution, and/or stability of protein targets [1–3]. Previous studies report SUMOylation of several substrates directly affects association with chromatin and consequently impacts normal cell physiology [4–6]. Consistently, studies evaluating SUMO dynamics at the chromatin have relevance to multiple diseases including Alzheimer's and cancer [2, 7].

Protein immunoprecipitation (IP) and subsequent immunoblot (IB) detection remains the gold standard for identification of SUMOylated proteins. Canonical IP methods are sufficient for enrichment of highly soluble cytoplasmic and nuclear proteins. However, chromatin-bound proteins solubilize poorly and are lost readily with conventional IP preparation techniques. Hence, identification of SUMOylated chromatin-bound proteins requires ectopic expression of SUMO-promoting components, i.e., SUMO isoforms, E2-Ubc9, E3-ligase. Concentrating SUMOylated chromatin-bound substrates in native conditions remains a major challenge for researchers.

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Previous studies demonstrate that sucrose-based hypotonic cytoskeleton buffer can be used to lyse cells and isolate chromatin-bound proteins [8–10]. Addition of slow-speed centrifugation concentrates the insoluble protein-DNA fraction. Finally, treatment with micrococcal nuclease (MNase) dissociates the chromatin-bound proteins from DNA. This multistep approach, called chromatin-bound protein IP or CP/IP, successfully captures SUMO-modified endogenous substrates such as heterochromatin protein 1α (HP1α/CBX5).

Isolation of SUMOylated HP1α in human breast cancer cells is especially tricky due primarily to two factors. First, SUMOylated HP1α, as demonstrated with ectopic expression of SUMO3-fused HP1α constructs, readily associates with chromatin and persist at sites within and outside heterochromatin loci [11]. Hence conventional IP procedure does not significantly concentrate SUMOylated HP1α due to its strong chromatin binding property (Fig. 1). Second, HP1α's interaction with full-length SUMO isopeptidase SENP7L maintains the protein predominantly in the unmodified state. SENP7L is upregulated in breast cancer cells and therefore must be knockdown to observe SUMOylation of endogenous HP1α (Fig. 2).

The following protocol will provide details on the CP/IP method with chromatin-bound HP1α as the SUMO substrate. Key steps and potential limitations will be highlighted throughout the chapter.

2 Materials

Dissolve all reagents in deionized ultrapure Milli-Q water unless an alternative is specified. For cell culture studies, filter reagents and autoclave dishware/glassware prior to use to prevent contamination.

2.1 Cell Growth and Transfection

1. Cell line: Human mammary adenocarcinoma cell line MCF7 from ATCC (Manassas, VA).
2. Cell Growth Media: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 100 U/mL penicillin–streptomycin.
3. Cell Culture Conditions: humidified 5% CO_2 chamber set to 37 °C.
4. Phosphate-buffered saline (PBS).
5. 0.05% trypsin–EDTA, phenol red.
6. Vectors/Plasmids: pcDNA3-nHA-SUMO3 (available at Addgene), pcDNA3-nV5-HP1α (wt-HP1α), and pcDNA3-nHA-SUMO3-fused HP1α (SU-HP1α).

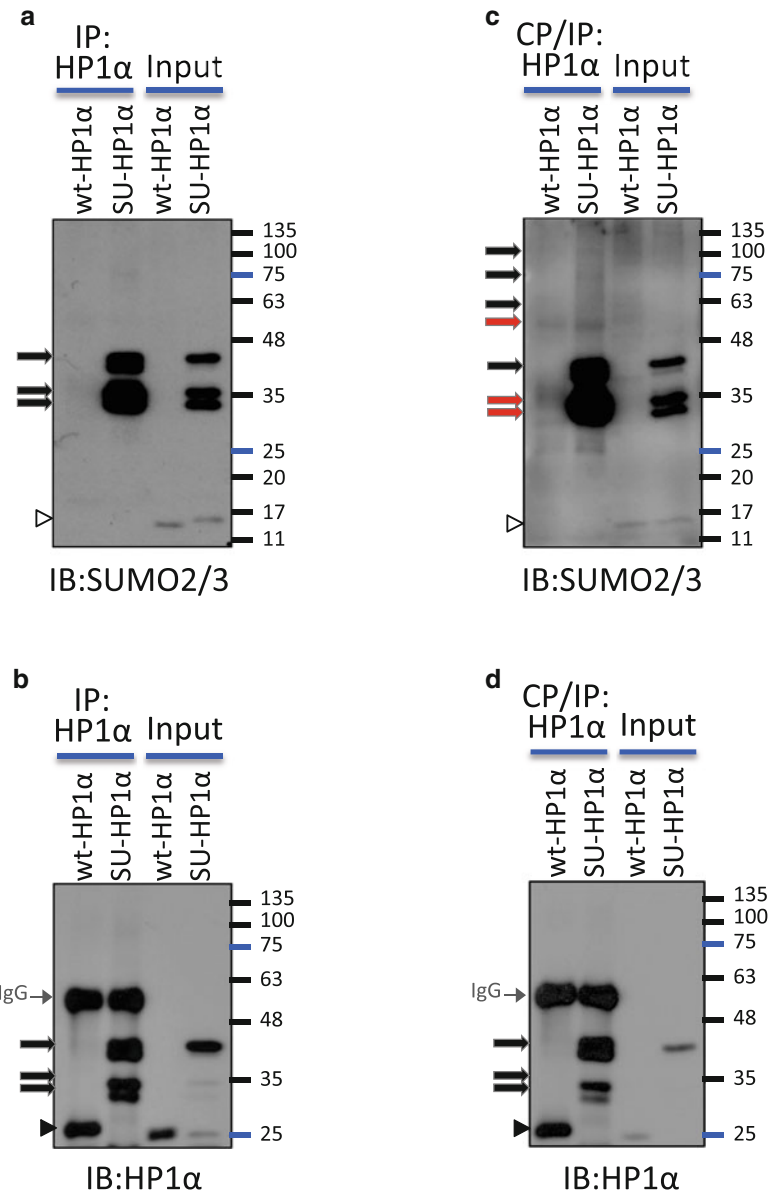

Fig. 1 Comparison of IP versus CP/IP for Isolation of Wild-type and SUMO3-fused HP1α. MCF7 cells were transiently transfected with either wild-type (wt-HP1α) or SUMO3-fused HP1α (SU-HP1α, **a**–**d**). After 24 h, harvested cells were prepared using either the IP (**a**, **b**) or CP/IP (**c**, **d**) protocol and precipitated with the anti-HP1α antibody. Membranes were probed with anti-SUMO2/3 (**a**, **c**) and blots with equivalent unconjugated SUMO2/3 (indicated with *white arrowhead*) are shown. Additional membranes were also incubated with the anti-HP1α primary antibody to evaluate pull-down efficiency of wt-HP1α and SU-HP1α in both IP and CP/IP conditions (**b**, **d**). *Black arrowhead* highlights unmodified wt-HP1α (**b**, **d**). *Black arrows* represent bands specific for SU-HP1α (**a**–**d**); higher molecular weight SUMO2/3 bands persist on SU-HP1α isolated from CP/IP but not IP (**a**, **c**). *Red arrows* indicate SUMO-conjugation bands shared by both wt-HP1α and SU-HP1α; these shared bands are present only in CP/IP samples (**c**)

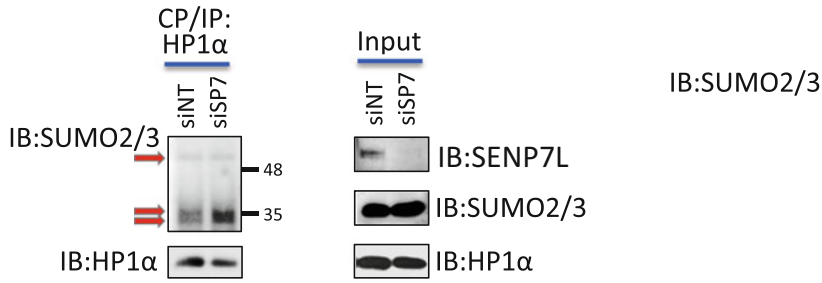

Fig. 2 SUMOylation of Endogenous HP1α Enhanced with Knockdown of SENP7L. MCF7 cells were treated with either non-targeting (siNT) or SENP7-targeting siRNA (siSP7) for 48 h. CP/IP protocols were followed to isolate endogenous HP1α and immunoblot for both SUMO2/3 and HP1α on independent PVDF membranes. *Red arrows* show SUMO-conjugation bands that increase with the targeted knockdown of SENP7

7. Small interference RNA (siRNA): siGENOME Control Non-targeting (siNT) # D-001210-02020 and siGENOME SMARTpool Human SENP7 (siSP7) # M-006035-01 (GE Dharmacon, Lafayette, CO).
8. Transfection Reagent: Lipofectamine-2000 # 11668019 (Thermo Fisher Scientific, Grand Island, NY) and Dharmafect-1 for siRNA #T-2001-03 (GE Dharmacon, Lafayette, CO).

2.2 Harvest Cell Samples

1. CP/IP Buffer 1: 10 mM HEPES/KOH [pH 7.4], 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, and 0.5 % Triton X-100, Protease Inhibitor (PI) cocktail, and 10 mM *N*-ethyl-maleimide # E3876-5G (NEM, Sigma, St. Louis, MO).
2. CP/IP Buffer 2: 50 mM Tris–HCl [pH 7.8], 1% Triton-100, 150 mM NaCl, 5 mM $CaCl_2$, PI Cocktail, 10 mM NEM and 1 U micrococcal nuclease (MNase).
3. CP/IP Buffer 3: 20 mM ethylene-di-amine-tetra-acetic acid (EDTA) [pH 8.0].
4. IP Buffer: 50 mM Tris–HCl [pH 7.8], 1% Triton-100, 150 mM NaCl, 5 mM $CaCl_2$, PI cocktail, and 10 mM NEM.

2.3 Immuno-precipitation of Proteins

1. Rabbit Polyclonal HP1α Antibody # NB110-40623 (Novus, Littleton, CO).
2. Protein A/G Plus Agarose Beads.
3. 2× Laemmli Sample Buffer with addition of β-mercaptoethanol.

2.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Handcast PAGE System for 1 mm thick gels with 15-well comb.
2. 10% Resolving Gel Buffer: 1.5 M Tris–HCl [pH 8.8], 30% (w/v) acrylamide/Bis-acrylamide, 10% (w/v) SDS, 10% (w/v) ammonium persulfate (APS), and *N*,*N*,*N*,*N*-tetrmethyl-ethylenediamine (TEMED).

3. Stacking Gel Buffer: 0.5 M Tris–HCl [pH 6.8], 30% (w/v) acrylamide/Bis-acrylamide, 10% (w/v) SDS, 10% (w/v) APS, and TEMED.
4. Prestained Protein Marker.
5. Vertical Electrophoresis Cell and Tank.
6. 10×-Running Buffer: 250 mM Tris–HCl, 1.92 M glycine, and 1% (w/v) SDS.
7. Power Supply.

2.5 Immunoblot for Protein Detection

1. Transfer Module with corresponding Tank, Cassettes, and Foam Pads.
2. Polyvinylidene fluoride membrane (PVDF).
3. Filter Paper.
4. Transfer Buffer: 25 mM Tris–HCl, 192 mM glycine, and 20% (v/v) methanol.
5. 10× Tris-buffered saline (TBS): 500 mM Tris–HCl [pH 7.5] and 1.5 M NaCl.
6. Wash Buffer: 1× TBS with 0.05% (v/v) Tween 20.
7. 2-Blot Boxes (9.0 × 6.5 × 2.5 cm).
8. Blocking Buffer: 5% (w/v) nonfat dry milk dissolved in 1×-TBS-T.
9. Primary Antibody: Mouse monoclonal SUMO2/3 antibody # ab81371 (Abcam, Cambridge, MA), Rabbit Polyclonal HP1α Antibody # NB110-40623 (Novus, Littleton, CO), and rabbit polyclonal SENP7L antibody generated in-house.
10. Secondary Antibody: Horseradish peroxidase (HRP)-conjugated goat anti-mouse # sc-2005 or HRP-conjugated goat anti-rabbit # sc-2004 (Santa Cruz Biotechnology, Dallas, TX).
11. Antibody Dilutions Buffer: 2.5% (w/v) nonfat dry milk dissolved in 1×-TBS-T.
12. Chemiluminescence Plus Reagent.
13. Blue X-ray Film.

2.6 Strip and Re-probe Membrane

1. Stripping Buffer: 62.5 mM Tris–HCl [pH 6.7], 2% (w/v) SDS, and 100 mM β-mercaptoethanol.
2. Precision Scientific Water Bath with Temperature Control.

3 Methods

3.1 Cell Growth and Transfection

1. Grow MCF7 cells in 100 mm dish (~1.0×10^7 cells) with 10 mL of growth media in normal cell-culture conditions. Sub-culture when cells are 90% confluent.

2. To sub-culture, remove growth media and wash adherent cells twice with 3 mL of PBS. Incubate cells at 37 °C for 3 min with 1 mL of trypsin–EDTA in normal culture conditions. View with an inverted microscope to ensure detachment of cells from the culture dish. If detached, inactivate trypsin–EDTA with addition of equivalent amounts (v/v) of growth media. Transfer cells to a fresh canonical vial and centrifuge at 125 × *g* for 5 min at 4 °C. Discard the trypsin–EDTA containing media without disturbing the cell pellet.
3. Resuspend the cell pellet in 5 mL of fresh, pre-warmed (37 °C) growth media. Plate cells in a 6-well dish at 1:10 ratio and incubate for 24 h in growth media under normal cell culture conditions to acquire 90% confluent cells (~8×10^5 cells/well, *see* **Note 1**).
4. Overexpression Studies (Fig. 1): Mix either wt-HP1α (1 μg) or SU-HP1α (1 μg) plasmid with Lipofectamine-2000 (3 μL/1 μg of plasmid) in 400 μL serum-free DMEM and place at room temperature for 20 min. Wash cells in a 6-well plate twice with cold PBS to remove residual serum prior to adding 2 mL of warm (37 °C) serum-free DMEM. Add appropriate plasmid/Lipofectamine solution to 1-well of cells and incubate in set culture conditions for 4 h. Replace the serum-free DMEM with growth media and incubate cells under normal culture conditions for 24 h prior to use.
5. Knockdown Studies (Fig. 2): Place either siNT (10 μM) or siSP7 (10 μM) with Dharmafect-1 (1 μL/2 μM siRNA) in 400 μL of serum-free DMEM at room temperature for 20 min. Wash a 6-well plate of cells twice with cold PBS and add 2 mL of warmed serum-free DMEM to each well. Add the siRNA/Dharmafect solution to cells and incubate for 6 h. Replace the siRNA/Dharmafect containing media with growth media and incubate cells in normal culture conditions for 48 h prior to use.

3.2 Harvest Cell Samples

1. Wash cells twice with ice-cold PBS. Add 250 μL of trypsin–EDTA per well to detach cells as described above (Subheading 3.1, **step 1**), stop trypsin activity with addition of equivalent volume of growth media, and transfer samples to a sterilized 1.5 mL tube. Centrifuge at 125 × *g* for 5 min at 4 °C and discard the trypsin-containing solution. Resuspend cell pellet in fresh ice-cold PBS and perform an additional centrifugation to completely remove trypsin. Discard the PBS-wash and place cell pellet on ice (*see* **Note 2**).
2. Sample Preparation for CP/IP: Reconstitute cell pellet in 100 μL of cold CP/IP Buffer 1. Allow cells to lyse in CP/IP Buffer 1 hypotonic solution for 2 min on ice. Subsequently centrifuge the sample at slow-speed to increase yield of poorly soluble chromatin-bound proteins; concentrate chromatin-fractions with

centrifugation at 2500 × *g* for 5 min at 4 °C. Discard the supernatant, which includes highly soluble proteins. Resuspend pellets in 500 μL of CP/IP Buffer 2 at 37 °C for 15 min with rotation to ensure adequate exposure of sample to buffer. Add CP/IP Buffer 3 to inactivate MNase. Then, gently vortex the sample and incubate on ice for 2 min. Pass samples through a 27-gauge needle ten-times and centrifuge at high-speed (18,000 × *g*) at 4 °C for 10 min. Collect chromatin-bound proteins in the 500 μL supernatant and transfer to a clean 1.5 mL tube on ice.

3. Sample Preparation for IP: Resuspend harvested cell pellets in 500 μL of IP buffer for 30 min at 4 °C with constant rotation. Clear samples through a 27-gauge needle ten-times to disrupt intact cells and reduce viscosity. Subsequently subject lysates to high-speed centrifugation at 18,000 × *g* for 10 min at 4 °C. Place the resulting 500 μL supernatant in a clean 1.5 mL tube on ice.

3.3 Immunoprecipitation of Protein

1. Transfer 50 μL of supernatant to a new 1.5 mL tube and utilize in subsequent steps as the Input lysate; store at 4 °C until required. Use the remaining 450 μL aliquot for protein precipitation as the CP/IP or IP sample.
2. Add 1 μg of anti-HP1α antibody to the CP/IP or IP lysate (*see* **Note 3**). Incubate samples for 2 h at 4 °C on a slow rotating platform to promote adequate interaction between the antibody and HP1α protein (*see* **Note 4**).
3. Add 25 μL of Protein A/G agarose resin to the antibody-containing lysate and incubate samples for 1 h at 4 °C with agitation. Collect agarose beads with centrifugation (1000 × *g* for 5 min at 4 °C) and discard the supernatant.
4. For either CP/IP or IP protocols, resuspend beads in 500 μL of IP Buffer with PI cocktail and NEM (*see* **Note 5**). Incubate samples with slow rotation for 5 min, centrifuge at 1000 × *g* to pellet beads, and discard supernatant. Repeat wash four more times.
5. Add 50 μL Laemmli Sample Buffer to Input and CP/IP (or IP) samples.
6. Boil samples at 95 °C for 5 min (*see* **Note 6**). Cool to room temperature. Centrifuge CP/IP samples at 1000 × *g* for 5 min at 4 °C and transfer supernatant (minus beads) to new 1.5 mL tubes for easier loading of samples.

3.4 SDS-PAGE

1. Assemble the gel-casting frame with 1 mm thick spacer plates and secure on the casting stand. Prepare a 10% resolving gel by mixing: 1.3 mL-1.5 M Tris–HCl [pH 8.8], 1.7 mL-30% acrylamide/Bis-acrylamide, 50 μL-10% (w/v) SDS, 50 μL-10% APS, 2 μL-TEMED, and 1.9 mL-Milli-Q water. Pour approximately 4 mL resolving buffer into the casting assembly and

allow the gel matrix to polymerize at room temperature for 30 min. Remove excess water using filter paper and insert 15-well comb into the cassette.

2. Over the solidified resolving gel, dispense about 1.5 mL of stacking solution: 250 μL-0.5 M Tris–HCl [pH 6.8], 330 μL-30% acrylamide/Bis-acrylamide, 20 μL-10% SDS, 20 μL-10% APS, 2 μL-TEMED, and 1.9 mL-Milli-Q water. Allow the gel to polymerase at room temperature for 45 min.
3. Dilute the 10×-Running Buffer tenfold. Fit the gel plate into the electrophoresis cell. Remove the well-comb, place cell in the tank, and fill the chambers with 1×-Running Buffer to the indicated levels.
4. Use a syringe with 27-gauge needle to purge residual acrylamide from the wells with running buffer. For detection of SUMO-conjugates, load 12 μL of each CP/IP sample, corresponding Input sample, and a pre-stained molecular-weight protein marker independently into each well from left-to-right. Prepare an additional gel in an analogous manner to identify the substrate protein.
5. Place the lid on the tank and connect electrodes to the power source. Run samples through the stacking gel for 15 min at a constant 80 V. Then, continue separation of protein samples for an additional 1 h at a constant 120 V. Stop electrophoresis when the dye-front runs-off the gel.

3.5 Immunoblot for Protein Detection

1. Cut the PVDF membrane and two-sheets of filter paper to fit the Transfer module (8.6×6.7 cm) and pre-soak in transfer buffer 15 min prior to use.
2. Dissemble electrophoresed gel from the PAGE cell and place in transfer buffer. Assemble transfer cassette as follows: foam pad, filter paper, PVDF gel, membrane, filter paper, and foam pad. Place transfer sandwich with the gel on the cathode side and the membrane on the anode side.
3. Put the cassette inside the transfer module and add the module to tank prefilled with cold transfer buffer. To ensure that the tank remains cool throughout the transfer process, add the cooling unit to the tank. Also include a stir-bar and place the tank on a stir-plate in the cold room. Transfer proteins at a constant voltage of 90 V for 1.5 h.
4. Remove the transfer cassette from the tank, open with the gel-side down, and remove the top filter paper to expose the PVDF membrane. Without disturbing the membrane-gel contact, use a razor to trim-off regions of the membrane that do not adhere to the gel; this minimizes the amount of antibody that will be utilized in subsequent steps. Separate membrane from gel and mark the membrane surface that was in direct contact with the gel.

5. Block membrane with 5% nonfat milk for 1 h at room temperature with constant gentle agitation in an appropriate size blot-box.
6. Dilute SUMO2/3 antibody 1:1000 in 3 mL of 2.5% milk. Place blocked membrane with the diluted antibody in the cold room (4 °C) on a rotating platform. Ensure that a tightly sealed blot-box is used for overnight (~16 h) incubation of the membrane with the antibody.
7. Move the membrane to a new blot-box and rinse four-times with TBS-T washing buffer; for each wash, submerge the membrane in 8 mL of TBS-T for 5 min with moderate agitation.
8. Dilute an appropriate species-specific secondary antibody 1:2000 in 3 mL of wash 2.5% milk. Put the washed membrane in a blot-box and incubate with the secondary antibody for 1 h at room temperature.
9. To reduce nonspecific bands, wash the membrane eight times in TBS-T as described above.
10. Mix luminol and oxidizing reagents in equal parts (v/v) to prepare 5 mL of active chemiluminescence solution per membrane. Incubate the membrane on a rotating platform with the chemiluminescence solution for 1 min. Subsequently, drain off the solution on the membrane onto a paper towel and place the membrane in an X-ray film cassette to protect it from light.
11. Expose the membrane to X-ray film for varying intervals between 30 s and 1 h depending on the detection antibody (*see* **Note 7**). For HP1α, SUMO-conjugation bands are visible after 30 s in ectopic conditions (Fig. 1) and 1 min in native conditions (Fig. 2) (*see* **Note 8**).

3.6 Strip and Re-probe Membrane

1. Wash the chemiluminescence solution off the membrane with TBS-T; specifically, incubate with 8 mL of TBS-T for 5 min with modest agitation, decant wash, and repeat this process three more times.
2. Warm 50 mL of Stripping Buffer (per membrane) to 52 °C.
3. In a blot box, submerge the membrane in the warmed Stripping Buffer. Place the box in a 52 °C water bath for 30 min; gently agitate every 10 min.
4. Wash the membrane six times with TBS-T as described in the previous step.
5. Expose the stripped membrane to an active chemiluminescence solution (as described for Subheading 3.5, **steps 10–11**) and ensure that no signal is retained from the first antibody. If original signal persists, than incubate membrane with fresh stripping buffer for an additional 15 min and repeat **steps 2–3**. When the membrane is free of residual signal, reprobe the membrane for additional proteins (*see* **Note 9**).

4 Notes

1. Like conventional IP studies, the number of cells required to observe SUMOylation of a chromatin-bound substrate with CP/IP is dependent upon certain factors. First, more cells are required if the protein target is endogenous versus ectopic. Second, the SUMO dynamics need to be considered; SUMOylation of select proteins are highly regulated in specific cell-type and/or cell cycle. For HP1α, either 1- or 2-wells (~8–16×10^5 MCF7 cells) is required to observe SUMO-modification of overexpressed wt-HP1α (Fig. 1c) or endogenous HP1α (Fig. 2), respectively.
2. At this point, the cell pellet can be either used for CP/IP or snap-frozen for storage at −80 °C. Snap-freezing cell pellets efficiently maintains SUMOylated HP1α; this is likely because the process reduces protease activity, prevents ice-crystal formations, and inhibits protein degradation. When using stored samples, thaw the cell pellet on ice for 5 min prior to adding CP/IP buffers.
3. Reverse CP/IP with an anti-SUMO2/3 antibody can be used to validate SUMOylation of target-protein but results could prove more difficult to interpret. First, IP with the anti-SUMO antibody will pull-down proteins that form both a covalent SUMOylation bond and a noncovalent bond through SUMO-interaction motif (SIM). Since covalent bond formation with SUMO may produce only an 11 kDa shift in the target's molecular weight, it may be hard to differentiate between covalent versus noncovalent SUMO–substrate interactions when conducting anti-SUMO IP and subsequent IB for target proteins. Second, anti-SUMO antibody IP concentrates the population of hyperSUMOylated target-proteins; proteins that exist predominantly in the SUMO-modified state. Detection of poorly expressed or hypoSUMOylated proteins may be lost due to the copious interaction between SUMO-antibody and hyper-SUMOylated targets. Approaches proposed in **Note 8** could be a better alternative for evaluating SUMO-conjugation bands.
4. For endogenous experiments, samples are incubated with the HP1α antibody overnight (~16 h) to ensure adequate precipitation of the protein.
5. NEM is a cysteine protease inhibitor and not a select SUMO-protease inhibitor. Greater than 100 de-ubiquitylating proteases or DUBs also are cysteine protease family members and consistently inhibited via NEM treatment. A substantial cross talk exist between SUMO and ubiquitin PTM; SUMOylation of select targets can either potentiate or antagonize ubiquitin-mediated degradation of the protein [12, 13]. Since NEM regulates both PTMs efficiently, studies solely dependent on NEM need to be interpreted with caution.

6. Don't "over"-boil your samples. Excessive boiling (greater than 5 min) reduces SUMO-conjugation band for HP1α. It is likely that the size of SUMO and HP1α is responsible for this observation; smaller proteins degrade easily with exposure to high temperatures.
7. SUMOplot and GPS-SUMO are two databases that help identify the number of potential SUMO-acceptor sites. Consistently, the number of bands to expect can be based on this prediction database. For example, a single SUMO3 moiety is 103 amino acids or 11 kDa and therefore conjugation of a single SUMO3 moiety increases the molecular weight of the protein by 11 kDa. Since the SUMO3 forms a heteromeric poly-chain with SUMO2, the bands should persist in increments of 11 kDa.
8. Additional experiments with either knockdown or overexpression of select SUMO components can be conducted concurrently to validate protein SUMOylation. Previously, SENP7L and catalytically inactive SENP7L mutant was transiently overexpressed to validate HP1α SUMO-conjugation bands present in chromatin fractions [11]. Along with SENP7L, SUMOylating-enzymes Ubc9, PIAS4, and PC2 as well as SUMO-protease SENP6 are readily chromatin-bound [14–19]. Consequently these SUMO components can be targeted in additional CP/IP experiments. Alternatively, lysine-deficient protein constructs can also be generated to confirm protein SUMO-PTM. However, SUMO-accepting sites can be promiscuous [20, 21], and therefore, mutation of multiple lysine residues may be required to observe complete loss of SUMOylation.
9. Stripped membranes can be used to detect additional interacting partners; especially, with highly specific antibodies. However, accurate assessment of SUMO or other PTMs may be difficult due to residual nonspecific bands that may persist after stripping. It is recommended that IB for SUMO and other PTMs be performed on fresh membranes.

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Part IV

Procedures to Study Protein SUMOylation In Vivo

Chapter 16

Identification of Substrates of Protein-Group SUMOylation

Ivan Psakhye and Stefan Jentsch

Abstract

Protein modification by conjugation to the ubiquitin-related protein SUMO (SUMOylation) regulates numerous cellular functions and is reversible. However, unlike typical posttranslational modifications, SUMOylation often targets and regulates proteins of functionally and physically linked protein groups, rather than individual proteins. Functional studies of protein-group SUMOylation are thus particularly challenging, as they require the identification of ideally all members of a modified protein group. Here, we describe mass spectrometric approaches to detect SUMOylated protein groups in *Saccharomyces cerevisiae*, yet the protocols can be readily adapted for studies of SUMOylation in mammalian cells.

Key words SUMO, Protein-group SUMOylation, Protein complex, Stable isotope labeling by amino acids in cell culture (SILAC), Affinity purification, Mass spectrometry, Quantitative proteomics

1 Introduction

Posttranslational modifications (PTMs) greatly expand the range of functions of proteins. The majority of such modifications are attached functional groups, which change the properties, activities or localization of proteins. Typically, PTMs are reversible and are thus ideal for providing switch-like functions. Because PTMs alter protein properties or mediate precise regulation, PTMs characteristically target individual proteins. Indeed, high substrate specificity appears to be an outstanding feature of most PTMs, and this property is usually tightly controlled at multiple levels.

The traditional view that a single PTM on a given protein mediates a specific function led experimentally in case of the SUMO pathway often to a dead end. However, a paradigm shift occurred with the discovery that the SUMO pathway often targets functionally and physically connected protein groups (termed "protein-group modification"; [1, 2]) rather than an individual protein. Protein-group modification also differs substantially from other multiple PTM events like phosphorylation bursts, as SUMO modifications are often functionally additive or redundant, and the modification reactions do not proceed by a reaction cascade [1, 2].

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SUMOylation is particularly important for nuclear functions where it regulates processes like transcription, chromatin remodeling, and DNA repair, and also establishes and controls nuclear assemblies like PML bodies [3–6]. As shown initially for DNA repair, triggers that activate a particular pathway often induce protein-group SUMOylation. For example, in case of double-strand break (DSB) repair and nucleotide excision repair (NER), protein group SUMOylation of repair proteins can be experimentally induced by exposing cells to either DSB-inducing agents (e.g., methyl methanesulfonate, MMS) or reagents that generate bulky DNA lesions (e.g., UV light), respectively [1]. Substrates modified by protein-group SUMOylation can thus be identified by comparing the levels of SUMO-conjugates from treated and untreated cells with SILAC-based mass spectrometry [7] (Figs. 1a and 2).

Alternatively, for functions that are virtually continuously active, like chromatin remodeling or basal transcription, SUMOylated protein groups may be identified by a SILAC-based comparison of SUMO substrates isolated from wild-type cells and cells defective in one crucial component of the respective pathway (Fig. 1b). For example, experimental down-regulation of an early-acting component of the transcription initiation machinery causes a specific depletion of SUMOylated proteins acting in transcription (Psakhye and Jentsch; unpublished).

A third potentially powerful method is to fuse the deSUMOylation enzyme Ulp1 domain to a known SUMOylated protein of a pathway of interest (UD fusion; [8]), with the aim to deSUMOylate not only the respective protein fusion but also its physical interaction partners (Fig. 1c). Again, a SILAC-based comparison of SUMOylated proteins of a control strain and of a strain expressing the UD fusion may identify a SUMOylated protein group.

2 Materials

2.1 Yeast Strains

1. A yeast *Saccharomyces cerevisiae* strain auxotrophic for lysine and arginine (*see* **Note 1**) expressing the N-terminally 7His-tagged Smt3 (^{HIS}SUMO) either from its endogenous or the *ADH1* promoter (*see* **Note 2**) can be used for the detection of protein-group SUMOylation induced by a specific stimulus (Figs. 1a and 2) [1]. If the alternative experimental setup is employed (Fig. 1b, c), the starting strain needs to be further genetically modified according to its specific requirements (see below).
2. If protein-group SUMOylation occurs with proteins that function in pathways acting virtually continuously (e.g., basal transcription or chromatin remodeling), blocking of an early-acting event might also prevent the formation of a functional protein group and concomitantly its SUMOylation (Fig. 1b).

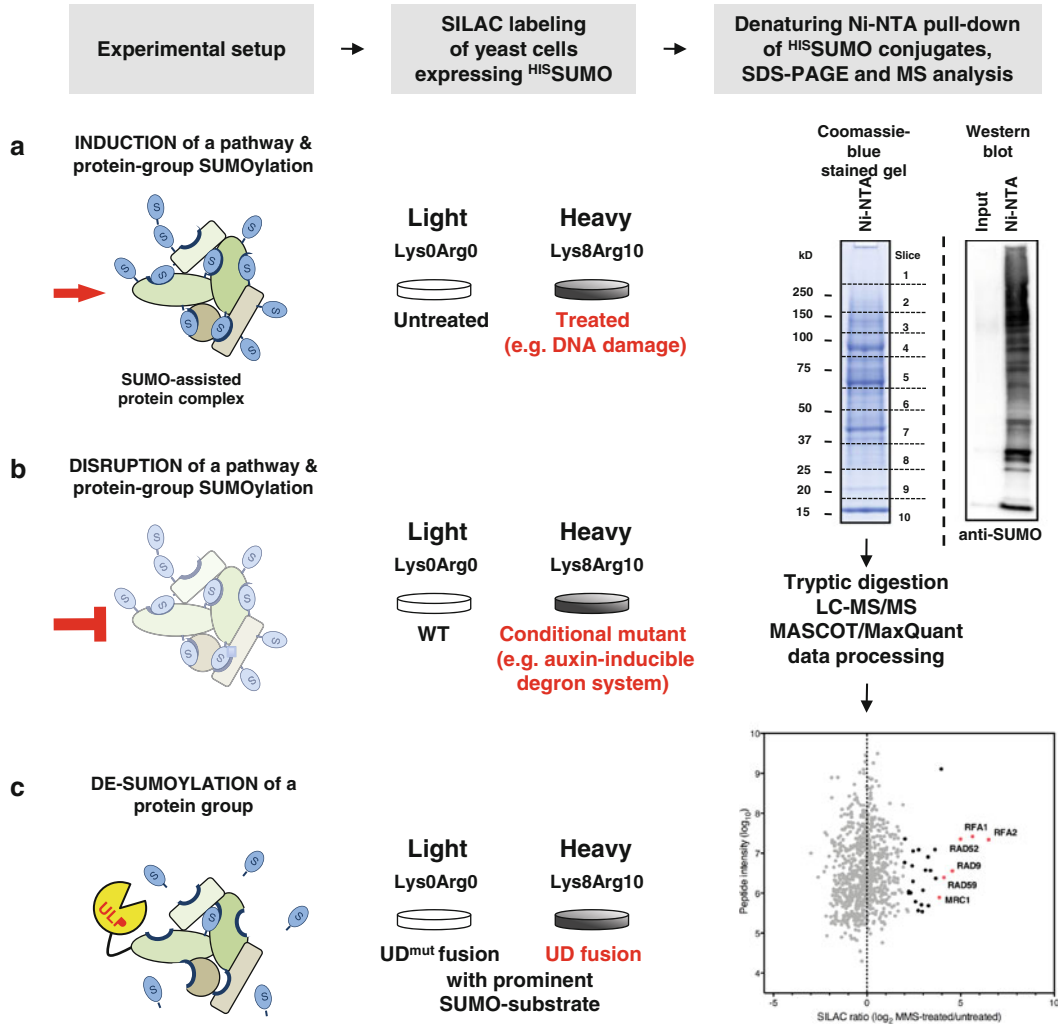

Fig. 1 Workflow for the SILAC-based Identification of Substrates of Protein-group SUMOylation. Three experimental setups for the SILAC-based identification of substrates of protein-group SUMOylation: (**a**) by induction, (**b**) by disruption of SUMO-regulated pathway, protein-group assembly and SUMOylation, or (**c**) by deSUMOylation of modified protein group by the use of a fused Ulp1 catalytic domain (UD fusion) to one of its prominent components. For the SILAC analysis, yeast cells auxotrophic for lysine and arginine (*lys1Δ arg4Δ*) expressing His-tagged Smt3 (^{HIS}SUMO), and further genetically modified for the experimental setups (**b**) and (**c**), are grown in media containing "light" or "heavy" isotope-labeled versions of the amino acids Lys0, Arg0 and Lys8, Arg10. Differentially labeled yeast cultures of the above setups (**a**–**c**) are mixed in a 1:1 ratio immediately after harvesting, lysed and proteins precipitated with trichloroacetic acid. ^{HIS}SUMO conjugates are isolated using Ni-NTA affinity chromatography under fully denaturing conditions to prevent deSUMOylation by cellular deconjugating enzymes in the sample. Following SDS-PAGE of isolated SUMO substrates (and control of ^{HIS}SUMO pull-down efficiency using western blot, WB), the corresponding lane is cut to slices, proteins are in-gel digested with trypsin, and the resulting peptide mixtures are subjected to LC-MS/MS analysis for protein identification and SILAC-based relative protein quantification, as described in detail [7, 12, 13]

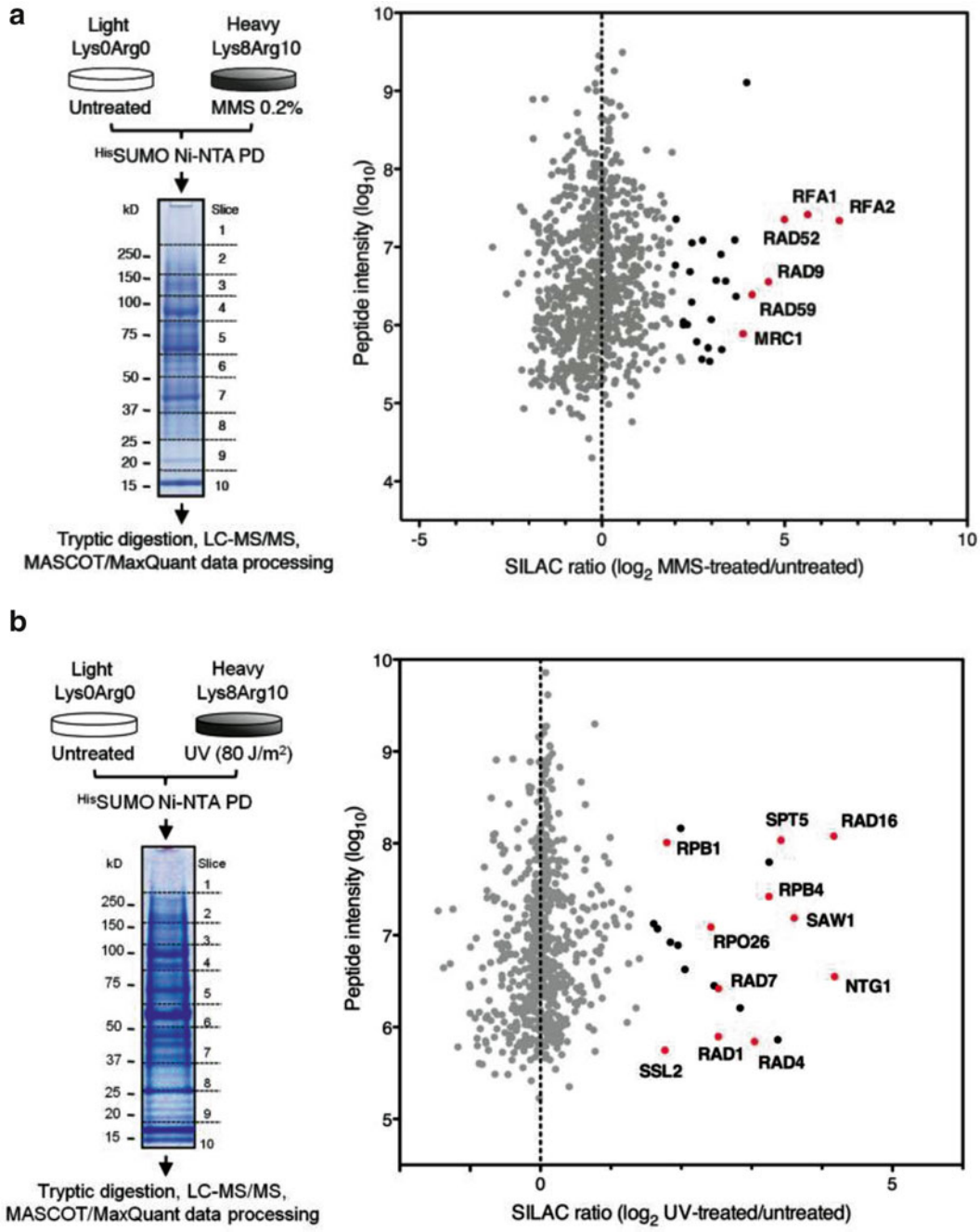

Fig. 2 Proteins Acting in the Same DNA Repair Pathway are Collectively SUMOylated Upon a Specific Stimulus. (**a**) Outline of a SILAC experiment performed to detect SUMOylated substrates enriched after MMS-induced DNA damage (*Left*). SILAC ratios (MMS-treated versus untreated) for 844 quantified proteins plotted against the sum of the relevant peptide intensities (*Right*). Proteins are colored according to values of MaxQuant Significance(B): *gray*, Significance(B) $> 10^{-2}$; *black*, SUMOylated proteins enriched after DNA damage with Significance(B) $\leq 10^{-2}$; *red*, proteins with Significance(B) $< 10^{-4}$ that are involved in homologous recombination and checkpoint activation. (**b**) Following UV-light treatment, specifically factors implicated in nucleotide excision repair (NER) become increasingly SUMOylated. Same as in (**a**), but cells grown in heavy media were

To this end, conditional mutants abolishing a pathway of interest must be introduced in the starting strain expressing ^{HIS}SUMO. For robust conditional depletion of a protein that initiates protein-group assembly and SUMOylation, it is desirable to use an auxin-inducible degron (AID) system [9] in combination with either a transcriptional shut-off (by replacing the endogenous promoter with the inducible *GAL1* promoter [10]), or a translational shut-off by using the metabolically inert tetracycline-repressible system [11] (*see* **Note 3**).

3. For the identification of a SUMOylated protein group with the help of deSUMOylation enzymes, a fusion of the catalytic domain of the deSUMOylation enzyme Ulp1 to a SUMOylated protein of interest (POI) needs to be constructed and expressed in cells (Fig. 1c). The Ulp1 catalytic domain (amino acids 418–621) attached via a flexible linker containing for example a 3HA tag (UD fusion, [8]) to a POI mediates deSUMOylation also of modified physically interacting partners. A SILAC comparison of the abundance of SUMO-conjugates isolated from a strain expressing the UD fusion and a control strain expressing catalytically inactive Ulp1$^{(F474A,C580S)}$ domain (UD^{mut}) fusion to POI leads to the identification of its SUMOylated interacting partners (*see* **Note 4**).

2.2 Yeast Culture Media

1. YPD or YPGal agar plates: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose or galactose, and 2% (w/v) agar.
2. "Light" and "heavy" SC media for SILAC labeling: 6.7 g/l yeast nitrogen base (YNB) without amino acids, 20 g/l glucose or galactose, supplemented with selected amino acids and nucleobases (20 mg/l each l-histidine-HCl, l-tryptophan, l-methionine, adenine sulfate, and uracil; 30 mg/l each l-leucine and l-tyrosine; 50 mg/l each l-phenylalanine and l-threonine; 30 mg/l l-lysine; 20 mg/l l-arginine); "light" medium contains $^{12}C_6$,$^{14}N_2$-lysine (Lys0) and $^{12}C_6$,$^{14}N_4$-arginine (Arg0), "heavy" medium is supplemented with $^{13}C_6$,$^{15}N_2$-lysine (Lys8) and $^{13}C_6$,$^{15}N_4$-arginine (Arg10) (*see* **Note 5**). Any other drop out mix lacking lysine and arginine further supplemented either with Lys0, Arg0 or Lys8, Arg10 will also work. Concentration of arginine can be lowered to 5 mg/l; low concentrations minimize arginine to proline

Fig. 2 (continued) UV-irradiated (80 J/m^2) instead of MMS-treatment. SILAC ratios (UV-treated versus untreated) for 717 quantified proteins plotted against the sum of the relevant peptide intensities. Proteins are colored according to values of MaxQuant Significance(B): *gray*, Significance(B) > 10^{-7}; *black*, SUMOylated proteins enriched after UV-irradiation with Significance(B) ≤ 10^{-7}; *red*, proteins with Significance(B) ≤ 10^{-8} that are involved in NER (both transcription-coupled and global genome repair) and base-excision repair. Reproduced from *Protein group modification and synergy in the SUMO pathway as exemplified in DNA repair. Cell 151, 807–820 (2012)*, with permission from Elsevier

conversion [12]. In case conversion of Arg10 to Pro6 still occurs, unlabeled proline can be added to the media to obtain accurate data.

3. Media supplements for conditional depletion of proteins using an auxin-inducible degron (AID) system and tetracycline-regulated translational shut-off system: dissolve auxin (indole-3-acetic acid; IAA) in ethanol (100 mg/ml), and tetracycline in water (20 mg/ml).

2.3 Cell Lysis and Denaturing Ni-NTA Pull-Down of ^{HIS}SUMO Conjugates

1. Lysis Buffer: 1.85 M NaOH, 7.5% β-mercaptoethanol (add directly prior to use); ice-cold.
2. 55% (w/v) trichloroacetic acid (TCA, ice-cold).
3. Buffer A: 6 M guanidine-HCl (Merck), 100 mM NaH_2PO_4, 10 mM Tris–HCl; adjust to pH 8.0 with NaOH (*see* **Note 6**).
4. Buffer B: 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl; adjust to pH 6.3 with HCl (*see* **Note 7**).
5. Tween 20.
6. Imidazole (stock solution, e.g., 1 M).
7. Ni-NTA agarose (Qiagen).
8. Empty 10 ml polypropylene gravity-flow chromatography columns with 30 μm filters and bottom caps (e.g., Poly-Prep, Bio-Rad, or equivalent).

2.4 SDS-Polyacrylamide Gel Electrophoresis

1. HU Sample Buffer: 8 M urea, 5% (w/v) SDS, 200 mM Tris–HCl (pH 6.8), 0.05% (w/v) bromophenol blue, and 1.5% (w/v) DTT (*see* **Note 8**).
2. NuPAGE Novex 4–12% Bis-Tris pre-cast 1.5 mm, 10 well protein gradient gels (commercially available from Thermo Fisher Scientific).
3. MOPS Running Buffer: 50 mM MOPS, 50 mM Tris–HCl, 1 mM EDTA, 0.5% SDS (w/v), pH 7.7 (*see* **Note 9**).
4. Prestained molecular weight marker (e.g., PageRuler prestained protein ladder 10–180 kDa; Thermo Fisher Scientific).
5. A Coomassie blue-based staining solution for protein gels compatible with mass spectrometry (MS) analysis (e.g., Instant Blue, Expedeon).

3 Methods

3.1 Growing Yeast Cultures for SILAC Labeling

For efficient metabolic labeling of proteins by SILAC, yeast cultures should be passaged for at least ten generations in "heavy" SC medium to reach full isotopic incorporation before sampling (*see* **Note 10**). Same culture volumes (typically 200 ml) of studied and control strains are required.

1. Streak out yeast strains selected for the experiment (*see* Fig. 1 and Subheading 2.1) on separate YPD or YPGal (if a repressible *GAL1* promoter is used) plates and incubate for 2–3 days at 30 °C (or the necessary permissive temperature for the selected mutant).
2. First starter culture: per yeast strain, inoculate 5 ml of SC media (glucose- or galactose-containing) in culture vials with cells from fresh YPD/YPGal plates and incubate overnight at 30 °C with shaking. Grow one strain in "heavy" SC medium and the other in "light" medium (*see* **Note 11**).
3. Second starter culture: transfer first starter culture to 250 ml flasks containing 20 ml of "light" or "heavy" SC media to reach a final OD_{600} of 0.2. Cells are further incubated overnight under the same conditions.
4. Main cultures: "heavy" and "light" SC media in 1 l flasks are inoculated with cells of the second starter cultures to an OD_{600} of 0.2 in a final volume of 200 ml per flask. Cultures are further incubated and cell density is monitored until OD_{600} of 0.7–0.8 is reached. At this point, cells can either be directly harvested (if experimental setup presented in Fig. 1c was selected) or treated as described below.
5. Depending on the experimental setup (Fig. 1a, b), appropriate yeast cultures are: (a) treated with a specific stimulus for protein-group SUMOylation (e.g., DNA damage; Figs. 1a and 2); (b) supplemented simultaneously with auxin (indole-3-acetic acid; IAA) at a final concentration of 0.2 mg/ml and either tetracycline at a final concentration of 0.6 mM (if translational shut-off with a tetracycline-repressible system [11] is used), or glucose at a final concentration of 2% (if transcriptional shut-off with the glucose-repressible *GAL1* promoter is used) for conditional depletion of the desired SUMOylated protein-group-forming component (Fig. 1b). The duration of treatment depends on the selected stimulus or the depletion efficiency of the utilized conditional mutant, but does not typically exceed 3 h (*see* **Note 12**).
6. Measure cell density (should be around $OD_{600} = 1$) and harvest yeast cells at 4 °C by centrifugation for 4 min at 1700 × *g* (*see* **Note 13**).
7. Wash harvested cells with ice-cold deionized water and combine equal amounts of "light" and "heavy" labeled yeast cells in one 50 ml conical centrifuge tube based on the cell density determined earlier (*see* **Note 14**).

3.2 Cell Lysis and Denaturing Ni-NTA Pull-Down of ^{HIS}SUMO Conjugates

1. Resuspend collected cells in 12 ml of ice-cold Lysis Buffer by vigorous vortexing, chill on ice for 15 min.
2. Add equal volume (12 ml) of ice-cold 55% trichloroacetic acid (TCA), vortex vigorously and chill on ice for another 15 min.

3. Centrifuge the tubes at 4 °C for 15 min at 2500 × *g* and discard the supernatant.
4. Wash the pellet with 50 ml of prechilled deionized water without resuspending, spin at 4 °C for 5 min at 2500 × *g*, and discard the supernatant (*see* **Note 15**).
5. Resuspend the pellet in 24 ml of Buffer A (add Tween 20 to the buffer to a final concentration of 0.05 % directly prior to use) by vigorous vortexing or pipetting with a 10 ml pipette; incubate at room temperature for at least 1 h in a rotator (*see* **Note 16**).
6. Transfer the contents to 50 ml Nalgene polypropylene copolymer or polycarbonate centrifuge tubes and spin at 4 °C for 20 min at 23,000 × *g*.
7. Carefully transfer the supernatant (approximately 24 ml) to a clean 50 ml conical centrifuge tube and add imidazole to a final concentration of 10–20 mM (*see* **Note 17**).
8. Add 250 µl of Ni-NTA agarose slurry after its complete resuspension to the protein solution; incubate overnight at 4 °C in a rotator with gentle mixing.
9. Following overnight incubation, pass the protein solution with Ni-NTA agarose beads through an empty 10 ml polypropylene gravity-flow chromatography column (e.g., Poly-Prep, Bio-Rad, or equivalent) with 30 µm bottom filters packing it (*see* **Note 18**); do not let the beads dry out.
10. Wash the 125 µl bed volume of Ni-NTA agarose beads first with 20 ml *Buffer A* followed with 20 ml Buffer B (add Tween 20 to the buffers to a final concentration of 0.05 % directly prior to use) on the column and by occasionally resuspending the beads by pipetting.
11. Remove remaining Buffer B from the beads by applying a negative pressure to the bottom of the column with a syringe and seal the column bottom with a cap.
12. Add imidazole to Buffer B to a final concentration of 250 mM and use 1 ml to resuspend the beads directly in the column in order to elute ^{HIS}SUMO conjugates from the Ni-NTA matrix. Incubate for 10 min, allowing the beads to precipitate.
13. Uncap the column and collect the eluate, dividing it equally into two 2 ml microcentrifuge tubes.
14. Precipitate eluted proteins by adding 1 ml of 55 % TCA to each of the tubes and incubate on ice for 30 min.
15. Centrifuge the tubes at 4 °C for 20 min at 18,000 × *g* and carefully remove the supernatant by aspiration (*see* **Note 19**). The precipitated proteins should now become visible as a white film at the bottom of the tubes.

16. Add 40 µl of freshly prepared HU Sample Buffer to one of the two tubes containing precipitated proteins, incubate at 65 °C for 10 min with maximal shaking. Spin briefly and transfer the contents to the second sample tube and repeat incubation (*see* **Note 20**).

3.3 SDS-Polyacrylamide Gel Electrophoresis and MS Analysis

1. For commercially available NuPAGE Novex 4–12% Bis-Tris pre-cast 1.5 mm, 10 well protein gradient gels, the XCell SureLock Mini-Cell system from Thermo Fisher Scientific is used (*see* **Note 21**).
2. Prepare 1× MOPS Running Buffer from 20× stock solution and fill the buffer tank until gel cassettes are covered. Carefully rinse each well with MOPS Running Buffer and load 30–40 µl of the Ni-NTA pull-down samples as well as 10 µl of the prestained molecular weight marker leaving empty lanes between them (*see* **Note 22**).
3. Run gel with constant voltage of 140 V until the dye front just runs off the gel (approximately 100 min).
4. Disassemble and carefully open the gel cassette. Avoid contaminating the gel and work as sterile and clean as possible.
5. Place gel into a clean culture dish of appropriate size and rinse the gel with deionized water.
6. Stain proteins with a Coomassie blue-based staining solution compatible with MS analysis (e.g., Instant Blue, Expedeon) for 1 h with gentle shaking.
7. Wash gel several times with deionized water to remove background staining (*see* **Note 23**).
8. Scan the gel and mark slices destined for MS analysis on the printout. The entire lane containing isolated ^{HIS}SUMO conjugates is further cut into 10–12 slices, proteins are in-gel digested with trypsin, and resulting peptide mixtures are subjected to LC-MS/MS analysis for protein identification and SILAC-based relative protein quantification, as described in detail [7, 12, 13].

4 Notes

1. *S. cerevisiae* wild-type cells are able to metabolically synthesize all amino acids. To ensure complete labeling of yeast proteins with “heavy” stable isotope-labeled lysine (Lys8) and arginine (Arg10) for efficient SILAC analysis, yeast strains auxotrophic for the amino acids chosen for metabolic labeling (*lys1Δ arg4Δ*) should be used and generated using common yeast genetic tools [10].

2. N-terminally His-tagged Smt3 (^{HIS}SUMO) can be expressed either as the only source of SUMO in cells by replacing the endogenous *SMT3* gene with a His-tagged variant, or as an additional copy upon transformation with a linearized integrative plasmid carrying ^{HIS}SUMO under the control of the *ADH1* promoter [14, 15].
3. The auxin-inducible degron (AID) system [9, 16] exhibits variations in the efficiency of degradation that depend on the nature and the basal levels of the tagged proteins of interest (POI). Chromatin-bound proteins sometimes seem to escape efficient AID-mediated destruction. To ensure rapid and complete depletion, we suggest combining the AID system with either a translational or a transcriptional expression shut-off protocol. Translational shut-off is accomplished by a PCR-based strategy that replaces the endogenous promoter of the POI and introduces also tetracycline-binding aptamers into its 5′ UTRs [11], which immediately prevent translation of the target mRNAs upon addition of tetracycline to the culture. Transcriptional shut-off can be easily achieved by replacing the endogenous promoter of the POI with the glucose-repressible *GAL1* promoter or its variants using common yeast genetic tools [10]. In this case, yeast culture media has to be supplemented with 2% (w/v) galactose instead of glucose prior to glucose-mediated transcriptional shut-off.
4. A UD fusion could be active already directly after translation and deSUMOylate also unrelated substrates in the vicinity of the tagged POI (e.g., SUMOylated chaperones). Consider this possibility when analyzing the SILAC data.
5. Stable isotope-labeled amino acids are available from Cambridge Isotope Laboratories (Andover, MA, USA), Sigma-Aldrich, or Silantes (Munich, Germany).
6. Reagents for sample preparation and subsequent LC-MS analysis should be HPLC grade; water should be of Milli-Q purity.
7. Control pH of Buffer B, and if necessary adjust to pH 6.3 directly prior to use as buffer storage can lead to pH changes.
8. HU Sample Buffer without DTT may be stored (in aliquots) at −20 °C. Add DTT to the thawed sample buffer immediately before use.
9. A 20× stock solution may be prepared and stored at 4 °C.
10. The amino acid composition of the SC medium used for SILAC labeling influences the metabolism of the cells. This can be used to suppress Arg10 to Pro6 conversion and to ensure accurate SILAC-based protein quantification. However, one has to take into account that yeast cells grow much slower in SILAC media and require an adequate inoculation cell density (at least of $OD_{600} = 0.2$) during passaging.

11. The metabolic labeling of the yeast strain pair should be swapped between replicates to exclude possible artifacts caused by incorporation of the heavy amino acids.
12. The efficiency of conditional depletion of the generated mutant may vary depending on the nature of the protein, and has to be determined experimentally prior to performing SILAC labeling. Typically, combining the auxin-inducible degron (AID) system with the transcriptional or translational shut-off should lead to complete depletion of the targeted protein within 60–90 min [17, 18].
13. In order to evaluate the efficiency of SILAC labeling and assess the level of arginine to proline conversion, collect additionally 1 unit of $OD_{600} = 1$ of cells cultured in "heavy" SC medium for further MS analysis of extracted proteins.
14. Collected cells (around 350–400 units of $OD_{600} = 1$) can be frozen in liquid nitrogen and stored at −80 °C.
15. Pellets may be frozen at −80 °C.
16. If the pellet is difficult to resuspend, leave the 50 ml tube with the pellet and Buffer A added on a roller or tube rotator at room temperature for 15 min and then repeat modest vortexing until the pellet is fully dissolved.
17. Imidazole helps to prevent nonspecific binding to the Ni-NTA matrix of endogenous yeast proteins that have histidine clusters (poly-His stretches) and compete with ^{HIS}SUMO conjugates. At least 10 mM final concentration of imidazole should be used. Increasing the concentration to 20 mM may further reduce unspecific binding, but depending on the batch of Ni-NTA agarose, it may also result in a complete loss of ^{HIS}SUMO conjugates binding to beads. When performing SILAC-based relative protein quantification, unspecific background proteins should exhibit an abundance ratio of 1 according to the mixing of equal amounts of "light" and "heavy" labeled yeast cells and may serve as internal "loading controls."
18. Avoid trapping of air bubbles at the bottom filter of the chromatography column, because they might limit flow. Remove bubbles by passing Buffer A through the column using a syringe prior to packing.
19. Urea in Buffer B can crystallize upon chilling or centrifugation at 4 °C. Dissolve crystals by incubating at 37 °C with shaking, add another 0.5 ml of 55 % TCA, leave on ice for 30 min and continue with the centrifugation step.
20. If the color of the sample (bromophenol blue dye and a pH indicator) changes to yellow (indicating low pH due to the presence of TCA traces in the tube) add 5–10 µl of 1 M Tris–HCl, pH 8.0 until the color of the sample switches to blue.

21. Any other gel system allowing for protein separation across a broad range of molar masses can be used.
22. To monitor the efficiency of the Ni-NTA pull-down, take an aliquot of the input material and aliquot following the affinity purification step. Analyze the samples by SDS-PAGE and subsequent immunoblotting using an antibody against yeast Smt3 (e.g., y-84, sc-28649; Santa Cruz Biotechnology).
23. Process the gel for LC-MS analysis as soon as possible. At this point, the gel may be submerged in deionized water and stored at 4 °C in a sealed culture dish.

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Chapter 17

Tools to Study SUMO Conjugation in *Caenorhabditis elegans*

Federico Pelisch and Ronald T. Hay

Abstract

The cell biology of sumoylation has mostly been studied using transformed cultured cells and yeast. In recent years, genetic analysis has demonstrated important roles for sumoylation in the biology of *C. elegans*. Here, we expand the existing set of tools making it possible to address the role of sumoylation in the nematode *C. elegans* using a combination of genetics, imaging, and biochemistry. Most importantly, the dynamics of SUMO conjugation and deconjugation can be followed very precisely both in space and time within living worms. Additionally, the biochemistry of SUMO conjugation and deconjugation can be addressed using recombinant purified components of the *C. elegans* sumoylation machinery, including E3 ligases and SUMO proteases. These tools and reagents will be useful to gain insights into the biological role of SUMO in the context of a multicellular organism.

Key words SUMO, *Caenorhabditis elegans*, Live imaging, Cell division, Chromosomes

1 Introduction

Small ubiquitin-related modifier (SUMO) conjugation is essential for development in mammals [1, 2] and in the nematode *Caenorhabditis elegans* (*C. elegans*) [3–6]. SUMO is conjugated to substrate proteins through the action of an E1-activating enzyme (the Sae1/Sae2 heterodimer in humans), an E2-conjugating enzyme (Ubc9 in humans), and SUMO-specific E3 ligases [7, 8]. The most studied type of SUMO E3 ligase is the SP-RING E3 ligase family, which includes PIAS proteins in vertebrates and their yeast homologs Siz1 and Siz2 [9, 10] and the related protein mms21 [11]. Regulation is also achieved at the level of desumoylation by SUMO-specific isopeptidases: SENP1, 2, 3, 5, 6, and 7 in vertebrates [12, 13]. While mammals contain three different SUMO proteins [7], in *C. elegans* there is one SUMO ortholog, SMO-1 (hereafter CeSUMO). The Ubc9 ortholog is UBC-9, while the PIAS and *mms21* orthologs are GEI-17 [14] and ZK1248.11.1 (hereafter

Manuel S. Rodriguez (ed.), *SUMO: Methods and Protocols*, Methods in Molecular Biology, vol. 1475,
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Table 1
Comparison of sumoylation pathway components between the yeast *Saccharomyces cerevisiae*, human, and the nematode *C. elegans*

Protein	*Saccharomyces cerevisiae*	*Homo sapiens*	*Caenorhabditis elegans*
Modifier	Smt3p	SUMO1, 2, 3	SMO-1 (CeSUMO)
E1 (activating enzyme)	Aos1p/Uba2p	SAE1/SAE2	AOS-1/UBA-2
E2 (conjugating enzyme)	Ubc9p	UBC9	UBC-9
SP-RING SUMO ligases	Siz1p, Siz2p	PIAS1, 2, 3, 4	GEI-17
	Mms21p	NSE2	MMS-21
SUMO proteases	Ulp1p	SENP1	ULP-1
		SENP2	
		SENP3 and SENP5	ULP-5
	Ulp2p	SENP6 and SENP7	ULP-2 and ULP-4

MMS-21), respectively (Table 1). Four SUMO proteases (ubiquitin-like proteases, ULPs), ULP-1, ULP-2, ULP-4, and ULP-5, have been recognized in *C. elegans* (Table 1) while ULP-3 is the putative Nedd8 protease (NEDP1) ortholog. SUMO has been shown to play many roles in *C. elegans* including gonadal and vulval development [15, 16], regulation of translesion synthesis DNA polymerase POLH-1 stability [17], cytoplasmic intermediate filament assembly [18], and Hox gene expression [19]. In the case of Hox gene regulation, a SUMO protease is also involved [19] and other examples have shown that SUMO proteases play important roles in *C. elegans*. The SUMO protease ULP-4 regulates mitotic cell cycle progression in *C. elegans* embryos, affecting chromosome structure, congression, and segregation [20]. Additionally, HMGS-1, the ortholog of human HMGCS1 enzyme that mediates the first committed step of the mevalonate pathway, undergoes an age-dependent sumoylation that is temporally balanced by the activity of ULP-4 [21]. More recently, a role for the SUMO protease ULP-2 has been described in the regulation of adherens junction assembly and dysregulation of ULP-2 activity impairs epidermal morphogenesis [22]. Altogether, *C. elegans* is emerging as an extremely useful system to address the biological function of sumoylation and desumoylation. Nevertheless, the field could benefit from the development of novel reagents and techniques. We provide here a set of tools and reagents to study the role of sumoylation in *C. elegans* both in vivo and in vitro.

2 Materials

2.1 Strains

2.1.1 C. elegans Strains

The strains used so far have been generated by the bombardment method, as described in [23] (Table 2). Even though current strains are being generated by either transposon-mediated single-site insertions (MosSCI [24]) or genome editing through CRISPR/Cas9 [25, 26], the strains described in Table 2 are established and reproduce the localization pattern of the endogenous SUMO. *C. elegans* N2 Bristol is used as wild type and the strain HT1593 [unc-119(ed3)] III is used for bombardment. Worms are grown in NGM plates (0.3% (w/v) NaCl, 2% agar (w/v), 0.25% (w/v) peptone, 5 mg/l cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM potassium phosphate, pH 6).

2.1.2 Bacterial Strains

The bacterial strains used for feeding worms are the *E. coli* OP50 and BH101 strains, available from the Caenorhabditis Genetics Center (CGC). For RNAi, we use the Ahringer library [27]. HT115(DE3) bacteria are used, allowing for RNA induction upon IPTG addition. RNAi clones related to the SUMO pathway are depicted in Table 3.

Table 2
Strains expressing different versions of CeSUMO

Strain	Strain	Genotype
mCherry-CeSUMO(GG)	FGP1	*fgpIs20*[*pAA64*; *Ppie-1 mCherry*::*smo-1*(*GG*) *unc-119*(+)], *unc-119* (*ed3*)
mCherry-CeSUMO(GA)	FGP2	*fgpIs21*[*pAA64*; *Ppie-1 mCherry*::*smo-1*(*GA*) *unc-119*(+)], *unc-119* (*ed3*)
GFP-CeSUMO(GG)	FGP3	*fgpIs23*[*pIC26*; *pie-1/GFP-TEV-S-Tag*::*smo-1*(*GG*) *unc-119*(+)], *unc-119* (*ed3*)
GFP-CeSUMO(GA)	FGP4	*fgpIs24*[*pIC26*; *pie-1/GFP-TEV-S-Tag*::*smo-1*(*GA*) *unc-119*(+)], *unc-119* (*ed3*)
mCherry-CeSUMO(GG)/GFP-AIR-2	FGP5	*ltIs14*[*pASM05*: *pie-1*::*GFP-TEV-STag*::*air-2* + *unc-119*(+)], *fgpIs20*[*pAA64*; *Ppie-1 mCherry*::*smo-1*(*GG*) *unc-119*(+)], *unc-119* (*ed3*)
mCherry-CeSUMO(GG)/GFP-β-tubulin	FGP7	*ruIs57*[*pie-1*::*GFP*::*tubulin* + *unc-119*(+)], *fgpIs20*[*pAA64*; *Ppie-1 mCherry*::*smo-1*(*GG*) *unc-119*(+)], *unc-119* (*ed3*)
mCherry-CeSUMO(GG)/GFP-H2B	FGP8	*ruIs32* [*pie-1*::*GFP*::*H2B* + *unc-119*(+)], *fgpIs20*[*pAA64*; *Ppie-1 mCherry*::*smo-1*(*GG*) *unc-119*(+)], *unc-119* (*ed3*)
6×His-CeSUMO(GG)	FGP14	*fgpIs35*[*pRH21*; *Psmo-1*::*6xHis*::*smo-1*(*GG*)::*smo-1 3′ UTR unc-119*(+)], *unc-119* (*ed3*)

Table 3
RNAi clones used to knock down components of the sumoylation pathway in *C. elegans*

GenePairs name	SourceBioscience location	Gene
W02A11.4	I-6B15	*uba-2*
K12C11.2	I-1O13	*smo-1*
F29B9.6	IV-2K06	*ubc-9*
W10D5.3	I-4D09	*gei-17*
T10F2.3	III-2N21	*ulp-1*
Y38A8.3	II-4K17	*ulp-2*
C41C4.6	II-6A07	*ulp-4*
K02F2.4	I-3I02	*ulp-5*

2.2 Plasmids

The vector pHISTEV30a is used to express 6×His-tagged proteins in bacteria [28] (Fig. 1). The primers used for cloning are detailed below. The protein sequences shown correspond to the recombinant product after TEV protease cleavage, with amino acids coming from the polylinker and/or TEV cleavage site underlined.

1. pHISTEV30a-CeSUMO(GG).

   Primers:CeSUMONcofwd:5′-ACTGGCCATGGCC GATGATGCAGCTCAAG-3′

   CeSUMOHindrev:5′-ACTGAAGCTTTCATCCG CCCAGCTGCTC-3′

   Protein sequence: GAMADDAAQAGDNAEYIKIKVV GQDSNEVHFRVKYGTSMAKLKKSYADRTGVAVNSL RFLFDGRRINDDDTPKTLEMEDDDVIEVYQEQLGG

2. pHISTEV30a-YFP-CeSUMO(GG): In this case, the CeSUMO(GG) cDNA was cloned into pHISTEV30a-YFP [29].

   Primers: YFPCeSUMOBamfwd: 5′-ATCGGGATCCG CCGATGATGCAGCTCAAG-3′

   YFPCeSUMONotrev: 5′-ATCGGCGGCCGCTCA TCCGCCCAGCTGCTC-3′

   Protein sequence: GAMAMVSKGEELFTGVVPILVELDG DVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPVPW PTLVTTLGYGLQCFARYPDHMKQHDFFKSAMPEG YVQERTIFFKDDGNYKTRAEVKFEGDTLVNR IELKGIDFKEDGNILGHKLEYNYNSH NVYITADKQKNGIKANFKIRHNIEDGGVQL ADHYQQNTPIGDGPVLLPDNHYLSYQSALSKDPN EKRDHMVLLEFVTAAGITLGMDELYKGSADDAAQAG

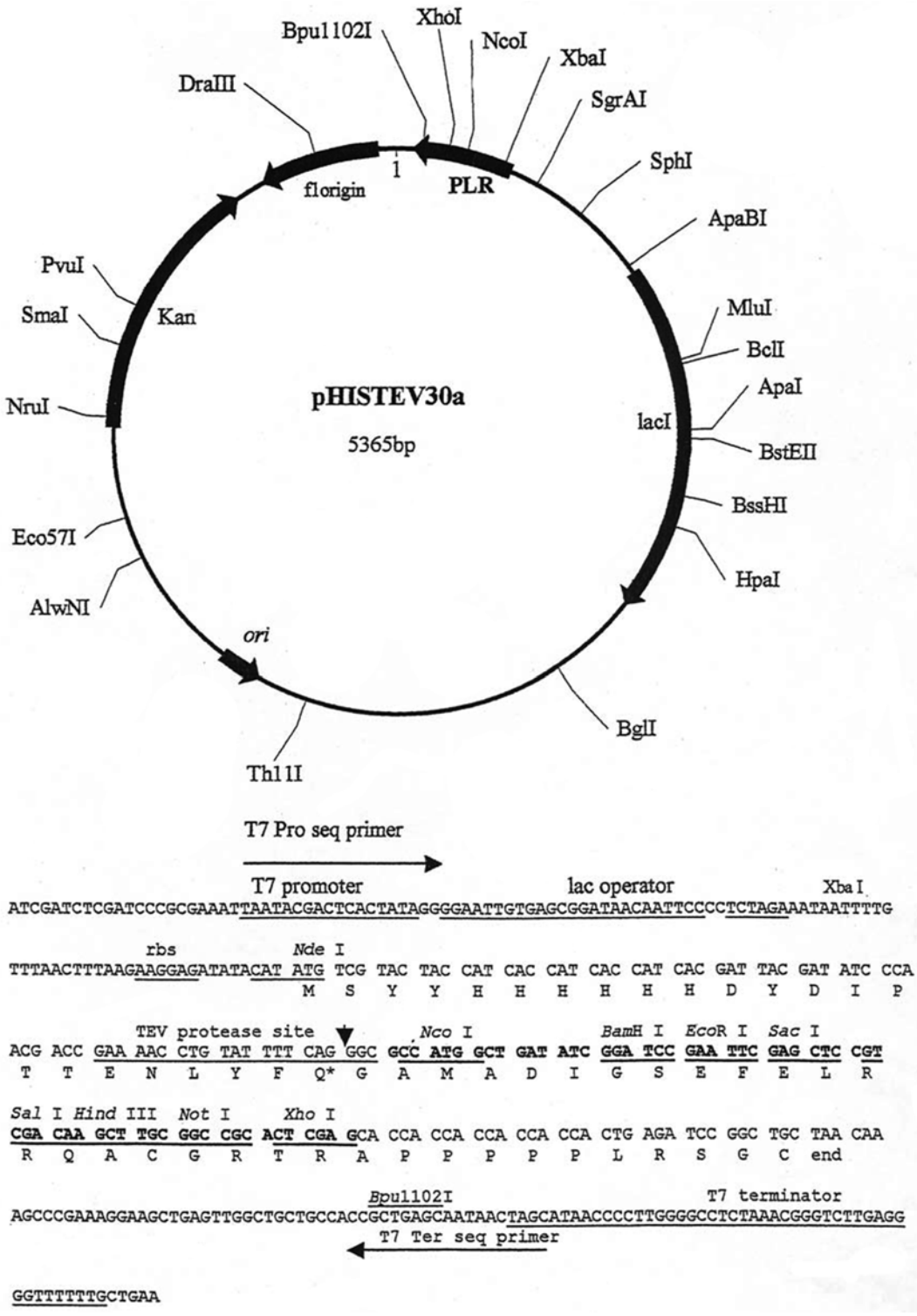

Fig. 1 Map of the pHISTEV30a vector is depicted with the detailed sequence of the multiple cloning site (MCS) below

DNAEYIKIKVVGQDSNEVHFRVKYGTSMAKLKKSYAD
R T G V A V N S L R F L F D G R R I N D D D T P K T L E M
EDDDVIEVYQEQLGG

3. pHISTEV30a-CeSUMO-HA.

   Primers: CeSUMONcofwd: 5′-ACTGGCCATGGCCGATG
   ATGCAGCTCAAG-3′

   CeSUMOLGG-HAHindrev:5′-ATCGAAGCTTCTAAGCGT
   A A T C T G G A A C A T C G T A T G G G T A G
   AATCCGCCCAGCTGCTCTTG-3′

   Protein sequence: GAMADDAAQAGDNAEYIKIKVVGQ
   DSNEVHFRVKYGTSMAKLKKSYADRTGVAVNSLRFL
   F D G R R I N D D D T P K T L E M E D D D V I E V Y Q E Q
   LGGFYPYDVPDYA

4. pHISTEV30a-UBC-9.

   Primers: C.e. ubc-9 for: 5′-ATCGCCATGGGA*TCG*
   GGAATTGCTGCAGGAC-3′

   C.e. ubc-9 rev: 5′-ATCGAAGCTTCTACTCGAGCATTT
   GCTTC-3′

   Protein sequence: GAMGSGIAAGRLAEERKHW
   RKDHPFGFIAKPVKNADGTLNLFNWECAIPGRKDTIW
   EGGLYRIRMLFKDDFPSTPPKCKFEPPLFHPNVYPSGTV
   CLSLLDENKDWKPSISIKQLLIGIQDLLNHPNI

   EDPAQAEAYQIYCQNRAEYEKRVKKEAVKYAAELVQ
   KQMLE

5. pHISTEV30a-GEI-17 (133–509 in isoform f, NP_001021678.3)

   Primers: gei-17133Ncofwd: 5′-ACTGCCATGGGACA
   ACAAATGATGGCGTCAC-3′

   gei-17509Notrev: 5′-ACTGGCGGCCG*CTA*
   AAGAGAGTTCATTATACCTC-3′

   These primers are predicted to amplify other isoforms as well. From N2 worms, we have been able to clone isoforms c and f, and the protein sequence below corresponds to the isoform f.

   Protein sequence: GAMGQQMMASHHSHLQQQHHPS
   TPKKMYADNFEPLPLPFYDVISVLLKPVELHSSDSPT
   L K Q T K Q L Q F P F L L T A E H I S K I S Y R A D V T
   PLPRYELQLRFFNLTEPVQGPQKDDFPLNCYARV
   D D S V V Q L P N V I P T N K T N A E P K R P
   S R P V N I T S N M N R Y K K E H T V A V E W L A D K R V
   WAAGVYFVHRVNSDILFKRLNQNVSRHRSLEVTKQ
   EVIKKLSGGEDDIAMDRLNISLLDPLCKTRMTTP
   SRCQDCTHLQCFDLLSYLMMNEKKPTWQCPVCSS
   NCPYDRLIVDDYFLDMLAKVDKNTTEVELKE

D G S Y D V I K E E A F C I S D D D D D D D V V P A T V N G T A S C S S T N G N G L A N E A A K K K P A D D D I I T LSDDDDEELNRGIMNSL

6. pHISTEV30a-ULP-4 (145–333)

   Primers: ulp-4catNcofor 5′-ATCGCCATG*GAT*CTTCTC AACGATACGATG-3′

   ulp-4catHindrev 5′-ATCGAAGCTTTTAAAAGTCGAAGT TGTCGAGATC-3′

   Protein sequence: GAMDLLNDTMIDFYLNHI VEHVLPDSNGSNVTVLPSIFWHNLSLRQHAFDSEDE KMMSDEQKMDLKFGDLHDFVADFDLQDFDYIV VPVNEWEHWSLAVICHPFTAQARTVIFDSQLTA DLNNLQNMATLIESFMKYSYEKRTGNAMPFPL P C I L P Q R M P Q Q T N N F D C G I F I A E F A RRFLLSPPKDLDNFDF

7. pHISTEV30a-ULP-4 (145–382)

   Primers: ulp-4catNcofor 5′-ATCGCCATG*GAT*CTTCT CAACGATACGATG-3′

   ulp-4Hindrev 5′-ATCGAAGCTT*TTA*AAGTG CACGATGAGGTG-3′

   Protein sequence: GAMDLLNDTMIDFYLNHIVEHVLP DSNGSNVTVLPSIFWHNLSLRQHAFDSEDEKMMS DEQKMDLKFGDLHDFVADFDLQDFDYIVVPVNE WEHWSLAVICHPFTAQARTVIFDSQLTADLN NLQNMATLIESFMKYSYEKRTGNAMPFPLPC ILPQRMPQQTNNFDCGIFIAEFARRFLLSPP KDLDNFDFAREYPDFSTATKRTEMQRVVLSL STNRARWRPLVELLNGYSTAAPHRAL

2.3 Antibody Methods

1. The complete list of antibodies is described in Table 4. Please *see* **Note 1**.
2. Binding buffer: 0.02 M Sodium phosphate, pH 7.0.
3. Elution buffer: 0.1 M Glycine-HCl, pH 2.5.
4. Neutralising buffer: 1 M Tris–HCl, pH 9.0.
5. *N*-hydroxy-succinimide (NHS) beads.
6. Coupling buffer: 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3.
7. NHS buffer A: 0.5 M NaCl, 0.5 M ethanolamine, pH 8.3.
8. NHS buffer B: 0.5 M NaCl, 0.1 M sodium acetate, pH 4.

2.4 Recombinant Proteins

1. Lysis buffer: 50 mM Tris–HCl, 500 mM NaCl, 10 mM imidazole, complete protease inhibitor cocktail tablet, EDTA-free (Roche, 1 tablet per 50 ml), pH 7.5.

Table 4
List of antigens used for the generation of antibodies against components of the *C. elegans* sumoylation pathway

Antigen	Species	Antibody clonality
CeSUMO (full length)	Mouse (6F2)	Monoclonal
CeSUMO (full length)	Sheep	Polyclonal
UBC-9 (full length)	Sheep	Polyclonal
GEI-17 (aa 133–509, isoform f)	Rabbit	Polyclonal
ULP-1 "1.1" (SDSRSKEFISPTPD-Cys)	Rabbit	Polyclonal
ULP-1 "1.2" (Cys-ASRRTTPRFTQKNM)	Rabbit	Polyclonal
ULP-2 "2.1" (Cys-CKNLKMPKINSEPNM)	Rabbit	Polyclonal
ULP-2 "2.2" (Cys-MPITRLVRRLRIPE)	Rabbit	Polyclonal
ULP-4 "4.1" (Cys-PKDLDNFDFAREYP)	Rabbit	Polyclonal
ULP-4 "4.2" (YGSFQDSDVSMRED-Cys)	Rabbit	Polyclonal
ULP-4 (aa 145–382)	Rabbit	Polyclonal

2. Binding buffer: 50 mM Tris–HCl, 500 mM NaCl, 10 mM imidazole, pH 7.5.
3. Washing buffer: 50 mM Tris–HCl, 500 mM NaCl, 30 mM imidazole, pH 7.5.
4. Elution buffer: 50 mM Tris–HCl, 150 mM NaCl, 150 mM imidazole, 0.5 mM TCEP, pH 7.5.
5. Dialysis buffer: 50 mM Tris–HCl, 150 mM NaCl, 0.5 mM TCEP, pH 7.5 (*see* **Note 2**).
6. Guanidine cleaning buffer: 6 M Guanidine hydrochloride, 0.2 M acetic acid.
7. Ni^{2+}-charged agarose beads: We use Ni-NTA beads from QIAGEN but beads from other providers should work as well.
8. 6×His-TEV protease is produced in-house by expressing MBP-TCS-6×His-TEV in BL21(DE3) bacteria, where TCS stands for TEV cleavage site. After induction with 1 mM IPTG, the target protein 6×His-TEV is cleaved from MBP in vivo owing to the presence of the TCS between MBP and 6×His. On 4–12 % Novex gels, the cleavage products are as follows: 6×His-TEV (~24 kDa) and MBP (~42 kDa).

2.5 Live Imaging

2.5.1 In Utero Live Imaging

1. M9 buffer: 20 mM KH_2PO_4, 40 mM Na_2HPO_4, 80 mM NaCl, 1 mM $MgSO_4$. Anesthetic solution: M9 buffer containing tricaine (0.1%) and tetramisole (0.01%) [30].
2. Standard microscope slides and cover slips.
3. 2% Agarose in M9 buffer.
4. Petroleum jelly.
5. Heat block set at 75 °C.
6. Toothpick with an eyelash attached to an end.
7. While in our hands spinning disk confocal microscope is the best suited for in utero imaging, standard wide-field/deconvolution microscopy is good enough for many strains.

2.5.2 Ex Utero Live Imaging

1. Blastomere culture medium: 60% L-15 medium, 25 mM HEPES pH 7.4, 0.5 mg/ml inulin, and 20% heat-inactivated FCS. This medium is used for meiotic recording. M9 buffer is suitable for mitotic recordings.
2. Standard microscope slides and cover slips.
3. 2% Agarose in M9 buffer.
4. Petroleum jelly.
5. Heat block set at 75 °C.
6. Toothpick with an eyelash attached to an end.
7. Confocal or wide-field microscopes work well for ex utero imaging.

2.6 Proximity Ligation Assay

Proximity ligation assays [31] and labeling of primary antibodies are performed with commercially available kits (OLINK Biosciences). We describe here its adaptation for use in *C. elegans*.

1. PLA wash buffer I: PBS + 0.5% Triton X-100.
2. PLA wash buffer II: PBS + 0.05% Tween-20.
3. Hoechst solution: Hoechst 33258 at 1.5 μg/ml in PBS + 0.1% Tween-20.
4. Mounting medium: 4% *n*-Propyl-gallate, 90% glycerol, in PBS. Commercial, anti-fade reagents are also a good choice.

2.7 Purification of 6×His-CeSUMO Conjugates

1. GuHCl lysis buffer (pH 8): 6 M GuHCl, 10 mM Tris–HCl 10 mM, 100 mM Na_2HPO_4/NaH_2PO_4, 5 mM imidazole, 10 mM IAA.
2. Urea I (pH 8): 8 M Urea, 10 mM Tris–HCl 10 mM, 100 mM Na_2HPO_4/NaH_2PO_4, 5 mM imidazole, 10 mM IAA.
3. Urea II (pH 6.3): 8 M Urea, 10 mM Tris–HCl 10 mM, 100 mM Na_2HPO_4/NaH_2PO_4, 0.2% Triton X-100, 5 mM imidazole, 10 mM IAA.

4. Urea III (pH 6.3): 8 M Urea, 10 mM Tris–HCl 10 mM, 100 mM Na_2HPO_4/NaH_2PO_4, 0.1% Triton X-100, 5 mM imidazole, 10 mM IAA.
5. Elution buffer: 50 mM Tris–HCl pH 6.8, 30% glycerol, 2% SDS, 100 mM DTT, 200 mM imidazole, 0.01% bromophenol blue.

3 Methods

3.1 Polyclonal Antibody Preparation

The basic protocol consists of affinity purifying the antibodies from a serum of choice. Dot blotting is used to determine the best-responding serum for each antibody. Afterwards, the serum is depleted of bacterial-reacting antibodies by passage through an HT115 lysate column. Finally, specific antibodies are affinity-purified. In the case of CeSUMO antibodies, a conjugation reaction is first performed to increase immunogenicity. This way, an extra step is added later on to deplete the serum from substrate-recognizing antibodies. Figure 2 details the protocol as used for anti-CeSUMO antibodies generated in sheep, but can be used for other protein antigens (*see* Table 4 for all the antigens used).

Recombinant CeSUMO needs to be conjugated in vitro to murine IRF2 (Fig. 2b).

3.1.1 Test Sera with Specific and Nonspecific Antigens in Dot-Blot Assays

1. Draw with a pencil as many rows of circles on a piece of nitrocellulose as required and spot the desired amount of recombinant protein using the circles as guide (we typically use between 1 and ~200 ng of protein) (*see* **Note 3**).
2. Block the membrane in 5% nonfat dry milk for 1 h at room temperature.
3. As a starting point, dilute the sera 1:1000 and incubate membranes for 1 h at room temperature or overnight at 4 °C.
4. Wash 3× with TBS + 0.1% Tween-20 and incubate membranes with secondary antibody at the appropriate concentration for 1 h at room temperature.
5. Wash 3× with TBS + 0.1% Tween-20 and develop using enhanced chemiluminescence.

3.1.2 Coupling of Proteins/Peptides/Bacterial Lysate to NHS Beads

1. Dialyze 40 mg of protein/peptide at 1–2 mg/ml against coupling buffer (*see* **Note 4**).
2. Wash 5 ml of NHS beads on a sintered glass funnel with 50 ml of ice-cold 1 mM HCl.
3. Wash beads with 100 ml of ice-cold coupling buffer.
4. Transfer NHS beads to a 15 ml Falcon tube.
5. Measure the protein concentration of the dialyzed protein and add to NHS beads overnight at 4 °C.

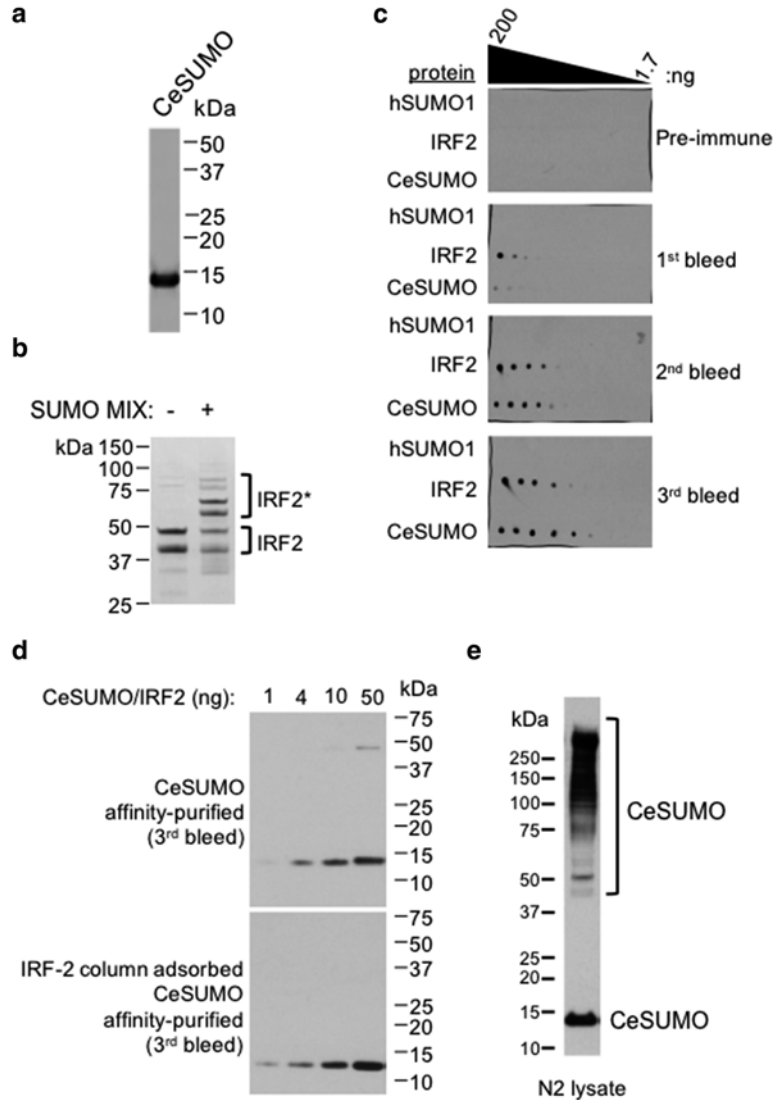

Fig. 2 Basic steps during polyclonal anti-CeSUMO antibody purification and testing. (**a**) CeSUMO was first expressed in bacteria and purified as described in this chapter. Subsequently, CeSUMO was conjugated to murine IRF2 in vitro and the resulting product was used to immunize sheep (**b**). (**c**) The different bleeds were tested for reactivity and specificity in dot blot assays using recombinant purified human SUMO-1 (hSUMO1), IRF2, and CeSUMO (200, 100, 50, 25, 12.5, 6.25, 3.13, and 1.7 ng). (**d**) The efficiency of the anti-IRF2 antibody depletion was tested in western blot assays using the indicated amounts of CeSUMO and IRF2. (**e**) The final product, anti-IRF2-depleted, affinity-purified, sheep anti-CeSUMO, recognizes both free and conjugated CeSUMO from worm lysate

6. Take an aliquot of the sample, pellet NHS beads, and measure protein concentration of the supernatant.
7. If >85% of the protein is bound, continue with the protocol.
8. Pour protein/NHS bead mix on a column and wash with 25 ml of coupling buffer.
9. Wash with 50 ml of NHS buffer A.
10. Wash with 50 ml of NHS buffer B.
11. Add 50 ml of NHS buffer A and let stand for 30 min.
12. Wash with 50 ml of NHS buffer B.
13. Wash with 50 ml of NHS buffer A.
14. Wash with 50 ml of NHS buffer B.
15. Store column in PBS, 0.5 M NaCl, and 0.1% sodium azide.
16. Adsorb the sera with HT1115 bacterial lysate-coupled NHS beads and use the flow-through for the following steps (*see* **Note 5**).

3.1.3 Affinity Purification

1. Pass 50–100 ml coupling buffer over column.
2. Dilute 15 ml of serum with 135 ml PBS and pass through a 0.2 μM filter.
3. Pass diluted serum over column two times (keep diluted serum in case of problem).
4. Wash column with 50–100 ml 10 mM Tris–HCl pH 7.5, and 0.5 M NaCl (keep wash in case of problem).
5. Elute with 0.1 M glycine pH 2.25, collecting 20 1 ml fractions into 1.5 ml disposable tubes containing 100 μl 1 M Tris–HCl pH 8.
6. Mix immediately and pool antibody-containing fractions.
7. Add glycerol to 10%, aliquot, and store at −80 °C.

Figure 2d highlights the efficiency of the anti-IRF2 antibody depletion procedure used for CeSUMO antibodies. The resulting antibody was tested by detecting SUMO conjugates from an N2 lysate (Fig. 2e).

3.2 Monoclonal Antibody Preparation

CeSUMO was conjugated in vitro to mIRF2 and the mixture was used to immunize mice. After selection of CeSUMO reactive sera by ELISA and dot blot, five different lines are isolated, re-cloned, and characterized (Table 5).

3.2.1 Hybridoma Supernatant Preparation

Hybridoma cells are maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and glutamine. High-titer tissue culture supernatants are obtained with the CELLine CL 1000 Bioreactor (Sartorius) as per the manufacturer's instructions (*see* **Note 6**). This high-concentration supernatant (usually ranging between 1 and 3 mg/ml) is ready to use for western blotting,

Table 5
List of hybridoma clones expressing different anti-CeSUMO antibodies

Parental	Re-cloning	In-house name	Isotype
6F2	D1	SUMO1.1	IgG1k
	D6	SUMO1.2	
6A4	A8	SUMO2.1	IgG1k
	C9	SUMO2.2	
5A3	B10	SUMO3.1	IgMk
	A4	SUMO3.2	
8A1	D10	SUMO4.1	IgG1k
	D11	SUMO4.2	

immunofluorescence, and immunoprecipitation. Add sodium azide to 0.1 % and keep at 4 °C (*see* **Note 7**).

The monoclonal antibodies were tested against recombinant CeSUMO (Fig. 3a) or worm lysate (Fig. 3b) in western blot assays. For immunoprecipitation tests, we used YFP-CeSUMO and performed either WB (Fig. 3c) or just irradiated the beads with UV light (Fig. 3d). Monoclonal antibodies are available at DSHB http://dshb.biology.uiowa.edu/SUMO-6F2.

3.2.2 Protein G Purification

For labeling the antibody either with PLA probes or fluorescent dyes, antibody purification from the supernatant is recommended.

1. Dialyze antibody solution overnight against wash/binding buffer with at least two buffer exchanges. Remove any particulate matter from the sample by centrifugation or filtration through a 0.45 μm filter.
2. Pour protein G slurry into column and allow column to flow by gravity to pack the column bed.
3. Equilibrate the packed affinity resin with 10 column volumes (CV) of the wash/binding buffer.
4. Gently apply sample to the column by layering onto the top of the resin. Be careful not to disturb the bed surface.
5. Wash column with 10 CV of the 1× wash/binding buffer, or until the absorbance of eluate at 280 nm approaches the background level.
6. Before beginning the elution step, set up enough tubes to collect the entire elution volume as 1 ml fractions (5 CV will be used to elute the antibody). To each collection tube add 250 μl

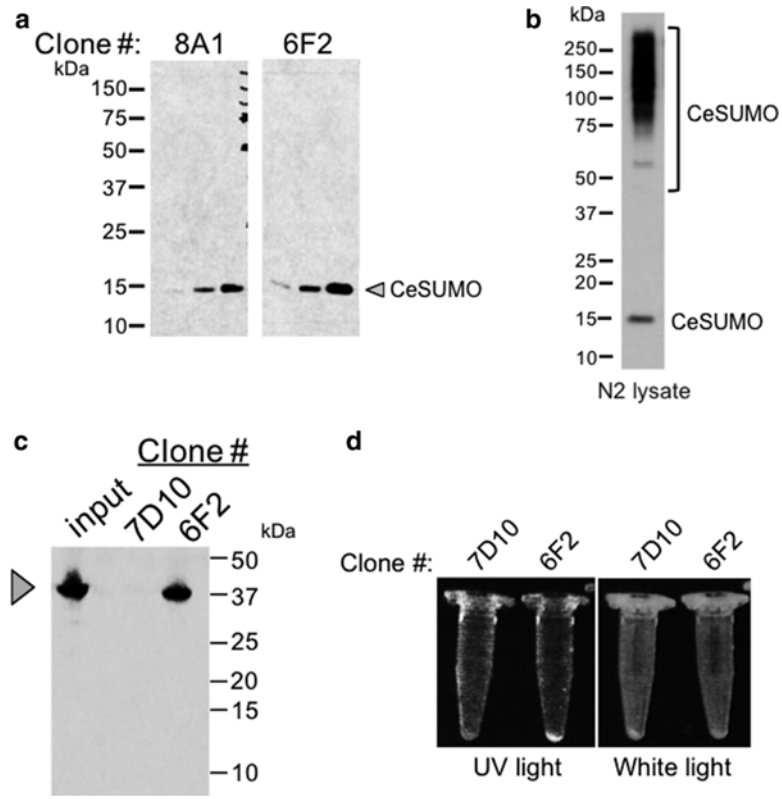

Fig. 3 Monoclonal antibody testing. (**a**) Two different clones (8A1 and 6F2) were tested on western blot assays with recombinant CeSUMO. Supernatants from each hybridoma cell line were used at 1/500 dilution. (**b**) The clone 6F2 was also tested in western blot assays using N2 lysate. Free CeSUMO can be observed at ~15 kDa while a strong smear corresponding to CeSUMO conjugates can be seen above 75 kDa. (**c**) Two supernatants were tested in immunoprecipitation assays against recombinant purified YFP-CeSUMO. While clone 6F2 immunoprecipitated YFP-CeSUMO, clone 7D10 did not. This can also be tested by irradiating beads with UV light, as seen in (**d**)

of 1 M Tris–HCl pH 9. To elute the antibody, gently add 1 ml of 1× elution buffer to the top of the resin, collecting the eluate in a prepared collection tube. Repeat until the entire volume has been collected, up to 4 column volumes.

7. Pool antibody-containing fractions, dialyze against the desired buffer, aliquot, and store at −80 °C.

3.3 Ex Utero Live Imaging

3.3.1 Preparing the Worms

1. Worms at the L4 stage are plated on RNAi plates for 24–36 h before imaging. Bacterial (HT115) clones expressing dsRNA for feeding strains are obtained from the Ahringer library [27].
2. Bacteria are grown at 37 °C to OD_{600} of 0.8, shifted to 20 °C, supplemented with 1 mM IPTG, and further incubated for 2 h.

3. Then, they are spread on 6 cm NGM plates supplemented with 1 mM IPTG and incubated for 12 h at 20 °C.
4. L4 worms are then added to plates and fed for 24–36 h before analysis.

3.3.2 Meiotic Recording

1. Worms are dissected in 4 μl L-15 blastomere culture medium on a 22 × 22 mm cover slip.
2. A ring of petroleum jelly is deposited around the drop of medium to serve as a spacer and prevent compression of the embryos.
3. A slide is placed on top to seal the chamber and prevent evaporation during filming.
4. Live imaging is conducted at 20–23 °C using a spinning-disk confocal microscope (MAG Biosystems) mounted on a microscope (IX81; Olympus) with a 100×/1.45 Plan Apochromat oil immersion lens (Olympus), a camera (Cascade II; Photometrics), spinning-disk head (CSU-X1; Yokogawa Electric Corporation), and MetaMorph software (Molecular Devices).

3.3.3 Mitotic Recording

Recording mitotic divisions is easier due to the presence of the eggshell. Worms are dissected in M9 buffer and dissected embryos can be placed on a 2% agarose pad; gently position a cover slip on top and image. Figure 4a shows still images from a time-lapse of embryos expressing GFP-tubulin and mCherry-CeSUMO (FGP7) acquired using a spinning disk confocal microscope. Images start prior to nuclear envelope breakdown and continue until late anaphase. The accumulation of CeSUMO on metaphase is conjugation dependent as it is abolished by the knockdown of GEI-17 (Fig. 4b) [20].

3.4 In Utero Live Imaging

1. Place healthy worms with a single row of embryos in anesthetic solution for 30 min.
2. Pipet worms onto a 2% agarose pad and covered with a cover slip.
3. You may seal the specimen with petroleum jelly.
4. Image worms immediately.

In our case, live imaging is conducted at 20–23 °C using a spinning-disk confocal microscope (MAG Biosystems) mounted on a microscope (IX81; Olympus) with a 100×/1.45 Plan Apochromat oil immersion lens (Olympus), a camera (Cascade II; Photometrics), spinning-disk head (CSU-X1; Yokogawa Electric Corporation), and MetaMorph software (Molecular Devices). As a standard, image stacks are obtained at 1 μm z-steps at 20-s intervals using 2 × 2 binning (*see* **Note 8**). Worms expressing GFP-H2B and mCherry-CeSUMO (FGP8) are imaged as described above and the green channel is shown on the left panel of Fig. 4c. The embryo going through metaphase of mitosis is enlarged and the red channel (CeSUMO) shown on the right.

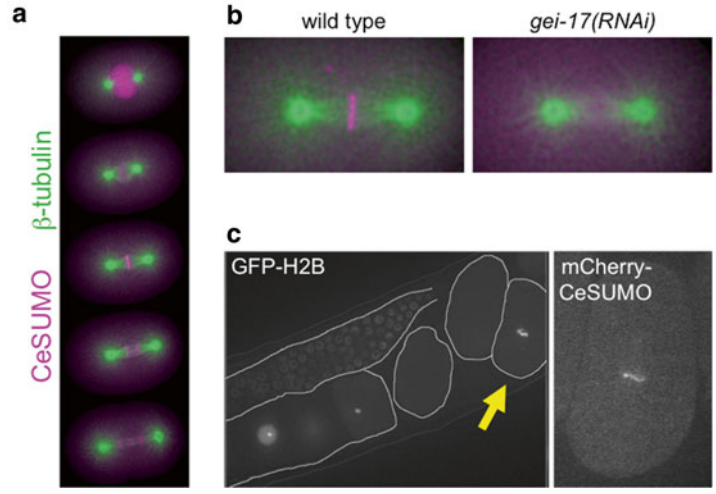

Fig. 4 Time-lapse microscopy using fluorescently labeled CeSUMO strains. (**a**) Ex utero live imaging of the first embryonic mitotic division of an embryo expressing mCherry-CeSUMO and GFP-tubulin. Images were acquired using a spinning disk confocal microscope. (**b**) Same as in (**a**), but comparing embryos fed control RNAi ("wild type") with others fed *gei-17(RNAi)*. (**c**) In utero image from a time-lapse movie using a strain expressing mCherry-CeSUMO and GFP-H2B. On the *left* panel, under the green channel, the pachytene region can be seen, three oocytes below it, and three embryos. The third embryo, indicated with a *yellow arrow*, is going through metaphase of the first mitotic cycle. An enlarged image of the red channel corresponding to the indicated embryo is shown on the *right*

3.5 Proximity Ligation Assay

Duolink® in situ Proximity Ligation Assays (PLA®) are performed using primary antibodies directly coupled to the PLA probes or using secondary antibody PLA probes (Sigma-Aldrich). When using the secondary antibody approach the theoretical maximum distance between the epitopes in the two target proteins is 30–40 nm to be able to create a signal [31]. Resolution could be taken down by a few nanometers if the assay is performed using directly labeled primary antibodies.

3.5.1 Making PLA Probes with Duolink® In Situ Probemaker

Using only primary antibodies for PLA assays is useful, as it will shorten the distance required for the probes to become ligated and amplified. Thus, the antigens will need to be in closer proximity than with the indirect method. Coupling of primary antibodies to PLA probes is carried out exactly as described by the manufacturer.

3.5.2 PLA

The protocol below has been optimized for detection of the interaction between CeSUMO and AIR-2 [20], but has since been used for other proteins.

1. 35 worms are placed on a drop of 4 µl of M9 worm buffer in a poly-D-lysine-coated slide and a cover slip is gently laid on top.
2. Once the worms extruded the embryos, slides are placed on a metal block on dry ice for 10 min.
3. The cover slip is taken off with a scalpel blade, and the samples are fixed in methanol at −20 °C for 30 min (optimal fixation time will depend on each antibody).
4. Three sequential washes of 5 min each are performed with PLA wash buffer I, PLA wash buffer II, and PBS.
5. Slides are incubated with monoclonal α-CeSUMO (6F2/D1, 10 µg/ml) and α-AIR-2 (10 µg/ml), both previously coupled to the PLA oligonucleotide arms using the Duolink® in situ Probemaker overnight at 4 °C. For indirect PLA, the same primary antibodies are used (unlabeled, at 1 µg/ml) and after an overnight incubation at 4 °C, slides are incubated with anti-mouse and anti-rabbit secondary antibodies coupled to the PLA oligonucleotide probes.
6. Ligation and amplification are performed for 30 min and 90 min, respectively, at 37 °C in a humid chamber.
7. Wash with PLA wash buffer I for 5 min.
8. Slides are incubated in Hoechst solution for 5 min.
9. Slides are mounted in mounting medium and imaged (*see* **Note 9**).

3.6 Purification of 6×His-CeSUMO Conjugates

For this procedure, we use a strain expressing a 6×His-tagged version of CeSUMO driven by its own promoter and 3′UTR (FGP14) (*see* **Note 10**).

1. If necessary, synchronize worms by bleaching and plate at ~4000 worms/9 cm plate. If this is not necessary, go to **step 7**.
2. Wash worms from plates with 6.5 ml M9 buffer and add 1 ml sodium hypochlorite solution (6–14%) and 2.5 ml 1 M NaOH (10 ml total).
3. Let stand for 4 min mixing from time to time.
4. Centrifuge for 1 min at 1,000 × *g*.
5. Wash two times with M9 and let hatch overnight.
6. Grow 6×His-CeSUMO worms at 20–25 °C until the desired stage.
7. Wash worms with M9 + 10 mM iodoacetamide (*see* **Note 11**).
8. Centrifuge for 1 min at 1,000 × *g* and discard the supernatant (leaving 100 µl).
9. Take 10 µl as input and add 1 ml GuHCl lysis buffer to the reminder.

10. Heat samples at 90 °C for 10 min and sonicate the GuHCl lysate 6 × 30″ with 30″ rest at MAX setting using a Bioruptor water bath sonicator (Diagenode).
11. Centrifuge for 30 min at 13,000 × *g* at 4 °C and transfer supernatant to a new tube.
12. Add 20 μl of Ni-NTA beads (~50 %, equilibrated in GuHCl lysis buffer) and incubate at room temperature for 2 h at room temperature or overnight at 4 °C.
13. Wash with: 1× GuHCl lysis buffer, 1× urea pH 8, and 1× urea pH 6.3 (1 ml each).
14. Add 30 μl of Ni-NTA loading buffer and incubate for 10 min shaking at RT.
15. Incubate at 100 °C for 10 min, spin down beads, and load supernatant on a gel.

3.7 Protein Purification

3.7.1 CeSUMO, UBC-9, GEI-17, and ULP-4

The purification of recombinant full-length or fragment *CeSUMO*, *ubc-9*, *gei-17*, and *ulp-4* cDNAs (as described above) is expressed from pHISTEV30a vector as N-terminal 6×His-tagged protein with a TEV protease site between the tag and the ORF. *E. coli* strain BL21(DE3) Rosetta is used for protein expression.

1. 20 ml of LB medium supplemented with kanamycin (50 μg/ml) and chloramphenicol (35 μg/ml) is inoculated with a single colony from a freshly streaked LB + kanamycin plate and the bacterial culture is incubated overnight at 37 °C with shaking at 220 rpm.
2. The following day, 5 ml of the overnight culture is used to inoculate 500 ml of LB medium with kanamycin (50 μg/ml) in a 2 l flask and cells are grown at 37 °C with shaking at 220 rpm until OD_{600} reaches ~0.6–0.8.
3. The cell culture is then cooled down in ice-cold water for 10–15 min.
4. 100 μM IPTG (final concentration) is added to induce protein expression and the cell culture is incubated at 20 °C with shaking at 220 rpm overnight (15–19 h) (*see* **Note 12**).
5. Bacteria are harvested by centrifugation (6200 × *g* for 20 min at 4 °C) and the cell pellet is resuspended in 35 ml of lysis buffer supplemented with protease inhibitors (Roche).
6. Purification is carried out immediately. Alternatively, the cell suspension can be flash-frozen in liquid nitrogen and stored at −80 °C until further use.
7. Bacteria are lysed by sonication (Digital Sonifier, Branson): big tip, 4 × 20″ pulses at 50 % amplitude, with a 20″ cooling period between pulses. Samples are kept on ice during sonication to prevent heating.

8. Samples are centrifuged (30,000 × *g* for 45 min at 4 °C) to remove any insoluble material.
9. The supernatant is loaded onto a column with 6 ml Ni-NTA agarose beads (packed volume, QIAGEN), washed with water, and pre-equilibrated with binding buffer (*see* **Note 13**).
10. The column is washed successively with binding buffer (~10 column volumes) and washing buffer (~10 column volumes) and the fusion protein is then eluted with elution buffer. Keep fractions for SDS-PAGE analysis.
11. Fractions containing 6×His-tagged protein are pooled and quantified with the Bradford reagent by measuring absorbance at 595 nm.
12. TEV protease is added (1 mg of TEV protease per 100 mg of the fusion protein) and samples are dialyzed overnight at 4 °C against 50 mM Tris–HCl, 150 mM NaCl, and 0.5 mM TCEP, pH 7.5.
13. In the meantime, the Ni-NTA column is cleaned using guanidine cleaning buffer (~7 column volumes), followed by extensive washing with water.
14. Once most of the fusion protein is cleaved (usually ~16 h at 4 °C, check by SDS-PAGE and Coomassie staining), imidazole is added to the final concentration of 10 mM (30 mM for GEI-17).
15. Samples are centrifuged (3900 × *g* for 15 min at 4 °C) to remove any precipitated material and the supernatant is then passed through the Ni-NTA agarose column pre-equilibrated with 50 mM Tris–HCl, 150 mM NaCl, 10 mM imidazole (30 mM for GEI-17), and 0.5 mM TCEP, pH 7.5.
16. A flow-through fraction is collected. This step removes free 6×His-tag, any uncleaved 6×His-tagged protein, and the 6×His-TEV protease.
17. The flow-through fraction is dialyzed overnight at 4 °C against 50 mM Tris–HCl, 150 mM NaCl, and 0.5 mM TCEP, pH 7.5.
18. Samples are concentrated using a centrifugal concentrator (Sartorius) with a molecular weight cutoff of 10,000 for GEI-17, 5000 for UBC-9 and ULP-4, and 3000 for CeSUMO.
19. Purified proteins are aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C.
20. This protocol yields high amounts of >90% pure protein. If further purification is needed, proteins can be subject to size-exclusion chromatography (Superdex 75 for CeSUMO, UBC-9, and ULP-4 CD; Superdex 200 for GEI-17). Alternatively, CeSUMO, GEI-17, and ULP-4 can be further purified using anion exchange (monoQ), while UBC-9 may be further purified through cation exchange (SP).
21. In Fig. 5, an aliquot of all the steps was taken from a UBC-9 preparation.

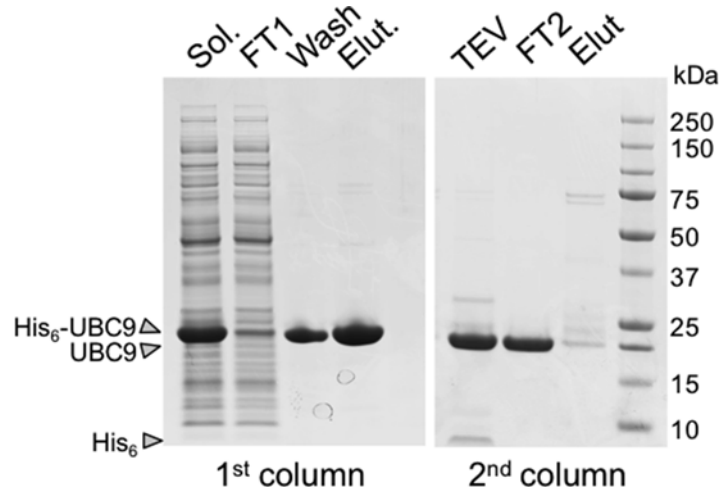

Fig. 5 Basic protocol for recombinant protein purification using the pHISTEV30a vector. After induction with IPTG soluble material (Sol.) was obtained and an aliquot loaded on a gel. An aliquot of the flow-through (FT1) from the Ni-NTA column was loaded on the gel. After washing with buffer containing 30 mM imidazole (wash), the protein was eluted with 200 mM imidazole (Elut.). After treatment with 6×His-TEV protease (TEV), a second flow-though (FT2) was taken consisting of the untagged purified recombinant protein

3.7.2 Expression and Purification of TEV Protease

1. BL21(DE3) cells containing pRK793 are grown at 37 °C in LB containing 100 μg/ml ampicillin and 25–30 μg/ml chloramphenicol.
2. Induce production of the fusion protein (MBP-TEV) by adding IPTG to a final concentration of 1 mM at $A_{600} = 0.8$ (after chilling cultures in ice water for 10 min). At the same time, lower the temperature to 25 °C unless using a bench-top shaker for induction step.
3. Harvest the cells by centrifugation after a minimum 4 h of induction (overnight expression is fine).
4. Resuspend the cell pellet in 5–10 ml of 50 mM sodium phosphate buffer pH 8, 150 mM NaCl, 10% glycerol, and 25 mM imidazole (lysis buffer) per 1 g of wet cell paste. **Add protease inhibitor** (we use complete EDTA-free, Roche) (*see* **Note 14**).
5. Lyse the cells (sonication) and remove the cell debris by centrifugation (20,000 rpm, 30 min). Filter the supernatant (0.2 μm filter).
6. Load the sample onto an appropriately sized Ni-NTA column (expect 30 mg TEV/L cell culture) equilibrated with lysis buffer.
7. Wash the column with 20 column volumes of lysis buffer; elute the TEV protease in successive ½ column volume fractions of

50 mM sodium phosphate pH 8, 150 mM NaCl, and 300 mM imidazole; and analyze fractions by SDS-PAGE.

8. Pool the appropriate fractions and desalt/dialyze against 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA, and 1 mM DTT. You may notice some precipitation here, but do not worry; just spin down and filter before proceeding.
9. Concentrate the protease to 1–2 mg/ml. Add glycerol to 50% and snap-freeze and store in 1 ml aliquots at −80 °C. Once raised from −80 °C, store at −20 °C.

3.8 SUMO Conjugation

Conjugation assays contained 50 mM Tris–HCl pH 7.5, 5 mM dithiothreitol, 5 mM $MgCl_2$, 2 mM ATP, 100 ng of SAE1/SAE2, 1 μM UBC-9 (reduced to 100 nM for E3-dependent conjugation), ~1 μg of substrate protein, and 5 μg of SUMO and are incubated at 37 °C for 4 h. Figure 6a shows a typical time course of an in vitro conjugation using RanGAP1 as a test substrate.

3.9 Processing of SUMO and Chain Editing

CeSUMO chains are assembled on GEI-17 and purified by size-exclusion chromatography (SEC) using a Superdex 200 column (Fig. 6b). SUMO processing assays contained 150 mM NaCl, 0.5 mM TCEP, 50 μM SUMO, and 100 nM SENP1 or ULP-4 recombinant catalytic domains (CD), and reactions are incubated at 30 °C for 60 min. Chain editing assays are performed by adding 0.5, 1, and 4.5 μM of the catalytic domain of ULP-4 (aa 145–333 in NP_495703.2) for the indicated times, whereas CeSUMO processing is performed for 2 h at 37 °C using 1 μM ULP-4 CD- and CeSUMO-modified GEI-17. The effect of pH and salt on ULP-4 activity is shown in Fig. 6c, d.

4 Notes

1. Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.45 μm filter before use.
2. Salt concentration and pH will need to be optimized for other proteins.
3. Make sure to let the protein(s) dry on the membrane before proceeding to the blocking step.
4. The procedure can be scaled down as required, provided that small amounts of antibody are to be purified.
5. In the case of the CeSUMO antibody, deplete serum of anti-IRF2 antibodies by passing through a column of IRF2 coupled to NHS beads and use the flow-through for the following steps. The same strategy is used if a tagged protein is used as antigen, to deplete of anti-tag antibodies.

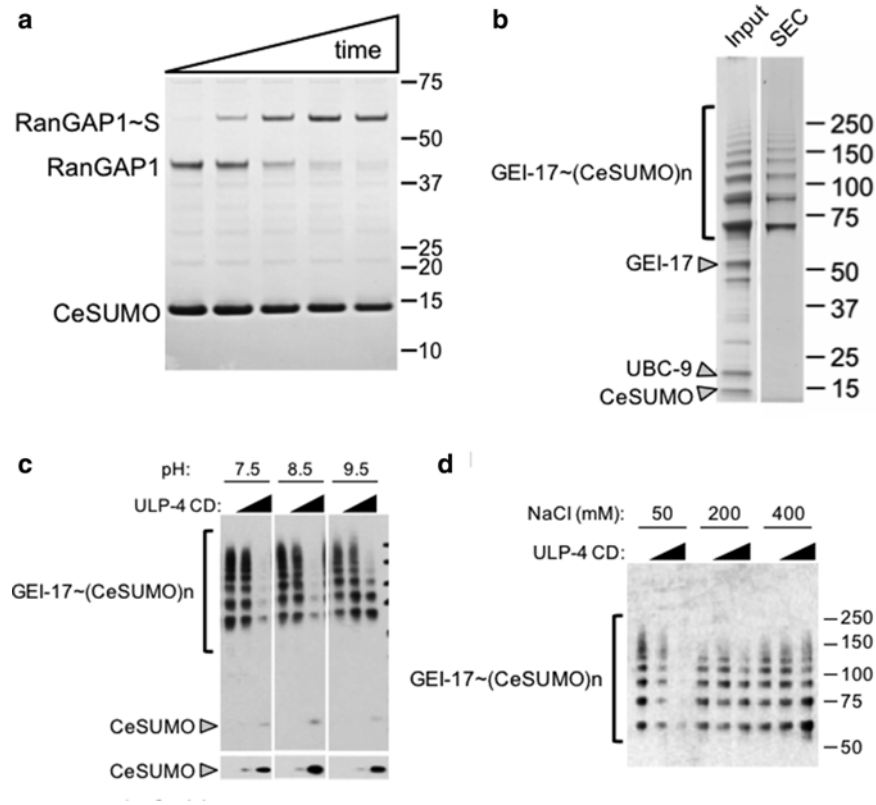

Fig. 6 In vitro conjugation and protease activity assays. (**a**) RanGAP1 was used as a substrate in conjugation reactions and aliquots were taken at 0, 2, 5, 15, and 30 min. Samples were analyzed by SDS-PAGE and Coomassie staining. (**b**) An in vitro conjugation reaction was performed using GEI-17 as the substrate. After 4 h, the reaction was diluted and fractionated over a Superdex 200 SEC column. Fractions containing SUMO-modified GEI-17 were pooled and concentrated. SUMO protease activity of ULP-4 was tested under different pH conditions (**c**) and salt concentrations (**d**)

6. These type of commercially available bioreactor chambers are very useful as the concentration of antibody in the supernatant is, in our experience, always >1 mg/ml.
7. Some applications may require or benefit from a cleaner antibody preparation. In those cases, which include the labeling for PLA assays, protein G purification from the hybridoma supernatant is performed.
8. Parameters such as amount and depth of z-stacks, exposure time, binning, and Δt should be determined for each strain with extra care to avoid phototoxicity.
9. We have found for all of our PLA assays that the channel corresponding to the PLA signal SHOULD NOT be deconvolved. Deconvolution leads to "dirtier" images.

10. In this protocol, sumoylated proteins are enriched through a denaturing purification using Ni-NTA beads. This is crucial to make sure that you are not detecting non-covalent SUMO interaction as opposed to conjugation (covalent).
11. Iodoacetamide should be handled with care and preferably prepared fresh. Small 0.4 M aliquots can be stored at –20 °C.
12. IPTG concentration, induction temperature, and length should be optimized for each protein. We use 0.1 M IPTG at 20 °C for 16 h as the standard protocol.
13. Filtering the supernatant through a 0.45 μm filter prior to binding is optional but might help in keeping a good flow by avoiding clumps to get stuck in the column.
14. All handling post-lysis should be done at 4 °C and the protease should be frozen as soon as possible.

Acknowledgments

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Chapter 18

Purification of SUMO Conjugates from Arabidopsis for Mass Spectrometry Analysis

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Abstract

The posttranslational modification of proteins with small ubiquitin-related modifier (SUMO) is a rapid, robust, and reversible mechanism that impacts a host of eukaryotic processes important to both normal cellular functions and survival during various abiotic and biotic challenges. Essential to defining the breadth of events impacted by SUMOylation is the development of full catalogues of protein targets. Here, we describe a stringent affinity method to purify native SUMO conjugates from the model plant *Arabidopsis thaliana* based on the expression of modified SUMOs bearing epitope tags. When combined with standard and quantitative mass spectrometric methods, deep datasets of SUMOylated proteins can be acquired. Functional analysis of these lists links SUMO to numerous regulatory events, with an emphasis on those associated with transcription, DNA replication and repair, and chromatin assembly/accessibility.

Key words Affinity purification, *Arabidopsis thaliana*, Mass spectrometry, Plants, Small ubiquitin-like modifier, SUMO

1 Introduction

The covalent attachment of the SUMO polypeptide to other proteins provides an essential mechanism to control the activity, localization, and fate of many intracellular processes in eukaryotes [1–5]. Besides having a central role in development and cellular homeostasis under normal conditions, SUMOylation rapidly rises upon stress and is required for the survival against many environmental challenges. In the plant *Arabidopsis thaliana* for example, SUMO conjugation is essential to embryogenesis and has been associated with basal and acquired thermotolerance, resistance to cold, salt and drought; response to phosphate deficiency; and innate immunity [2, 6–16]. Some of these effects appear connected to signaling by the stress hormones salicylic acid and abscisic acid [6, 9–11, 17].

SUMOylation is driven by a three-step E1-E2-E3 enzymatic cascade akin to other posttranslational modifiers within the

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ubiquitin-fold family (Fig. 1a). In *Arabidopsis*, ATP-dependent activation is directed by the E1 heterodimer consisting of the SAE1 and SAE2 polypeptides, which then transfers the activated SUMO moiety to a single E2, SCE1 [1, 2]. With the help of at least four SUMO ligases (or E3s), SIZ1, MMS21, PIAL1, and PIAL2, a host of substrates become modified; the C-terminal glycine of the SUMO moiety is linked via an isopeptide bond to one or more lysines within the target (Fig. 1d) [7, 18–20]. In addition, SUMO is conjugated to other SUMOs to generate internally linked poly-SUMO chains [21, 22]. Bound SUMOs can also be subsequently modified with ubiquitin using a small collection of SUMO-dependent ubiquitin ligases; these SUMO + ubiquitin conjugates often become targets of 26S proteasome-mediated turnover [22–24].

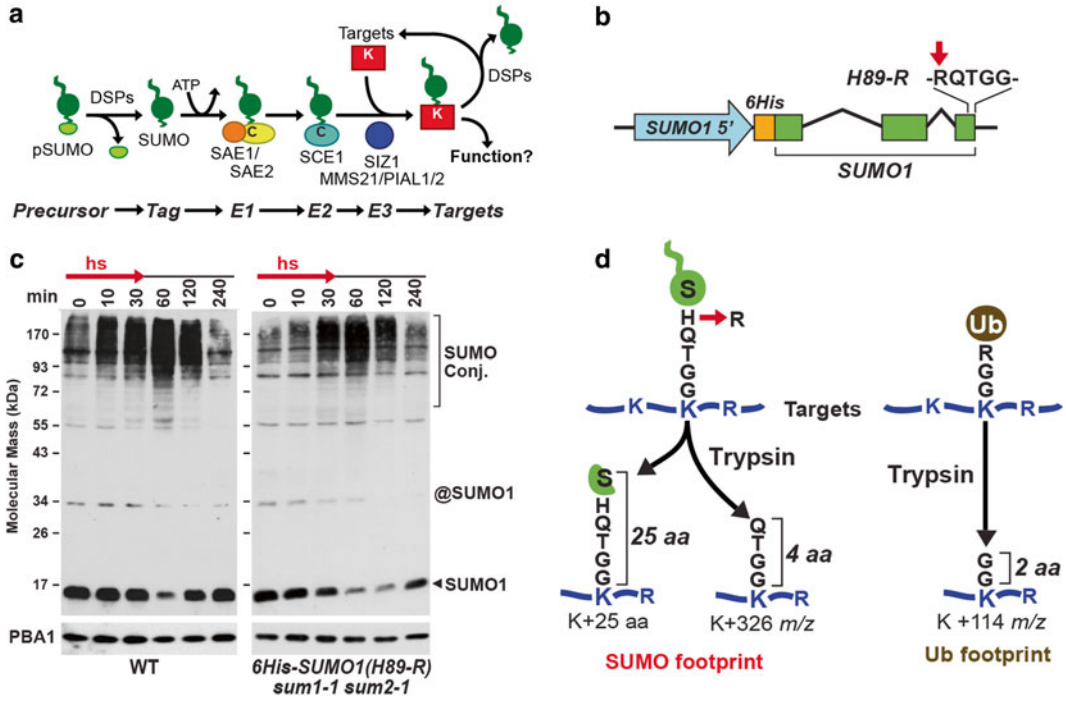


Fig. 1 Description of the SUMO pathway in *Arabidopsis* and use of tagged SUMO1 for affinity purification of SUMO conjugates. (**a**) Description of the SUMOylation pathway. The names of the various components from *Arabidopsis* are indicated. (**b**) Diagram of the *6His-SUMO1*(*H89-R*) transgene. The position of the H89-R mutation is shown (adapted from [22]). (**c**) Like wild-type SUMO1, the 6His-SUMO1(H89-R) protein becomes conjugated to other *Arabidopsis* proteins during heat stress. Seven-day-old wild-type (WT) or *6His-SUMO1*(*H89-R*) *sum1-1 sum2-1* seedlings grown at 24 °C were subjected to heat stress at 37 °C for 30 min and then returned to 24 °C for 3.5 h. At various time points, seedlings were collected. Crude seedling extracts were subjected to immunoblot analysis with anti-SUMO1 antibodies (adapted from [22]). (**d**) Generation of a detectable SUMO footprint following trypsinization of 6His-SUMO1(H89-R) conjugates as compared to wild-type SUMO1 and ubiquitin (Ub). The added mass of the SUMO/Ub fragments that remain attached to the modified lysines after trypsin cleavage as detected by tandem MS is indicated (adapted from [22])

SUMOylation is reversed by a family of deSUMOylating proteases (DSPs) that cleave just the isopeptide bond to release both proteins intact (Fig. 1a) [21, 25–27]. These cysteine proteases are also responsible for processing the SUMO precursors into their conjugatable forms by removing a short stretch of extra amino acids beyond the C-terminal di-glycine motif. Notably, DSPs are active in cell extracts upon tissue homogenization and thus require inactivation by thiol-modifying agents such as iodoacetamide (IAA) and *N*-ethylmaleimide (NEM) to stabilize SUMO conjugates during isolation [21, 28].

In most plant species, SUMO is expressed from a family of genes. *Arabidopsis* has eight loci predicted to encode the 100-amino-acid SUMO polypeptide, of which only SUMO1, SUMO2, SUMO3, and SUMO5 are transcriptionally active [1, 2, 29]. SUMO1 and SUMO2 are the canonical species; they share 93% amino acid sequence identity to each other and typically have >90% identity to orthologs in other plant species [1, 30a]. This pair is essential, as the homozygous double *sum1-1 sum2-1* null mutant arrests in early embryonic development [2]. SUMO1 and SUMO2 conjugates are present at low levels under normal growth conditions, but dramatically rise upon heat shock and other stresses, including ethanol, cold and hydrogen peroxide, thus implicating these isoforms as the main effectors in stress protection (Fig. 1b) [1, 7, 13, 22, 30a, 30, 31]. SUMO3 and SUMO5 are more divergent isoforms found in *Arabidopsis*. They share an ~50% identity with SUMO1, and are only 43% identical to each other [1, 30a]. Their functions are not yet known, but comparably dissimilar isoforms exist in other plant species, suggesting that they are important but have less structurally constrained roles within the SUMO system.

To fully appreciate the breadth of processes impacted by SUMOylation, deep catalogs of SUMO targets are clearly needed. The basic strategy is to enrich for the conjugates based on the SUMO moiety and then identify them in bulk by protein mass spectrometry (MS) methods. Typically, these protocols start with expression of a tagged SUMO, either stably or transiently, followed by affinity purification of the SUMO and its conjugates based on the tag sequence. Examples include the use of six-histidine (6His), HA, and FLAG tags followed by affinity enrichment with nickel chelate or anti-tag antibody columns [32–41]. Extractions often use buffers containing strong denaturants to avoid isolating proteins non-covalently associated with SUMO, and irreversible cysteine protease inhibitors such as IAA to help block DSPs.

First attempts at purifying SUMO conjugates employed a single affinity purification step using human cell culture lines transiently expressing a 6His-tagged SUMO construction combined with nickel chelate chromatography [32, 36]. The tagged SUMO was expressed in a background of wild-type SUMO, which

challenged efficient enrichment. Subsequent improvements in the yeast *Saccharomyces cerevisiae* involved replacing the endogenous SUMO with a 6His-tagged version such that the entire pool of SUMO was tagged [33]. To increase the purity of the preparations and thus provide more confidence of SUMO conjugate identification, tandem affinity methods were subsequently adopted in which two different affinity steps are employed sequentially. Examples include using modified SUMOs bearing two different tag sequences, such as 6His combined with a FLAG epitope, or a tandem affinity purification (TAP) tag consisting of a protein A domain followed by a calmodulin-binding protein [34, 38]. These arrangements allowed for nickel-nitrilotriacetic acid (Ni-NTA) chromatography followed by immunoprecipitation with anti-FLAG antibodies or for pull-down with nonspecific immunoglobulins and calmodulin, respectively. It is also possible to combine Ni-NTA columns with anti-SUMO antibody columns if a single 6His tag is employed [22]. Vertagaal and colleagues have recently exploited a poly-His tag containing ten histidines that bind tighter to Ni-NTA beads and thus can be washed under stronger denaturing conditions to reduce contaminants [42–45].

A number of proteomic studies have attempted to provide catalogs of SUMO conjugates from plants, mainly using *Arabidopsis thaliana* as the model. One of the main difficulties with isolating such conjugates is their low abundance under normal growth conditions with most of the SUMO pool present in a free form (Fig. 1b). Recent attempts combined the endogenous expression of a 6His-tagged SUMO1 with a single Ni-NTA chromatography step [40, 46]. To enrich for SUMO conjugates in vivo, Budhiraja et al. [40] employed a 6His-SUMO1 variant with a glutamine-to-alanine mutation at residue 90; the resulting conjugates are less readily disassembled by DSPs and thus more stable in extracts. Unfortunately, expression of this SUMO1(Q90-A) protein negatively affected the phenotype of the plants and resulted in the detection of few potential conjugates, leaving any list developed with this variant open to question. To improve conjugate detection, Park et al. [46] combined two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of the Ni-NTA-enriched fraction from 6His-SUMO1-expressing plants coupled with matrix-assisted laser-desorption ionization time-of-flight MS. However, this method is severely limited by the ability of 2D-PAGE to sufficiently separate individual proteins, and by its failure to detect low-abundance conjugates (which is typically the case) or conjugates that fall outside the optimum range for PAGE analysis (e.g., too acidic, basic, and/or hydrophobic or very large or small). An alternative approach was to identify SUMO targets by combining two-dimensional liquid chromatography fractionation of the whole plant proteome with an immunoscreen of the resulting fractions for those containing SUMOylated proteins, and then identify the

abundant proteins in each fraction by MS [47]. Unfortunately, this strategy failed to present support that the proteins in question were directly modified with SUMO as opposed to being more abundant contaminants that co-fractionated with actual targets. Collectively, the low number of targets identified, the lack of overlap among the datasets, and the presence of proteins that probably are not *bona fide* targets, provided little confidence that the resulting catalogs are not populated with contaminants.

To avoid the above complications, we recommend using a dual-purification strategy for plants that relies on a tagged SUMO that faithfully mimics the wild-type protein combined with two separate affinity methods to isolate SUMO conjugates based on the SUMO moiety. We also recommend employing strong denaturants during the various purification steps whenever possible to (1) slow disassembly of SUMO conjugates by DSPs, (2) minimize contaminants (especially Rubisco), and (3) avoid isolating proteins that interact with SUMO non-covalently as opposed to proteins directly modified with SUMO.

Our successful approach was to genetically replace the main SUMO isoforms (SUMO1 and -2) with a tagged version that is fully active in planta and amenable to affinity purification [22, 31]. Here, the *Arabidopsis sum1-1 sum2-1* double-null mutant was rescued with a 6His-tagged genomic SUMO1 construction bearing a histidine-89-to-arginine (H89-R) substitution, which was expressed under the control of the native *SUMO1* promoter. Importantly, this modified SUMO appears functional both by its ability to completely rescue the lethal phenotype of homozygous *sum1-1 sum2-1* embryos and by its ability to enter into the conjugation cycle. Like wild-type SUMO, this 6His-SUMO1(H89-R) variant becomes reversibly attached to other *Arabidopsis* proteins in vivo with the levels of these conjugates rising dramatically upon heat stress (Fig. 1c).

As a first step, SUMO conjugates are enriched based on the 6His sequence by nickel chelate affinity chromatography using a Ni-NTA resin under strong denaturing conditions (Fig. 2a). As a second step, the conjugates are further purified using beads coated with anti-SUMO1 antibodies generated against the *Arabidopsis* SUMO1/2 polypeptide sequence. And finally, the anti-SUMO antibodies, which unavoidably bleed from antibody columns, are removed by a second Ni-NTA chromatography step in the presence of 8 M urea. To reduce contaminants and the action of DSPs, initial tissue extractions are conducted at 55 °C with a 7 M guanidine-HCl containing buffer, and the first Ni-NTA step uses 8 M urea washes followed by elution with an imidazole buffer containing 6 M urea.

To help with the identification of SUMOylation sites on individual targets, we included the H89-R mutation in the 6His-SUMO1 moiety, which introduced a novel trypsin cleavage site.

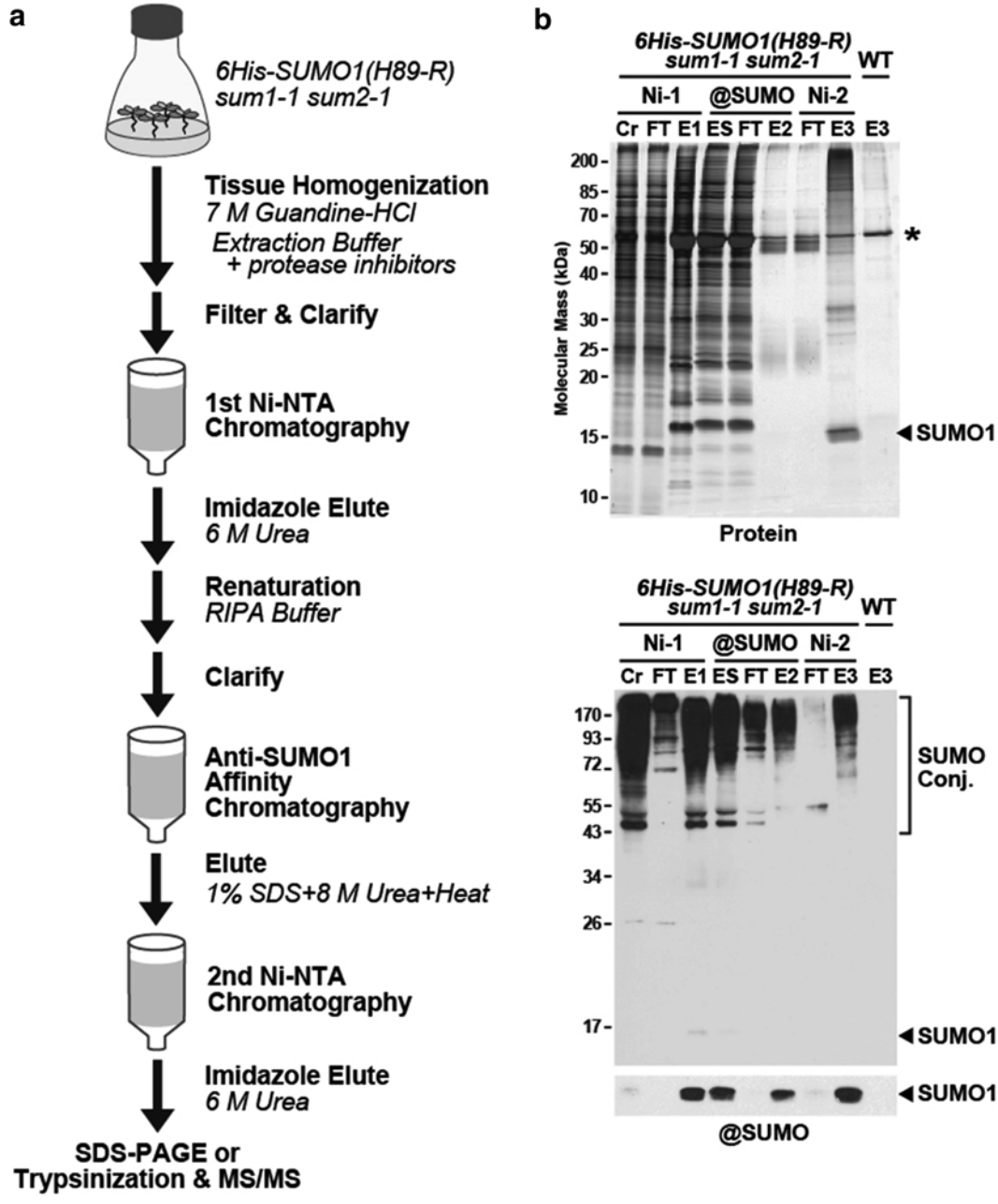


Fig. 2 Three-step affinity purification of SUMO conjugates from *Arabidopsis*. (**a**) Flow chart of the purification strategy using plants expressing 6His-SUMO1(H89-R). (**b**) SDS-PAGE of the first Ni-NTA, anti-SUMO affinity, and second Ni-NTA steps in the purification. *Bottom* panel shows an immunoblot of the samples using anti-SUMO1 antibodies. *Top panel* shows a gel stained for total protein with silver. *FT* flow-through, *E* eluate. SUMO conjugates, SUMO1/2, and Rubisco are indicated by the *bracket*, and closed and open *arrowheads*, respectively (adapted from [22])

This substitution enables identification of SUMO attachment sites by tandem MS as a lysine residue isopeptide modified with a 326-Da pyroQ-T-G-G addition (Fig. 1d). Importantly, the SUMO(H89-R) variant is functional and generates a "SUMO footprint" that is distinct from the Ub-binding site "footprint" (K+114 Da; K-G-G), thus allowing us to map SUMO and Ub attachment sites simultaneously (Fig. 1d).

As shown in Fig. 2b, the final preparations are substantially enriched for free SUMO and SUMO conjugates but contain few contaminants. The only major contaminant was the large subunit of Rubisco; remarkably some of this highly abundant protein remains in the final eluate despite the highly stringent extraction and wash conditions. When combined with protein identification MS methods, a list of highly confident SUMOylation targets is possible. Our initial studies identified over 350 SUMO targets in *Arabidopsis*, and by using a more sensitive mass spectrometer we recently increased this list to over 900, which concur with the current literature on number of SUMO targets in yeast and mammalian cells [22, 43]. Intriguing examples in our initial catalog from *Arabidopsis* are illustrated in Table 1 [22]. Notably, most SUMOylated proteins are found within the nucleus with many involved in various aspects of transcription, DNA repair, chromatin structure and accessibility, factors related to RNA processing and export, and components of the nuclear pore complex. A number of enzymes within the SUMO pathway are themselves SUMOylated; the function(s) of this modification remains to be discovered.

Our SUMO conjugate purification strategy is likely amenable to any plant species, assuming that mutants missing the appropriate SUMO isoform(s) are available and transformation methods to introduce the tagged SUMO have been developed. However, it might be possible to avoid the need for SUMO mutants if the transgenic SUMO can be expressed to levels high enough to successfully compete with the endogenous SUMO pool in conjugation to other proteins.

2 Materials

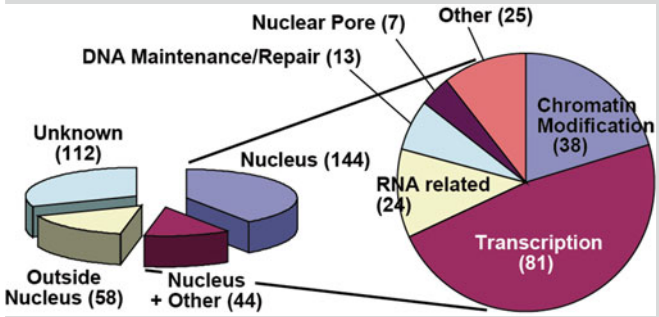
All solutions are prepared fresh with ultrapure water (18 ΩM at 25 °C). Buffers are prepared at room temperature (21 °C) unless otherwise noted.

2.1 Purification of Recombinant A. thaliana SUMO1

1. *Escherichia coli* strain expressing untagged *A. thaliana* SUMO1 polypeptide terminating after the C-terminal di-glycine motif involved in forming the covalent bond to targets.
2. Luria Broth (LB).
3. Terrific Broth (LB).

Table 1

Distribution of SUMOylated proteins and representative members in *Arabidopsis*[a,b]



Functional group	*SUMO substrate*
SUMO pathway	SUMO1, SAE2, SCE1, SIZ1, ESD4
Transcription factors	
WRKY family	WRKY3, WRKY4, WRKY6, WRKY33, WRKY72
HSF family	HSFA1D, HSFA2, HSFB2B
Global TF family	GT2, GTB1, GTE1, GTE4, GTE7, GTL1
ANACONDA family	ANACO50, ANACO51, ANACO81/ATAF2
Homeodomain	BEL1, BEL10, KNAT3, HB6, ANL2
Others of note	EIN3, ARR1, PHR1, ERF6, BIM1, HUA2, SEUSS
Transcriptional co-regulators	
TOPLESS family	TPL, TPR1, TPR2, TPR4, LEUNIG, LUH
Chromatin modifiers	
Chromatin methylation related	IDN2, MORC, SUVR1, SUVR2, SUVH2, SUVH9, KTF1, IBM1
SWI-SNF complex	SWI3A, SWI3C, SWI3D, PICKLE, PKR1, CHR11
Histone acetylation related	SNL2, SNL4, SNL5, HAC1, GCN5, HDA19(HD1), ADA2A, ADA2B, EML3
Histone 2b related	Histone2B, NRP1, UBP26, SPT16
DNA main/repair	LIG1, DRT111, KU80, POLD3, TOP1, TRB1
RNA related	RPS35, RPS41, STA1, PRH75, CCR2, NUC-L1, U2AF65A, SDN3, LA1, XRN3, DRH1, PRP40B, SERRATE, LACHESIS
Nuclear pore	IMP-6, IMPA1, WIP1
Cell cycle regulators	SYN4, ILP1, ILP2, RHL1, DPB, CDC5, CDC48, RPA1

[a]Adapted from Miller et al. [22]

[b]Number of unique proteins in each catagory is in parentheses in the pie chart

4. 1 M Isopropyl β-d-1-thiogalactopyranoside (IPTG).
5. *E. coli* lysis buffer: 50 mM Na_2HPO_4 (pH 7.4), 1 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA), and 1 mM phenylmethylsulfonyl (PMSF) at 4 °C (*see* **Note 1**).
6. 30 mL High-speed polycarbonate centrifuge tubes, such as Nalgene™ Oak Ridge (Thermo Scientific).
7. Ammonium sulfate ($(NH_4)_2SO_4$).
8. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 at 4 °C.
9. Dialysis tubing and clamps.
10. Other equipment: Oven, glass rod, stir bar and stir plate, Erlenmeyer flasks, 4 L beakers, thermometer, centrifuge and rotor, ice bucket and ice, 15 and 50 mL screw-top tubes, sterile pipettes, buffers and equipment necessary for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue or silver for staining proteins, Protein Assay (Bio-Rad), and bovine serum albumin (BSA).

2.2 Affinity Purification of Anti-SUMO Antibodies

1. Purified recombinant *A. thaliana* SUMO1 at 4 mg/mL (*see* **Note 2**).
2. 1 M Tris–HCl (pH 7.4).
3. Ice-cold water.
4. Affi-Gel 15 beads (Bio-Rad).
5. Protein Assay (Bio-Rad) and BSA protein standard.
6. 12 mL PolyPrep® chromatography columns and end caps (Bio-Rad).
7. Affi-Gel quenching buffer: 500 mM NaCl and 50 mM Tris–HCl (pH 7.2) at 4 °C.
8. Affinity wash buffer 1: 150 mM NaCl and 10 mM Tris–HCl (pH 7.2) at 4 °C.
9. Affinity wash buffer 2: 500 mM NaCl and 10 mM Tris–HCl (pH 7.2) at 4 °C.
10. Neutralization buffer: 1.5 M NaCl, 1 M Tris–HCl (pH 8.0), and 1 mM Na_2EDTA.
11. Gentle Ag/Ab Elution Buffer (Pierce) (*see* **Note 3**).
12. IgG Elution Buffer (Pierce) (*see* **Note 4**).
13. Glycerol.
14. Antibody dialysis buffer: 25 mM MOPS (pH 7.2), 150 mM NaCl, and 10% (v/v) glycerol at 4 °C.
15. Dialysis tubing and clamps.

16. Concentrating spin column with a 30 kDa molecular weight cutoff (MWCO) such as Vivaspin 20 GE Health Care or Amicon Ultra 15 EMD Millipore (*see* **Note 5**).
17. Other equipment: 5 mL Spin tube (Eppendorf), Parafilm, stir bar and stir plate, Erlenmeyer flask, centrifuge and rotor, ice bucket and ice, 15 and 50 mL screw-top tubes, sterile pipettes, ice bucket and ice, stir plates, clamp stands or other column supports, drip tray, pH strips, and buffers and equipment necessary for SDS-PAGE and immunoblotting.

2.3 Protein Extraction

1. Plant extraction buffer: 7 M Guanidine-HCl (*see* **Note 6**), 300 mM NaCl, 100 mM Na_2HPO_4, 10 mM Tris-HCl (pH 8), 10 mM IAA (*see* **Note 7**), 10 mM sodium metabisulfate, and 2 mM PMSF (*see* **Notes 1** and **8**). Adjust pH to 8.0 with NaOH (*see* **Note 9**).
2. Miracloth and cheese cloth.
3. Pestle and mortars.
4. 30 mL High-speed polycarbonate centrifuge tubes, such as Nalgene™ Oak Ridge (Thermo Scientific).
5. 1 M Imidazole in plant extraction buffer.
6. Ni-NTA Agarose (Qiagen).
7. Additional equipment: 250 mL Beakers, centrifuge and rotor, stir plate and stir bars, 1.5 mL tubes, 15 and 50 mL screw-top tubes, liquid nitrogen, plastic spoons/spatulas, plastic funnels, sterile pipettes, Parafilm, and shaking or rotating platform.

2.4 First Ni-NTA Affinity Purification

1. Ni-NTA wash buffer A: 6 M Guanidine-HCl, 100 mM Na_2HPO_4, 10 mM Tris–HCl (pH 8), 300 mM NaCl, 10 mM imidazole, 10 mM IAA, and 0.25 % (v/v) Triton X-100. Adjust pH to 8.0 with NaOH (*see* **Note 10**).
2. Ni-NTA wash buffer B pH 6.8: 8 M Urea, 100 mM Na_2HPO_4, 10 mM Tris–HCl (pH 8), 300 mM NaCl, 10 mM imidazole, 10 mM IAA, and 0.25 % (v/v) Triton X-100. Adjust pH to 6.8 with HCl (*see* **Note 10**).
3. Ni-NTA wash buffer B pH 8.0: 8 M Urea, 100 mM Na_2HPO_4, 10 mM Tris–HCl (pH 8), 300 mM NaCl, 10 mM imidazole, 10 mM IAA, and 0.25 % (v/v) Triton X-100. Adjust pH to 8.0 with HCl (*see* **Note 10**).
4. Ni-NTA elution buffer A: 6 M Urea, 100 mM Na_2HPO_4, 10 mM Tris–HCl (pH 8), 300 mM imidazole, and 10 mM IAA. Adjust pH to 8.0 with HCl.
5. Glass chromatography columns with 2.5 cm diameter, such as the Econo-Column by Bio-Rad.

6. Concentrating spin column, 10 kDa MWCO, such as Vivaspin 20 GE Health Care or Amicon Ultra 15 EMD Millipore (*see* **Note 5**).
7. Additional equipment: Centrifuge and rotor, clamp stands or other column supports, drip tray, 1.5 mL tubes, 15 and 50 mL screw-top tubes, sterile pipettes.

2.5 Anti-SUMO Immunoprecipitation

1. Ice-cold water.
2. Affi-Gel 10 beads (Bio-Rad).
3. Purified anti-SUMO antibodies at 1 mg/mL concentration (*see* **Note 11**).
4. Protein Assay (Bio-Rad) and immunoglobulin G (IgG) Standard (Pierce).
5. Affi-Gel quenching buffer: 500 mM NaCl and 50 mM Tris–HCl (pH 7.2) at 4 °C.
6. Phosphate-buffered saline (PBS): 100 mM NaCl and 50 mM Na_2HPO_4 (pH 7.2) at 4 °C.
7. RIPA buffer: 10 mM Tris–HCl (pH 7.2), 50 mM Na_2HPO_4, 100 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.5% (w/v) sodium dodecyl sulfate (SDS), 10 mM IAA, and 2 mM PMSF at 4 °C. Adjust pH to 7.2 if necessary using HCl.
8. 30 mL High-speed polycarbonate centrifuge tubes, such as Nalgene™ Oak Ridge (Thermo Scientific).
9. Small magnetic stir bar.
10. PBS with IAA: 100 mM NaCl, 50 mM Na_2HPO_4 (pH 7.2), and 10 mM IAA at 4 °C.
11. 12 mL PolyPrep® chromatography columns and end caps (Bio-Rad).
12. Affinity elution buffer: 8 M Urea, 300 mM NaCl, 100 mM Na_2HPO_4, 10 mM Tris–HCl (pH 8), and 10 mM IAA. Adjust pH to 8.0 with HCl.
13. Affinity elution buffer with 1% (w/v) SDS: Prepare by adding 500 ul 20% (w/v) SDS into 9.5 mL of prepared affinity elution buffer.
14. 1 M Imidazole in affinity elution buffer.
15. Additional equipment: Centrifuge and rotor, ice bucket and ice, stir plates, clamp stands or other column supports, drip tray, heat block or water bath, 1.5 mL tubes, 15 and 50 mL screw-top tubes, sterile pipettes.

2.6 Second Ni-NTA Affinity Purification

1. Ni-NTA wash buffer C pH 8.0: 6 M Urea, 300 mM NaCl, 10 mM imidazole, 100 mM Na_2HPO_4, 10 mM Tris–HCl (pH 8), and 10 mM IAA. Adjust pH to 8.0 with HCl (*see* **Note 10**).

2. Ni-NTA elution buffer B: 6 M Urea, 300 mM NaCl, 300 mM imidazole, 100 mM Na_2HPO_4, 10 mM Tris–HCl (pH 8), and 10 mM IAA. Adjust pH to 8.0 with HCl (*see* **Note 10**).
3. Ni-NTA Agarose (Qiagen).
4. 12 mL PolyPrep® chromatography columns and end caps (Bio-Rad).
5. Concentrating spin column, 10 kDa MWCO, such as Vivaspin 6 GE Health Care or Amicon Ultra 4 EMD Millipore (*see* **Note 5**).
6. Additional equipment: Centrifuge and rotor, clamp stands or other column supports, drip tray, 1.5 mL tubes, 15 and 50 mL screw-top tubes, sterile pipettes.

3 Methods

3.1 Purification of Recombinant A. thaliana SUMO1

For optimal immunoprecipitation of SUMO1 conjugates, a high-quality affinity-purified anti-SUMO1 antibody is necessary. To prevent the isolation of antibodies that might interact with the 6His epitope, we use wild-type SUMO1 without a tag to prepare the affinity column for IgG enrichment.

1. To express recombinant SUMO1, grow overnight a 250 mL LB culture of the *E. coli* strain expressing full-length SUMO1 coding region in a suitable expression vector (e.g., pET23, Merck Millipore). The next morning, inoculate 4 L of TB with the overnight culture. Grow at 37 °C until OD_{680} reaches 0.4–0.6. Induce expression by adding 1 M IPTG to a final concentration of 1 mM. Transfer culture to 30 °C and incubate for 4–5 h. Collect cells by centrifugation at 4000 × *g* for 20 min. Freeze cell pellet in liquid nitrogen.
2. Resuspend pellet with 40 mL ice-cold *E. coli* lysis buffer. Sonicate on ice for 15 min using 6 s on/off cycles (*see* **Note 12**).
3. Transfer the lysate to clean high-speed polycarbonate centrifuge tubes and clarify at 50,000 × *g* for 20 min at 4 °C.
4. Transfer the supernatant to a clean Erlenmeyer glass flask, and heat until lysate reaches 90 °C to denature most *E. coli* proteins. While heating, stir lysate occasionally using a glass rod. SUMO1 is relatively heat resistant and much of it remains soluble.
5. Cool to room temperature on bench top, and then place on ice to cool to 4 °C.
6. Add the cooled lysate to clean high-speed polycarbonate centrifuge tubes and clarify at 50,000 × *g* for 20 min at 4 °C. Transfer the supernatant to a clean glass flask containing a

sir bar. Save the pellet for SDS-PAGE analysis of the purification.

7. While stirring the supernatant on ice, add $(NH_4)_2SO_4$ to make a 70% (w/v) solution (for 100 mL of supernatant, add 47.2 g $(NH_4)_2SO_4$). Stir for 30 min on ice, and then continue stirring overnight at 4 °C.
8. Transfer the solution to clean high-speed polycarbonate centrifuge tubes and clarify at 10,000 × *g* for 20 min at 4 °C. Transfer the supernatant to a clean flask containing a sir bar. Save the pellet for SDS-PAGE analysis of the purification.
9. While stirring at room temperature, add $(NH_4)_2SO_4$ to make a 100% (w/v) solution (for 100 mL of supernatant, add 23.7 g $(NH_4)_2SO_4$). If not all the $(NH_4)_2SO_4$ dissolves, add *E. coli* lysis buffer dropwise until the crystalline $(NH_4)_2SO_4$ disappears. Finally, stir on ice for 20 min.
10. Add the $(NH_4)_2SO_4$ solution to a clean high-speed polycarbonate centrifuge tubes and collect the precipitate at 10,000 × *g* for 20 min at 4 °C. Remove the supernatant and save for SDS-PAGE analysis of the purification.
11. Resuspend pellet in 25 mL of lysis buffer.
12. Dialyze resuspended pellet in appropriate volume of PBS to remove residual $(NH_4)_2SO_4$. Add elute to a dialysis bag equilibrated in PBS for 1 h and perform dialysis at 4 °C in 3 L of dialysis buffer. Change buffer after 1 and 3 h. After second change of buffer, dialyze the sample overnight.
13. Check purification using SDS-PAGE, and quantify the final protein sample by using BSA as the standard.

3.2 Affinity Purification of Anti-SUMO Antibodies

This method is designed for purifying polyclonal rabbit antibodies generated against untagged SUMO1 described in Subheading 3.1. This purification should be completed at 4 °C or on ice to slow degradation of the antibodies.

1. Transfer 1 mL Affi-Gel 15 slurry (*see* **Note 13**) to a clean 5 mL spin tube using a cut pipette tip. If more than one sample is being processed, this volume should be scaled up accordingly.
2. Collect beads by centrifugation at 1000 × *g* for 1 min. Discard supernatant.
3. Resuspend beads in 1 mL ice-cold water.
4. Collect beads by centrifugation at 1000 × *g* for 1 min. Discard supernatant.
5. Repeat **steps 3** and **4** twice more (*see* **Note 14**).
6. Add 3 mL of the recombinant SUMO1 to reach a final concentration of 12 mg of protein per 1 mL of resin (*see* **Note 15**).

7. Incubate for 4 h at 4 °C while rotating.
8. Collect beads by centrifugation at 1000 × *g* for 1 min. Remove supernatant and save in a clean tube. To confirm efficient binding of the antibody to the beads, quantify the protein concentration remaining in the supernatant using BSA as the standard. Little to no protein should be detected, as more than 90 % of the recombinant protein should have bound to the Affi-Gel 15 beads.
9. Resuspend beads in 4 mL Affi-Gel quenching buffer and incubate for at least 1 h up to 3 h at 4 °C while rotating.
10. Transfer beads to a clean 12 mL PolyPrep® chromatography column that has been washed with 2 mL quenching buffer. Allow column to drain.
11. Wash column three times with 10 mL Affi-Gel quenching buffer. Do not allow resin to dry.
12. Wash column three times with 10 mL affinity wash buffer 2. Do not allow resin to dry.
13. Thaw 9 mL of frozen anti-SUMO1 antiserum, and clarify by spinning at 5000 × *g* for 10 min. Add 1 M Tris–HCl (pH 7.4) to a final concentration of 10 mM.
14. Transfer the antiserum to a column containing the SUMO Affi-Gel 15 resin. Cap and seal both ends of column with Parafilm. Rotate overnight at 4 °C.
15. Drain the flow-through and save in a clean 15 mL screw-top tube. Save the flow-through to test the success of the purification.
16. Wash column twice with 10 mL of affinity wash buffer 1.
17. Wash column three times with 10 mL of affinity wash buffer 2.
18. Elute with 4 mL gentle Ag/Ab elution buffer. Wash column with equal volume of affinity wash buffer 1. Collect elute and wash in the same 15 mL tube (*see* **Note 16**).
19. Wash column with 2× 10 mL affinity wash buffer 1 (*see* **Note 17**).
20. Elute with 4 mL IgG elution buffer. Collect elute in a new 15 mL tube containing 400 µL neutralization buffer. While collecting, swirl tube to more rapidly mix elute and neutralization buffer. Check the pH immediately after final elution. If the pH is below 7.0, add neutralizing buffer dropwise until pH is 7. Wash the column with 2 mL wash buffer 1. Collect elute and wash in the same 15 mL tube.
21. For both elutes, add 100 % glycerol to reach a final concentration of 10 % (v/v) (*see* **Note 18**).
22. Add elute to a dialysis bag equilibrated in antibody dialysis buffer for 1 h. Perform dialysis at 4 °C in 3 L antibody dialysis buffer. Change buffer after 1 and 3 h. After second buffer change, dialyze the sample overnight.

23. After dialysis add 100% glycerol to increase the concentration to 20% (v/v).
24. Concentrate the affinity-purified antibody to a final concentration of 1 mg/mL using a concentrating spin column with a 30 kDa MWCO. Before use, equilibrate the column by spinning the concentrator with 4 mL of dialysis buffer for 10 min. Remove residual buffer in concentrator before adding sample. Make appropriate sized aliquots and quick freeze at liquid nitrogen temperatures. Store for a long term at −80 °C.
25. To confirm that the affinity purification of anti-SUMO1 antibodies was successful, compare by immunoblot analysis signals from the crude antiserum and the purified antibody when used as the primary antibodies. Use 7-day-old *Arabidopsis* seedlings as the substrate of the immunoblot. The purified antibodies should have less background as compared to the antiserum, but should have the same signal intensity for free SUMO and high-molecular-mass SUMO conjugates.

3.3 Protein Extraction

To generate the transgenic 6His-tagged SUMO1(H89-R) expression line, the *A. thaliana* SUMO1 and SUMO2 null mutant (*sum1-1 sum2-1*) was rescued using the genomic form of *SUMO1*(*H89-R*) in which the coding sequence for a 6His epitope was placed after the start codon, and expressed under the native promoter [22]. We prefer to use the genomic sequence of SUMO1 driven by the native promoter to rescue the null mutant as it better mimics native expression levels. Care should be taken not to make the N-terminal epitope tag too long, as it may interfere with the function of SUMO in planta.

This protocol has been optimized for 50 g of 7-day-old seedlings either grown in liquid culture or on agar plates. However, we feel that this protocol can be amended to examine different plant tissues grown under a variety of conditions through the life of the plant. Note that a short heat-shock treatment (e.g., 30 min at 37 °C for *Arabidopsis* seedlings) increases SUMO conjugates and improves the yield for the purification, if the conjugates are below detection [1, 22, 31].

1. Prepare plant extraction buffer, and add PMSF just before use given its instability in aqueous buffers.
2. Pre-chill mortar and pestle with liquid nitrogen. Grind the frozen plant tissue to a fine powder, adding liquid nitrogen as needed to keep tissue frozen (*see* **Note 19**).
3. Add the required volume of extraction buffer (2 mL per gram of fresh weight) into a 250 mL glass beaker containing a stir bar. Add the pulverized tissue into beaker using a chilled plastic spoon or spatula (*see* **Note 20**). Mix on stir plate until all plant tissue has been suspended in the plant extraction buffer.

4. Incubate at 55 °C for 1 h in an oven or water bath. Halfway through the incubation period, mix plant extract on stir plate.
5. Remove from incubator and mix using stir plate.
6. Filter plant extract through two layers of Miracloth and two layers of cheesecloth into 50 mL screw-top tubes. Squeeze out excess liquid (*see* **Note 21**).
7. Add filtered extract to high-speed polycarbonate centrifuge tubes and clarify at 15,000 × *g* for 30 min (*see* **Note 22**).
8. Filter the supernatant through two new layers of Miracloth into clean 50 mL tubes. Save 200 µL of filtered supernatant as the starting sample to test efficiency of purification by SDS-PAGE (*see* **Note 23**).
9. Add 1 M imidazole in plant extraction buffer to supernatant to reach a final concentration of 10 mM imidazole.
10. To equilibrate the Ni-NTA agarose resin, add the appropriate amount of beads to a 50 mL screw-top tube (1 mL 50% Ni-NTA resin slurry per 5 g of tissue). If more than one sample is being processed, this volume should be scaled up accordingly.
11. Collect beads by centrifugation at 1000 × *g* for 1 min. Discard supernatant.
12. Resuspend beads in plant extraction buffer.
13. Repeat **steps 11** and **12** twice more.
14. Collect beads by centrifugation at 1000 × *g* for 1 min. Discard supernatant.
15. Add the appropriate amount of plant extraction buffer to make a 50% Ni-NTA slurry.
16. Add equilibrated Ni-NTA resin to plant protein extract. Seal tubes with Parafilm and rotate overnight at 4 °C.

3.4 First Ni-NTA Affinity Purification

1. Set up a clean glass chromatography column in an appropriate stand. Rinse column with 5 mL of Ni-NTA wash buffer A.
2. Apply the protein extract and Ni-NTA beads to the column and allow sample to flow through. Save 200 µL of the flow-through for SDS-PAGE analysis of the purification.
3. Wash the Ni-NTA column twice with 5 volumes of Ni-NTA wash buffer A (i.e., 5 mL buffer for 2.5 mL bead volume).
4. Wash the column twice with 5 volumes of Ni-NTA wash buffer B pH 6.8 (*see* **Note 24**).
5. Wash the column three times with 5 volumes of Ni-NTA wash buffer B pH 8.
6. To elute, add 1 volume of Ni-NTA elution buffer A. Collect flow-through into a 50 mL tube. Repeat elution four addi-

tional times. The elutes may be collected in the same tube (*see* **Note 25**). Save a 100 µL sample for SDS-PAGE confirmation of the purification.

7. Concentrate the elute 20-fold (*see* **Note 26**) using a concentrating spin column with a 10 kDa MWCO. Before use, wash the spin column by centrifuging the concentrator with 4 mL of Ni-NTA elution buffer A for 10 min. Remove remaining buffer in concentrator before adding sample.

3.5 Anti-SUMO Immunoaffinity Chromatography

1. Transfer 800 µL Affi-Gel 10 slurry (*see* **Note 27**) to a clean 1.5 mL tube using a cut pipette tip. If more than one sample is being processed, this volume should be scaled up accordingly.
2. Collect beads by centrifugation at 1000 × g for 1 min. Discard supernatant.
3. Resuspend beads in 1 mL ice-cold water.
4. Collect beads by centrifugation at 1000 × *g* for 1 min. Discard supernatant.
5. Repeat **steps 3** and **4** twice more (*see* **Note 14**).
6. Add 500 µL of the affinity-purified SUMO1 antibodies to a final concentration of 1 mg antibodies per 1 mL of beads (*see* **Note 28**).
7. Incubate for 4 h at 4 °C while rotating.
8. Collect beads by centrifugation at 1000 × *g* for 1 min. Remove supernatant and save in a clean tube. To confirm efficient binding of the antibodies to the beads, check the protein concentration of the supernatant using IgG as the standard. More than 90% of the antibodies should bind to the beads.
9. Resuspend the beads in 1 mL Affi-Gel quenching buffer and incubate for at least 1 h up to 3 h at 4 °C while rotating.
10. Collect beads by centrifugation at 1000 × *g* for 1 min. Discard supernatant.
11. Wash beads by resuspending in 1 mL quenching buffer.
12. Repeat **steps 10** and **11** twice more.
13. Collect beads by centrifugation at 1000 × *g* for 1 min. Discard quenching buffer.
14. Wash beads by resuspending in 1 mL PBS.
15. Repeat **steps 13** and **14**.
16. Collect beads by centrifugation at 1000 × *g* for 1 min. Discard supernatant.
17. Add appropriate volume of PBS to beads to make a 50% slurry. Keep the beads at 4 °C until ready to use.
18. Add the concentrated elute dropwise into 25 volumes of RIPA (i.e., 1 mL concentrated elute is added to 25 mL of RIPA) in

30 mL high-speed polycarbonate centrifuge tubes while stirring on ice. Incubate on ice for 30 min.

19. Centrifuged for 5 min at 15,000 × *g* at 4 °C. Transfer the supernatant to a clean 50 mL tube (*see* **Note 29**).
20. Add the appropriate amount of SUMO1 antibody bound to Affi-Gel 10 to the supernatant. Incubate overnight at 4 °C while rotating.
21. Equilibrate a clean 12 mL PolyPrep® chromatography columns by allowing 2 mL RIPA buffer to flow through the column. Cap the column.
22. Transfer the beads and supernatant to the column. Collect the supernatant in a clean 50 mL tube. Save a 100 µL sample for subsequent SDS-PAGE analysis of the purification.
23. Wash the column twice with 5 mL of RIPA for a total wash of 10 mL.
24. Wash the column ten times with 10 mL of PBS. Cap after final wash.
25. Bound SUMO conjugates are eluted in four steps from the antibody column to enable collection of even the most tightly bound conjugates. First, add 1 mL of affinity elution buffer with 1% (w/v) SDS, and gently resuspend the beads in the column using a cut 1000 µL tip. Transfer beads to a clean 15 mL screw-top tube, and incubate at 65 °C for 15 min on a heat block or in a water bath.
26. Add beads along with the buffer back into the column, and collect the elute. Cap column.
27. Repeat **step 26** and **28** once more, making sure to collect the second elute.
28. For the third elution step, add 6 mL of affinity elution buffer, and gently resuspend the beads in the column. Transfer beads to a 15 mL tube, and incubate at room temperature for 30 min while rotating to increase protein elution. Add beads along with buffer back to column, and collect the elute.
29. Elute the remaining conjugates from the resin bed by adding an additional 2 mL of affinity elution buffer and collecting the flow-through. Combine all of the four elutes in one 15 mL tube.

3.6 Second Ni-NTA Affinity Purification

The main purpose of this step is to remove the anti-SUMO1 antibodies eluted along with the SUMO conjugates in the prior antibody affinity step.

1. To the elute from the anti-SUMO immunoaffinity step, add 100 µL 1 M imidazole in affinity elution buffer to reach a final concentration of 10 mM imidazole.

2. To prepare the Ni-NTA agarose resin, add 700 µL of 50 % Ni-NTA resin slurry to a clean 5 mL tube. If more than one sample is being processed, this volume should be scaled up accordingly.
3. Collect beads by centrifugation at 1000 × *g* for 1 min. Discard the supernatant.
4. Resuspend beads in 5 mL affinity elution buffer to equilibrate.
5. Repeat **steps 11** and **12** twice more. Discard the supernatant.
6. Add appropriate volume of affinity elution buffer to beads to make a 50 % slurry.
7. Add equilibrated Ni-NTA resin to affinity elute and rotate at room temperature for 4 h (*see* **Note 30**).
8. Equilibrated a 12 mL PolyPrep® chromatography column by washing with 2 mL of affinity elution buffer. Cap the column.
9. Transfer the Ni-NTA beads and elute to the column. Allow the supernatant to drain. Collect the flow-through and save a 100 µL sample for subsequent SDS-PAGE analysis of the purification.
10. Wash the column twice with 5 mL of Ni-NTA wash buffer C. Wash the column four more times with 10 mL of wash buffer C for a total wash volume of 50 mL.
11. Elute four times with 500 µL of Ni-NTA elution buffer B. Collect the elutes in a 15 mL tube.
12. Check purification using SDS-PAGE and quantify final protein solution.
13. The final Ni-NTA elute can be concentrated to about 100 µL if a higher protein concentration is desired for downstream applications such as MS/MS. It is not necessary to concentrate the sample for SDS-PAGE analysis.

3.7 Analysis Methods for SUMOylated Proteins

SDS-PAGE and immunoblot analysis using anti-SUMO1 antibodies should be performed to confirm enrichment of SUMO conjugates (Fig. 2b). The immunoblot loads should be proportionally adjusted to allow direct comparison among the samples obtained at each step of the purification.

3.7.1 SDS-PAGE and Immunoblotting

Examination of the SDS-PAGE gel stained for proteins with silver should also be completed to ensure that the protocol successfully removed most contaminants.

3.7.2 Mass Spectrometric Identification

For MS analysis of the final elute, it should be concentrated to 20–100 ng/µL using a concentrating spin column with a 10 kDa MWCO. To quantify the amount of protein, absorbance at 280 nm can be measured with a NanoDrop Spectrophotometer or by using

a microcuvette in a standard spectrophotometer. Use the Ni-NTA elution buffer 2 as the blank for the 280 nm absorbance. An alternative is to employ the micro BCA protein assay (Pierce) using BSA as the standard. However, this assay requires the sample to be diluted 25-fold to reduce the concentration of imidazole and urea, which may reduce the protein concentration below that which can be measured accurately.

For MS analysis, approximately 2 μg of the final SUMO conjugate sample is reduced using dithiothreitol (DTT) and alkylated using IAA. In this case, it is important that IAA be excluded from the Ni-NTA elution buffer 2, as the IAA will react with the DTT and prevent proper reduction of proteins. Trypsin digestion is performed for approximately 36 h in 1 M urea with 25 mM ammonium bicarbonate (pH 8.3), using a 1:20 trypsin-to-protein ratio. Once desalted with an Agilent Omix C18 pipette tip, the digest is now ready for liquid chromatography separation combined online with tandem MS analysis after dissolution in appropriate MS buffers. It is preferred that the MS identification of SUMO conjugates includes a negative control. By using tissue not expressing the 6His-tagged SUMO1(H89-R) variant, but that underwent the same three-step purification as 6His-tagged expression tissue, a list of contaminants can be obtained.

3.7.3 Quantitative Mass Spectrometric Analysis of SUMO Conjugates

Because our stringent three-step purification protocol consistently yields highly enriched SUMO conjugate samples, quantitative MS analysis can be performed. We have successfully applied iTRAQ labeling to quantify the changes in SUMOylation upon heat shock (Fig. 3a, b). For more information on the iTRAQ methodology, see Miller et al. [31]. Alternatively, label-free quantification approaches based on peptide spectral matches (PSM), whose values are adjusted using distributed normalized spectral abundance factor (dNSAF), are possible for rough estimates (Fig. 3c). dNSAF calculations take into consideration polypeptide length since the number of possible peptides can increase PSM counts for longer proteins, and distribute shared peptides and unique peptides if protein families or individual isoforms are being measured. Both iTRAQ and dNSAF methods are usually limited to measuring only highly abundant proteins or proteins that are easily detected by MS in the mixture.

4 Notes

1. Add PMSF to extraction buffer right before use as it degrades quickly in aqueous solutions.
2. The recombinant protein must be in PBS or MOPS buffer to bind properly to the Affi-Gel 15.

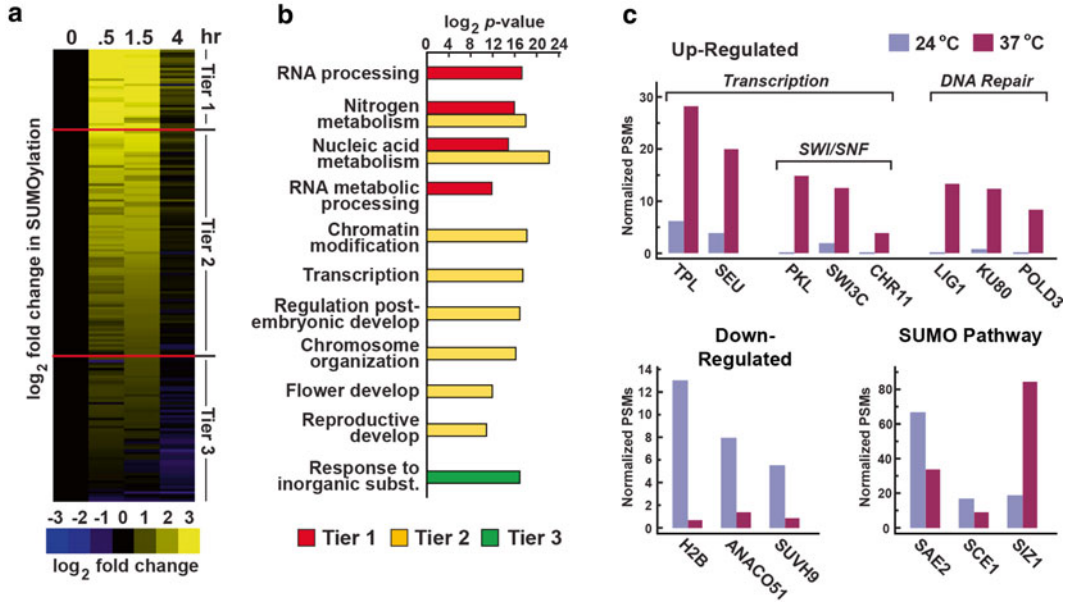


Fig. 3 Quantitative analysis of SUMO conjugates from *Arabidopsis*. (**a**, **c**) Dynamics of SUMO1 conjugation during and after heat shock as quantified by iTRAQ MS. (**a**) Relative changes in the SUMOylation status of 172 targets from *6His-SUMO1*(*H89-R*) *sum1-1 sum2-1* plants before and immediately after a 30-min heat shock at 37 °C followed by a recovery at 24 °C. The values are illustrated by a heat map where *yellow* denotes an increase in SUMOylation and *blue* a decrease. The three tiers cluster proteins displaying a >7-fold increase (tier 1), a 2–7-fold increase (tier 2), or a <2-fold increase (tier 3) in SUMOylation during the heat stress (adapted from [31]). (**b**) Gene Ontogeny (GO) functional enrichment for the targets clustered in each tier as a function of the $\log_2$(*p*-value) (adapted from [31]). (**c**) Use of peptide spectral matches (PSMs) to quantify the effects of heat shock on SUMO conjugates. Seven-day-old seedlings grown at 24 °C were subjected to a 30-min heat stress at 37 °C. Levels of each protein in the control and heat-shock MS datasets were estimated by the number of PSMs in the MS precursor scans for unique peptides from each target; the PSM values were then normalized by the total number of PSMs for each MS run. Representative targets include SUMOylated transcription and DNA repair components that increase in abundance during heat stress, SUMOylated proteins that decrease in abundance during heat stress, and members of the SUMO conjugation pathway (adapted from [48])

3. We found that using this gentle elution buffer greatly helps reduce precipitation of the antibody during elution and dialysis.
4. This buffer may be substituted with 100 mM glycine (pH 2.5).
5. Vivaspin 20 spin columns are preferred as we have found that their design minimizes protein precipitation.
6. To dissolve the guanidine-HCl, the buffer can be heated in a 55 °C water bath before adding IAA, sodium metabisulfate, and PMSF. Care must be taken to bring buffer back to room temperature before adjusting the pH.
7. IAA inhibits cysteine-based proteases, including deSUMOylating proteases.

8. PMSF and sodium metabisulfate serve as protease inhibitors to prevent cleave of target proteins and tag.
9. The Na_2HPO_4, Tris–HCl and NaCl may be prepared as a 4× buffer base, as they are used in most solutions in this protocol. Combine 1.5 M NaCl, 400 mM Na_2HPO_4, and 50 mM Tris–HCl (pH 8.0). The pH of the 4× buffer base does not have to be adjusted before use. To dissolve the salts faster, use water warmed to 37 °C to prepare the solution.
10. The 4× buffer base can be used to make this buffer (*see* **Note 9**).
11. The concentration of the antibodies is important for proper binding to the Affi-Gel 10/15 beads and for binding to the SUMO moieties, and should be at 1 mg/mL. The antibodies must be in PBS or MOPS buffer to bind properly to the Affi-Gel 10 resin.
12. Avoid heating up the sample during sonication, although the suspension may reach temperatures of up to 15 °C without affecting the purification.
13. The amount of Affi-Gel resin required per 9 mL bleed is 1 mL. 1.6 mL of Affi-Gel slurry will yield about 1 mL of beads.
14. The washing of beads with water should not take longer than 20 min to prevent a loss in binding capacity of the Affi-Gel. In addition, add the recombinant protein or antibody immediately after washing the beads.
15. For proper binding of the SUMO1 to the Affi-Gel, it is important that the volume of the recombinant proteins is no larger than three times the volume of beads.
16. This step is optional, but recommended as it increases the quality of the antibody purified. If not using the gentle elution buffer, skip **step 19** and use 8 mL IgG elution buffer instead.
17. This step is essential to remove all traces of the gentle elution buffer.
18. We have found that this prevents precipitation during dialysis.
19. The tissue should be ground until a homogeneous powder; otherwise protein extraction may not be complete. Be careful adding additional liquid nitrogen, as the ground tissue can aerosol and risk contaminating equipment and workplace.
20. To prevent tissue from sticking to spoon/spatula, pre-chill in liquid nitrogen.
21. This filtering step removes most large particles of plant material. Place funnel in 50 mL screw-top tube. Add two layers of Miracloth and then two layers of cheesecloth. Have additional tubes ready as needed to collect flow-through. Care should be taken when squeezing that the Miracloth does not rupture and add insoluble material back into the extract.

22. This centrifugation step removes large insoluble material from the protein extract. If the pellet is loose, or did not form, after 30 min of centrifugation, then additional centrifugation time (10–30 min) may be required.
23. The pellet may also be saved to confirm extraction of SUMOylated proteins. This is recommended, if any other tissue than 7-day-old seedlings is used. Resuspend pellet in an amount of plant extraction buffer equivalent to the volume of the supernatant.
24. This low pH wash helps remove a large fraction of contaminants from the column.
25. The elute can be flash frozen and stored at –80 °C before concentration.
26. It is important to avoid concentrating the sample too much to prevent precipitation of the proteins in the elute. The solution should stay clear; if a brown color is observed the sample is excessively concentrated. Thus, it is important to monitor the sample while concentrating.
27. The amount of Affi-Gel beads required per sample is 500 µL, and 800 µL of Affi-Gel slurry will yield about 500 µL resin.
28. For proper binding of the antibodies to the Affi-Gel, it is important that the volume of antibodies is equivalent to the volume of beads.
29. The insoluble pellet should be very small. When transferring the supernatant, be careful not to disturb the pellet as it is typically loose.
30. Rotating at room temperature prevents the SDS in the sample from precipitating. This incubation step should not be done longer than 4 h to prevent degradation of sample.

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Chapter 19

Detection of SUMOylation in *Plasmodium falciparum*

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Abstract

Reversible protein modification by small ubiquitin-related modifiers (SUMOs) regulates many cellular processes, including transcription, protein quality control, cell division, and oxidative stress. SUMOylation is therefore essential for normal cell function and represents a potentially valuable target for the development of inhibitors of pathogenic eukaryotic organisms, including the malaria parasite, *Plasmodium falciparum* (*Pf*). The specific and essential functions of SUMOylation in *Pf*, however, remain largely uncharacterized. The further development of antimalarial drugs targeting SUMOylation would benefit significantly from a more detailed understanding of its functions and regulation during the parasite life cycle. The recent development of antibodies specific for *Pf* SUMO provides a valuable tool to study the functions and regulation of SUMOylation. In preliminary studies, we have used immunoblot analysis to demonstrate that SUMOylation levels vary significantly in parasites during different stages of the red blood cell cycle and also in response to oxidative stress. Owing to the dynamic nature of SUMOylation and to the robust activity of SUMO isopeptidases, analysis of SUMOylation in cultured *Pf* parasites requires a number of precautions during parasite purification and lysis. Here, we outline methods for preserving SUMO conjugates during isolation of *Pf* parasites from human red blood cell cultures, and for their detection by immunoblot analysis using *Pf*SUMO-specific antibodies.

Key words SUMO, *Plasmodium falciparum*, Posttranslational modification, Immunoblotting, Malaria

1 Introduction

P. falciparum is an obligate intracellular parasite with life cycles spanning both mammalian host hepatocytes and erythrocytes, as well as the mosquito midgut. Adaptation to these disparate cellular environments requires complex developmental and morphological changes during the various life stages. The rapid spatial and temporal control of protein functions necessary for such remodeling can be regulated by changes in gene expression as well as posttranslational modifications (PTM) of cellular proteins [1]. SUMOylation is a reversible PTM that plays important roles during cell division, transcription, and stress response [2]. While the SUMO pathway has been identified in *P. falciparum*, its function in the parasite has not

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been well characterized [3–5]. We have previously shown that SUMO levels are variable between the asexual erythrocytic stages and are sensitive to changes in oxidation (unpublished) [4]. Moreover, disruption of SUMO de-conjugation blocks parasite replication, suggesting that dynamic SUMOylation is required to maintain viability [5]. The ability to monitor patterns of SUMOylation in the parasite provides an essential tool for understanding the regulation and functional consequences of SUMO dynamics.

SUMO conjugation to target proteins is regulated by an enzymatic cascade consisting of activating (E1) and conjugating (E2) enzymes, and E3 ligases, the actions of which are counteracted by SUMO-specific Cys-isopeptidases. As seen in other eukaryotes, the levels of target protein modification in *Pf* parasites vary depending on developmental stage, growth conditions, and stress treatments [4, 6–11]. Overall, however, most individual proteins are SUMOylated at very low steady-state levels, making detection of this PTM challenging [12].

Immunoblotting represents the most widely used method for detecting SUMO conjugates from cell lysates. Due to the dynamic nature of SUMOylation, however, monitoring SUMO conjugates by this method poses specific challenges, requiring specific precautions during sample preparation. In particular, SUMO proteases are highly active in parasites and must be inhibited by the irreversible cysteine protease inhibitor, *N*-ethylmaleimide (NEM). Existing methods of parasite lysate preparation lack this essential additive [3, 13]. Moreover, we have found that addition of NEM during both parasite harvesting and lysis is necessary to fully preserve SUMOylation levels (discussed below). In this chapter, we describe methods for harvesting parasites from red blood cells and subsequent lysis conditions that allow for optimal detection of SUMO-modified proteins via gel electrophoresis and anti-*Pf*SUMO immunoblotting.

2 Materials

Prepare all solutions using ultrapure water (resistivity of 18.2 MΩ cm at 25 °C). All stock solutions should be filtered using 0.22 μm filter units.

2.1 *P. falciparum* Culturing

1. Complete malaria culture media (MCMA): RPMI 1640, supplemented with 0.025 M HEPES, 0.2% sodium bicarbonate (w/v), 12.5 μg/mL hypoxanthine, 5 mg/mL Albumax I (Life Technologies). Sterile filter media and store at −80 °C in 40 mL aliquots.
2. Human O+ erythrocytes.
3. 3% CO_2, 3% O_2, 94% N_2 atmosphere.

2.2 Parasite-Harvesting Components

1. Protease inhibitors (PI): 100 μM Leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/mL aprotinin, 1 μg/mL pepstatin, 5 mM *N*-ethylmaleimide (NEM) (final concentrations). Add to designated solutions immediately before use.
2. $MCMA^{PI}$: MCMA supplemented with protease inhibitors.
3. PBS (1×): 0.137 M NaCl, 0.0027 M KCl, 0.0015 M KH_2PO_4 monobasic, 0.0081 M Na_2HPO_4 dibasic anhydrous. Prepare a working solution from a 10× stock by diluting 100 mL into 900 mL ultrapure water.
4. PBS^{PI}: PBS supplemented with protease inhibitors.
5. Saponin lysis buffer: PBS supplemented with 0.2% saponin and protease inhibitors. Prepare immediately before use.

2.3 Parasite Lysis Components

1. Parasite lysis buffer: PBS supplemented with 0.2 M NaCl, 1% NP-40, 0.1% SDS, 0.05% sodium deoxycholate, protease inhibitors. Prepare immediately before use.
2. Bioruptor water bath (Diagenode).
3. SDS sample buffer (5×): 0.313 M Tris–HCl, pH 6.8, 10% SDS, 50% glycerol, 3.5 M β-mercaptoethanol, 0.1% (w/v) bromophenol blue. Store stock solution at –20 °C, and working aliquots at room temperature.

2.4 SDS Polyacrylamide Gel Components

1. Stacking gel acrylamide: 30% Acrylamide, 0.44% Bis-acrylamide. Store at 4 °C protected from light.
2. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8. Store at 4 °C.
3. Resolving gel acrylamide: 33.5% Acrylamide, 0.3% Bis-acrylamide. Store at 4 °C protected from light.
4. Resolving gel buffer: 1 M Tris–HCl, pH 9.1. Store at 4 °C.
5. Ammonium persulfate: 3% Solution in ultrapure water. Store at –20 °C. Store working aliquots at 4 °C for 1 week.
6. *N*,*N*,*N*′,*N*′-tetramethylethane-1,2-diamine(TEMED) (BioRad).
7. SDS-PAGE running buffer (1×): 0.025 M Tris–HCl, 0.192 M glycine, 0.1% SDS. Prepare a working solution from a 4× stock by diluting 250 mL into 750 mL ultrapure water.
8. Mini-protean gel electrophoresis system (BioRad).
9. PageRuler Prestained Protein Ladder (Thermo Scientific).

2.5 Immunoblot Components

1. Transfer buffer (1×): 0.192 M Glycine, 0.025 M Tris–HCl, 0.01% SDS, 20% MeOH. Prepare a working solution from a 10× stock and add MeOH immediately before use.
2. Tris-buffered saline (TBS): 0.05 M Tris–HCl, 0.137 M NaCl, pH 7.4. Prepare a working solution from a 20× stock by diluting 50 mL into 950 mL ultrapure water.

3. TBST: TBS containing 0.05 % Tween-20.
4. Blocking solution: 5 % Nonfat powered milk in TBS.
5. Immobilon-P polyvinylidene difluoride (PVDF) 0.45 μm membrane (Millipore).
6. Classic X-ray film (RPI).
7. Mini-trans blot cell (BioRad).
8. Foam pads (BioRad).
9. Thick blot filter paper.
10. Thin Whatman filter paper, 6 × 9 mm.

2.6 Antigens and Conjugates

1. Monoclonal *Pf*SUMO antibody (Matunis Lab).
2. HRP-conjugated goat anti-mouse secondary antibody (Jackson).
3. ECL Prime chemiluminescent substrate (GE Healthcare).

3 Methods

3.1 Preparation of Protein Samples from P. falciparum

1. Transfer 15 mL of asynchronous parasite culture, at 2 % hematocrit and 10 % parasitemia, to a 50 mL conical tube (*see* **Note 1**).
2. Centrifuge infected erythrocytes at 500 × *g* for 5 min at room temperature (*see* **Note 2**). Aspirate and discard the supernatant.
3. Wash the erythrocyte pellet with 1.5 mL MCMA^{PI} (37 °C) (*see* **Note 3**). Centrifuge sample at 500 × *g* for 5 min at room temperature. Aspirate and discard the supernatant.
4. Resuspend sample in 1.5 mL saponin lysis buffer. Allow the erythrocytes to lyse for 3 min, inverting the tube 2–3 times. The culture will turn translucent red.
5. Add 10 mL ice-cold PBS^{PI} to quench lysis. Centrifuge sample at 5000 × *g*, 4 °C, for 10 min. Aspirate and discard the supernatant. Repeat wash.
6. Resuspend the parasite pellet in 1 mL ice-cold PBS^{PI}, and transfer to 1.5 mL tube (*see* **Note 4**). Centrifuge sample at 20,800 × *g*, 4 °C, for 5 min. Aspirate and discard the supernatant (*see* **Note 5**).
7. Store pellet at −80 °C until ready for parasite lysis.

3.2 Parasite Lysis

1. Remove parasite pellets from −80 °C and thaw quickly on ice.
2. Resuspend pellet in 100 μL parasite lysis buffer.
3. Place on ice for 5 min.
4. Lyse pellet on high, 30 s ON, 30 s OFF (on ice), for a total of 2.5 min, using a Bioruptor water bath sonicator (*see* **Note 6**). The sample will turn brown as the hemozoin is released.

5. Place sample on ice for 5 min, with occasional vortexing.
6. Pellet debris at 20,800 × *g*, 4 °C, for 10 min. The hemozoin will pellet and the lysed supernatant will mostly be clear.
7. Take 95 μL supernatant and place in a fresh 1.5 mL microcentrifuge tube. Add 23.75 μL 5× SB and aliquot into single-use tubes (17 μL each). Aliquoted sample can be stored at −20 °C (*see* **Note 7**).

3.3 12.5% SDS-Polyacrylamide Gel Electrophoresis

1. Prepare a 12.5% SDS-PAGE gel (*see* **Note 8**). For 10 mL resolving gel, add 3.75 mL resolving gel acrylamide, 3.8 mL resolving gel buffer, 100 μL 10% SDS, 2.1 mL MilliQ H_2O, 250 μL 3% APS, and 5 μL TEMED, and mix. Pour between 1.0 mm thick glass plates secured in a BioRad casting stand, leaving an approximately 20 mm space at the top of the short plate. Add 250 μL ultrapure water to the top of the resolving gel and allow to polymerize for 30 min at room temperature. Once polymerized, remove excess water. For 2.5 mL stacking gel, mix 325 μL stacking gel acrylamide, 602 μL stacking gel buffer, 25 μL 10% SDS, 1.5 mL MilliQ H_2O, 50 μL 3% APS, and 5 μL TEMED. Pour over the resolving gel to reach the top of the short plate. Add a 1.0 mm 15-well spacer comb, and allow gel to polymerize for 30 min at room temperature. Wrap extra gels in a wet paper towel, and store at 4 °C for up to 1 week.
2. Heat lysate samples on a 72 °C sand block for 10 min (*see* **Note 9**).
3. Spin sample at 20,800 × *g* for 5 min at room temperature.
4. Load 15 μL supernatant on a 12.5% SDS-PAGE gel, reserving a lane for 4 μL protein ladder.
5. Run SDS-PAGE at 100 V, room temperature, for approximately 2 h. Do not run the gel beyond the 10 kDa molecular weight marker.

3.4 Immunoblot

Perform blocking and antibody incubations while shaking the membrane at low speed, and washing steps at medium speed.

1. Pry the gel plates open using a spatula to release the gel.
2. Presoak a 0.45 μM PVDF Immobilon-P membrane (*see* **Note 10**) in 100% MeOH for 5 min. Allow the membrane to equilibrate in 1× transfer buffer as you assemble the cassette. Under a thin layer of transfer buffer, layer the cassette as follows: clear cassette, foam pad, thick blot paper, membrane, gel, and two thin Whatman papers (*see* **Note 11**). Remove air bubbles by rolling a conical tube across the Whatman paper. Add the remaining foam pad and close the cassette clamp. Place cassette in the transfer cell so that the black cassette faces the black cell. Attach the electrode cover and plug into power source.
3. Perform transfer at 100 V, 4 °C, for 2 h.

4. Block membrane in blocking solution for 30 min at room temperature.
5. Incubate membrane in primary monoclonal antibody, 1:1000 *Pf*SUMO, for 1 h at room temperature in 1× PBS, 2% BSA, and 0.02% NaN_3 (*see* **Note 12**).
6. Wash membrane with TBST buffer for 10 min. Repeat wash three times.
7. Incubate membrane in secondary HRP-antibody, 1:15,000 goat anti-mouse, in blocking solution for 30 min at room temperature.
8. Wash membrane with TBST buffer for 10 min. Repeat wash three times.
9. Develop with chemiluminescent substrate, and ECL Prime, and expose for various times (1–30 s, 1–2 min, and 5 min) (Fig. 1) (*see* **Note 13**).

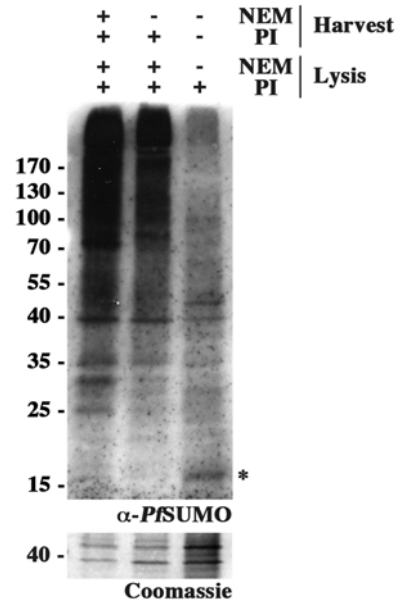

Fig. 1 Analysis of *Pf*SUMOylation by western blot. Anti-*Pf*SUMO blot of total cell lysates from harvested parasites. Note that the addition of protease inhibitors, including NEM, is necessary in both the harvest and lysis buffers to enhance the detection of total cellular SUMO conjugates. "*" denotes free *Pf*SUMO. Coomassie-stained membrane shows relative sample loading

4 Notes

1. Asynchronous cultures produce a characteristic SUMO smear; however, ring-, trophozoite-, and schizont-staged parasites have distinct SUMO banding patterns, with peak detection of high-molecular-weight conjugates during the trophozoite stage [4]. Dd2 strain parasites were used to obtain higher parasitemia levels and were cultured daily. The culture can be scaled down as needed; however, resuspending the parasite pellet from a 15 mL culture in 100 μL parasite lysis buffer during Subheading 3.2, **step 2**, provides an appropriately concentrated sample for subsequent immunoblotting.
2. SUMO isopeptidases remain active at 4 °C. Do not place samples at 4 °C until NEM has been added to the buffer.
3. In addition to lysis buffer, adding NEM to parasite harvesting buffers further enhances the detection of SUMO conjugates by immunoblot (Fig. 1).
4. The parasite pellet can stick to 1.5 mL microcentrifuge tubes. Avoid pipetting up and down.
5. Carefully aspirate residual erythrocyte ghosts on top of parasite pellet using a 10 μL pipette tip.
6. Water bath sonication provides a more gentle and homogeneous cell disruption than vortexing alone; however, we understand that there may be limitations in equipment. We have also lysed samples with cycles of 5-min vortexing, 5-min ice, for a total of 30 min, and observed similar results. However, solubilizing the parasite pellet by vortexing can be challenging.
7. Freeze-thaw cycles reduce the detection of high-molecular-weight SUMO conjugates. Limit freeze-thaw cycles by using samples immediately, if possible, and aliquot samples for future blots.
8. 12.5% Gels show clear separation between free SUMO and SUMO conjugates. SUMO shows altered gel mobility on SDS-PAGE and runs slightly larger (~17 kDa) than its predicted molecular weight (11.5 kDa).
9. Boiling of SUMO samples may reduce detection of high-molecular-weight SUMO conjugates. Re-thaw previously heated samples on ice.
10. The higher binding capacity of PVDF membranes is recommended over nitrocellulose to prevent transfer of free SUMO through the membrane.
11. It is recommended to use one piece of thick blot paper per cassette. Overly tight cassettes can lead to dimples in the membrane that pool antibody, leading to large overexposed circles once developed.

12. *Pf*SUMO 7E11 monoclonal antibody was further purified on diethylaminoethanol (DEAE) anion-exchange resin, and does not react with human SUMO-1, 2/3 [4]. Antibody is available upon request.
13. While asynchronous and trophozoite samples can be easily detected with ECL Prime, we often use Femto detection for ring and schizont cultures, or for samples obtained from low parasitemia cultures.

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Chapter 20

Systematic Localization and Identification of SUMOylation Substrates in Knock-In Mice Expressing Affinity-Tagged SUMO1

Marilyn Tirard and Nils Brose

Abstract

Protein SUMOylation is a posttranslational protein modification that is emerging as a key regulatory process in neurobiology. To date, however, SUMOylation in vivo has only been studied cursorily. Knock-in mice expressing His_6-HA-SUMO1 from the *Sumo1* locus allow for the highly specific localization and identification of endogenous SUMO1 substrates under physiological and pathophysiological conditions. By making use of the HA-tag and using wild-type mice for highly stringent negative control samples, SUMO1 targets can be specifically localized in and purified from cultured mouse nerve cells and mouse tissues.

Key words SUMOylation, Knock-in mice, Affinity purification, Immunoprecipitation, HA-tag, In vivo

1 Introduction

SUMOylation is a reversible, highly dynamic posttranslational protein modification [1]. The consequences of SUMOylation depend on the target protein, and include alterations of protein localization, enzymatic activity, solubility, stability, or interactions [2–4]. In view of this broad functional relevance of SUMOylation, immense efforts have focused over the past two decades on the biochemical enrichment of SUMO targets using anti-SUMO antibodies [5], heterologous expression of tagged SUMOs [6, 7], Ubc9 fusion-dependent SUMOylation [8], or SUMO-interaction motif domains [9, 10]. Combined with mass spectrometric identification of candidate proteins, these studies have provided a huge resource of information on SUMO substrates—often including the identification of relevant modified lysine residues—and established protein SUMOylation as a crucial posttranslational protein modification that operates in every eukaryotic cell to regulate its growth, proliferation, differentiation, and function [7, 11–13].

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Unfortunately, however, the analysis of endogenous SUMOylation in complex tissues and organisms, such as mouse brain, liver, or heart, has remained challenging. This is a substantial concern, not least because growing evidence indicates an important role of SUMOylation in human diseases that can partly be modeled in genetically modified mice, particularly in neurodegenerative disorders. Consequently, several mutant mouse models have been developed to study SUMOylation in vivo [14–21]. However, most methods described so far focus on enriching SUMOylated protein species for further proteomic analysis, and only few methods are available to combine the specific localization of endogenously SUMOylated protein species with their enrichment. As a consequence, the exact subcellular distribution of endogenous SUMO targets in cells—especially in neurons—is highly debated.

To allow for the precise analysis of the localization of endogenous SUMO1 targets and their stringent enrichment, we generated His_6-HA-SUMO1 knock-in (KI) mice that express His_6-HA-SUMO1 from the endogenous *Sumo1* locus, so that overexpression artifacts can be largely excluded [15]. Additionally, these KI mice facilitate the localization and enrichment of SUMO1 substrates because anti-HA antibodies usually have higher epitope affinities than anti-SUMO1 antibodies and thus provide better signal-to-noise ratios. Further, cells or tissues from wild-type (WT) mice provide highly stringent negative controls when compared to KI material, which boosts the confidence in corresponding results. Finally, the His_6-HA-SUMO1 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 of the His_6-HA-SUMO1 KI mice over other tools that make them a very useful model system for the analysis of SUMOylation.

Our own research focus is on SUMOylation in neurons for which we used the His_6-HA-SUMO1 KI model and WT controls. We thus describe step-by-step methods to (1) enrich SUMO1 substrates from His_6-HA-SUMO1 KI mouse brain for subsequent proteomic analysis, based on an anti-HA immunopurification protocol, and to (2) study SUMO1 localization in mouse neurons and brain sections. These methods are generally applicable and can be easily adapted to other cell types and tissues. The KI mice can be obtained from us freely, based on an MTA.

2 Materials

2.1 Immunoprecipitation

Radioimmunoprecipitation assay (RIPA) buffer, 150 mM NaCl, 20 mM Tris–HCl pH 7.4 (at 4 °C), 1% (w/v) Triton X-100, 0.5% (w/v) Na-deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS). RIPA should be made fresh and cooled at 4 °C. Protease inhibitors (see below) are added shortly before lysis.

Laemmli SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, 50 mM Tris–HCl pH 6.8, 2% SDS (w/v), 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 10 mM dithiothreitol (DTT, added freshly).

Glycine elution buffer, 0.1 M glycine-HCl pH 2.

Bead storage buffer, 20 mM Tris–HCl pH 7.5 (at 4 °C), 100 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.09% (w/v) NaN_3.

Protease inhibitors: Aprotinin dissolved in water as a 500 μg/ml stock solution and used at a final concentration of 0.5 μg/ml, leupeptine dissolved in water as a 1 mg/ml stock solution and used at a final concentration of 1 μg/ml, phenylmethylsulfonyl (PMSF) dissolved in isopropanol as a 17.4 mg/ml stock solution and used at a final concentration of 17.4 μg/ml, *N*-ethylmaleimide (NEM) dissolved in DMSO as a 1 M stock solution and used at a final concentration of 20 mM.

Ultrasonic homogenizer (e.g., Bandelin Sonopuls HD2200, tapered tip KE76).

100% (w/v) methanol.

100% (w/v) chloroform.

HA peptide (custom made).

Chromatography columns (10 ml reservoir).

Peristaltic pump.

Anti-HA beads.

50 ml Falcon tubes.

1.5 ml Eppendorf tubes.

Porcelain mortar and pestle.

Liquid N_2.

Precast gels (e.g., Invitrogen 4–12% BisTris).

Eppendorf Thermomixer.

Benchtop centrifuge (e.g., Eppendorf 5416, fixed-angle rotor).

Ultracentrifuge (e.g., Beckmann Coulter Optima L-70, rotor 50.2Ti).

Shaker.

2.2 Immunostaining

1× PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.

Blocking and antibody buffer, 1× PBS, 5% (v/v) goat serum, 0.3% (w/v) Triton X-100.

Slides (ThermoScientific, SuperFrost Plus).

Mounting medium containing DAPI.

Cover slips.

Anti-HA antibody.

Secondary goat anti-mouse Alexa-Fluo 555 antibody.

3 Methods

3.1 Immunoprecipitation

The following procedure has been optimized for mouse brain but can also be used in order to enrich SUMOylated protein species from other mouse tissues such as heart or liver. Detergent extraction conditions can be altered depending on the tissue and target proteins to be recovered.

3.1.1 Preparation of Brain Lysate and Chromatography Column

1. Kill mice by cervical dislocation.
2. On ice, quickly remove brains from His_6-HA-SUMO1 KI and WT mice and remove brainstem.
3. Flash-freeze brains in liquid N_2.
4. Grind each brain to fine powder using a precooled porcelain pestle and a precooled porcelain mortar filled with liquid N_2.
5. Transfer frozen powder to a 50 ml Falcon tube.
6. Once all liquid N_2 has evaporated, add 10 ml of fresh, ice-cold RIPA buffer supplemented with protease inhibitors.
7. Triturate samples by pipetting up and down until complete dissolution of the powder.
8. Sonicate samples on ice for 15 s, 8-pulsed cycles, 75 % of power.
9. Ultracentrifuge samples (100,000 × *g*, 1 h, 4 °C). Carefully remove supernatants for further use.
10. During ultracentrifugation, sediment 0.5 ml of anti-HA beads into a plastic chromatography column and wash with 10 ml of RIPA buffer to equilibrate the beads.

3.1.2 Immunoaffinity Binding and Washing of the Column

1. Put the supernatant obtained after ultracentrifugation into a 50 ml Falcon tube and add fresh NEM to a final concentration of 20 mM. Take a small aliquot and keep on ice for later analysis (Input sample, INP).
2. For efficient depletion of SUMOylated protein species from the lysates, pump samples over the column for 12 h at a flow rate of 1 ml/min in the cold room (*see* **Note 1**).
3. After 12 h, take an aliquot of the lysate and keep on ice for later analysis (flow-through sample, FT).
4. Drain the column of lysate until the meniscus of the lysate almost reaches the column bed (do not let column run dry). Then wash the beads with 40 ml of RIPA containing fresh protease inhibitors and NEM. Once all the washing buffer has passed through the column, start with the elution.

3.1.3 Elution

1. Take 3 mg of lyophilized HA-peptide from −20 °C and let it warm up to room temperature for 30 min.
2. Add 600 µl of water to make a stock solution of 5 mg/ml. Mix well until peptides are completely dissolved.
3. Add 5.4 ml of RIPA to make elution buffer at a peptide concentration of 0.5 mg/ml. Keep at room temperature.
4. Carefully resuspend the washed beads in the column with RIPA buffer and transfer them to a fresh 2 ml Eppendorf tube. Pellet the beads by centrifuging gently (1000 × *g*, 3 min, room temperature). Carefully remove with a 1 ml pipet all buffer on top of beads.
5. Add 1.5 ml of elution buffer to each bead aliquot and shake (1400 rpm) in the Thermomixer at 30 °C.
6. Pellet the beads by centrifugation (1000 × *g*, 3 min, room temperature), transfer the first eluate to a fresh tube, and keep on ice.
7. Add another 1.5 ml of elution buffer to the beads and repeat elution as described above. A syringe with a 24 G needle can be used to remove all the eluate without taking beads.
8. Pool both eluates and centrifuge at maximum speed (23,100 × *g*, 5 min, room temperature) to remove eventual residues of beads. Again, a syringe with a 24 G needle can be used to remove all the eluate without taking beads.
9. From here onwards, eluates can be precipitated and separated on SDS-PAGE (*see* Subheading 3.1.4) or eluates can be further submitted to Ni-NTA chromatography (*see* **Note 2**).

3.1.4 Precipitation of Proteins from Eluates

All steps are performed at the bench at room temperature.

1. Prepare 300 µl aliquots of pooled eluate in 1.5 ml Eppendorf tubes.
2. Add 400 µl of methanol and vortex for 10 s.
3. Add 200 µl of chloroform and vortex for 10 s.
4. Add 400 µl of distilled water and vortex for 10 s.
5. Centrifuge (5000 × *g*, 3 min, room temperature).
6. Remove upper phase but leave interphase undisturbed as it contains proteins.
7. Add 400 µl of methanol and vortex for 10 s.
8. Centrifuge (23,100 × *g*, 5 min, room temperature).
9. Remove supernatant and leave pellet undisturbed.
10. Let the pellet dry at room temperature (~10 min).
11. Pool all pellets in a final volume of 50 µl of Laemmli SDS-PAGE sample buffer.

3.1.5 Bead Recovery

1. After elution, resuspend beads in PBS and transfer back to the column.
2. Drain the PBS and add 20 bead volumes of glycine elution buffer.
3. Immediately re-equilibrate the beads with 20 bead volumes of PBS.
4. For storage, drain equilibration buffer and add 20 bead volumes of storage buffer, close the column tightly, and keep at 4 °C.
5. Beads can be reused 3–4 times for Western blot purposes (*see* **Note 3**).

3.1.6 Analysis of Purified Proteins

1. *Western blotting*

   The efficiency of the anti-HA affinity purification is determined by Western blotting (Fig. 1). In this example, proteins containing an HA tag were efficiently bound to the matrix as revealed by a reduced anti-HA signal in the flow-through (FT) fraction as compared to the input fraction (INP). Anti-HA substrates were enriched in eluate fractions of the His_6-HA-SUMO1 sample (EL KI) but not the WT sample (EL WT). Putative SUMO1-conjugated protein candidates are validated by a Western blotting approach as well. For example, SUMOylated forms of RanGAP1 and KAP1 were enriched in eluates from KI as compared to WT (Fig. 2).

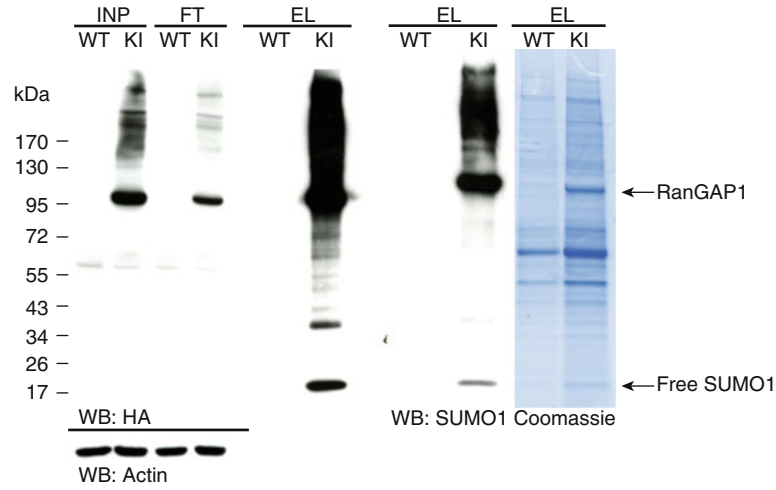

Fig. 1 Anti-HA affinity purification of HA-SUMO1 conjugates from P10 wild-type (WT) and His_6-HA-SUMO1 KI mouse brain (KI). Input (INP), flow-through (FT), and eluate fractions (EL) were analyzed by SDS-PAGE followed by either Coomassie staining (*right*) or Western blotting (*left*) using anti-HA and anti-SUMO1 antibodies

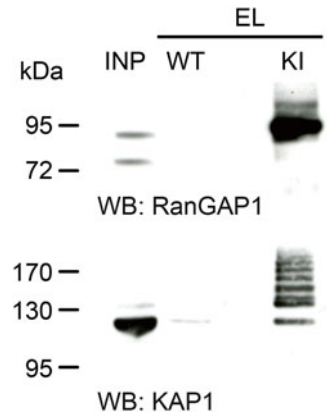

Fig. 2 Western blot analysis of the SUMO1 substrates RanGAP1 and KAP1 after anti-HA immunoaffinity purification from WT and His_6-HA-SUMO1 KI adult mice brain. Input material (from WT) and anti-HA peptide eluates of HA-immunopurified samples from WT and His_6-HA-SUMO1 KI were analyzed by Western blot using either anti-RanGAP1 (*top*) or anti-KAP1 (*bottom*) antibodies

2. *Mass Spectrometry*

   A possible follow-up procedure for proteomic analysis is described elsewhere [22]. Coomassie staining of SDS-PAGE gels loaded with HA peptide eluate fractions from WT mice reveals binding of contaminant proteins to the beads, but increased levels of protein material corresponding to enriched His_6-HA-SUMO1 targets are seen in eluate fractions from His_6-HA-SUMO1 KI mouse brain (Fig. 1).

3.2 Immunostaining

As regards the immunostaining of His_6-HA-SUMO1-conjugated proteins, we focus on the specific features of the KI mouse model. We only provide a summary of general routine techniques such as perfusion fixation of mice, neuron culture, and fixation of cultured neurons, and refer to the published literature for more details [23, 24].

3.2.1 Sample Preparation

1. *PFA fixation of mouse brain*

   His_6-HA-SUMO1 KI mice and WT littermate are first briefly anesthetized using isoflurane and then deeply anesthetized using Avertin. Mice are transcardiacally perfused with 4% cold PFA in 0.1 M PB. Brains are then post-fixed for 1 h in 4% PFA in 0.1 M PB at 4 °C and then placed in 30% sucrose in 0.1 M PB. Brains are then frozen either on dry ice or directly in the cryostat prior to cutting 30 μm thick sections (*see* **Note 4**). Sections are kept in PBS with 0.09% azide at 4 °C until further use.

2. *Primary neuron culture*

   Hippocampal or cortical neurons from His_6-HA-SUMO1 KI and WT littermates are prepared form newborn animals. Brain regions of interest (hippocampi or cortex) are carefully dissected out and digested for 45 min in a papain solution (25 units/ml) at

37 °C with gentle shaking. Papain is then inactivated by incubating the samples in stop solution containing 2.5 mg/ml bovine serum albumin, 2.5% (wt/vol) ovalbumin, and 10% (vol/vol) fetal bovine serum for 15 min at 37 °C with gentle shaking. Hippocampi or cortex pieces are then triturated in neurobasal medium complemented with B27. Neurons are then plated on poly-l-lysine-coated cover slips at a density of 13,000 cells per cm^2. After 14 days in vitro, neurons are fixed on ice for 10 min using 4% PFA in PBS with gentle shaking. Cover slips are then washed three times with PBS and kept in PBS at 4 °C until used.

3.2.2 Immuno-labeling HA

1. Incubate brain section or cover slip with neurons in 200 µl of blocking/permeabilization buffer for 1 h at room temperature with gentle shaking (*see* **Note 5**).
2. Remove blocking solution and incubate samples either overnight at 4 °C (brain sections) or for 2 h at room temperature (neurons on cover slips) with 200 µl blocking/permeabilization solution containing anti-HA primary antibody at a final dilution of 1:1000.
3. Carefully remove the primary antibody buffer and slowly add 0.5 ml of PBS. Shake gently for 10 min at room temperature.
4. Repeat the washing step described above (3) at least three times.
5. Remove washing buffer and incubate samples with 200 µl blocking/permeabilization solution containing Alexa-goat anti-mouse 555 at a final dilution of 1:2000 for 2 h (brain sections) or for 1 h (neurons on cover slips) at room temperature with gentle shaking.
6. Carefully remove the buffer with primary antibodies and add 0.5 ml of PBS. Shake gently for 10 min at room temperature.
7. Repeat the washing step described above (3) at least three times.

3.2.3 Mounting and Imaging

1. *Mounting free-floating sections*
   (a) Fill up a large glass petri dish with PBS and carefully transfer brain sections into it using a thin brush.
   (b) Submerge the glass slide below the brain section.
   (c) With a thin brush, mount and flatten the brain section on the slide.
   (d) Slowly remove the slide with the brain section from the PBS solution.
   (e) Let sample dry for a few minutes by holding the slide vertically.
   (f) Add a small drop of mounting medium to the partially dried brain section without touching.
   (g) Cover with a cover slip slowly, avoiding air bubble formation.

(h) Let dry overnight at 4 °C. Imaging can proceed on the next day.

2. *Mounting cover slips*

(a) Place a small drop of mounting medium on a slide.

(b) Carefully and slowly reverse the cover slip of stained neurons onto the drop of mounting medium, avoiding air bubble formation.

(c) Leave overnight at 4 °C to dry. Imaging can proceed on the next day.

3.2.4 *Imaging*

Image acquisition is performed as described [15]. Briefly, confocal laser-scanning microscope Leica SP2 or SP5 was used to acquire serial confocal images. Settings (gain and offset) were kept constant for a given staining and genotypes to allow for fluorescence intensity comparison. High-resolution analysis of anti-HA labeling of His_6-HA-SUMO1 brain sagittal sections (Fig. 3) or cultured neurons (Fig. 4) revealed a strong nuclear and nuclear envelope labeling of cells as compared to WT. Line scanning through cell bodies and dendrites of triple-labeled CA3 hippocampal neurons using anti-HA, anti-MAP2, and anti-Synapsin 1 antibodies showed that extra nuclear His_6-HA-SUMO1 conjugates are not localized at synapses (Fig. 3, white arrow), an observation that was further confirmed by double immunostaining of primary hippocampal neurons using anti-HA and anti-Synapsin 1 (Fig. 4, white arrow).

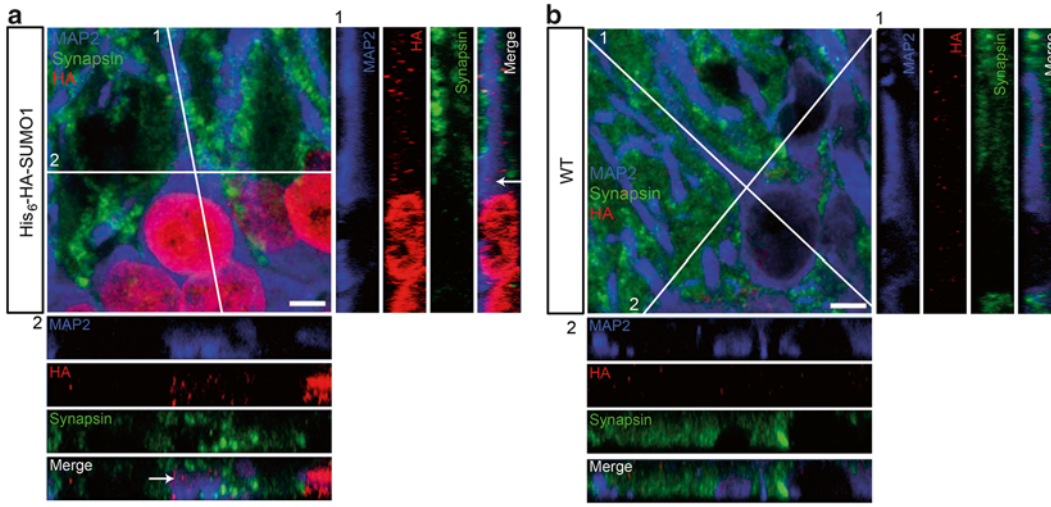

Fig. 3 Localization of His_6-HA-SUMO1 conjugates in the cytosol and the nucleus of CA3 hippocampal neurons of His_6-HA-SUMO1 mice. Sagittal brain sections from KI (**a**) and WT (**b**) mice were stained using antibodies to HA (*red*), Synapsin 1 (*green*; presynaptic terminals), and MAP2 (*blue*; neuronal dendrites). The *white line* shows the orientation of the scan used to generate the image stacks shown in side view on the *right* and *bottom*. Scale bar, 10 μm. The white arrows indicate that extra nuclear His_6 -HA-SUMO1 conjugates are not localized at synapses

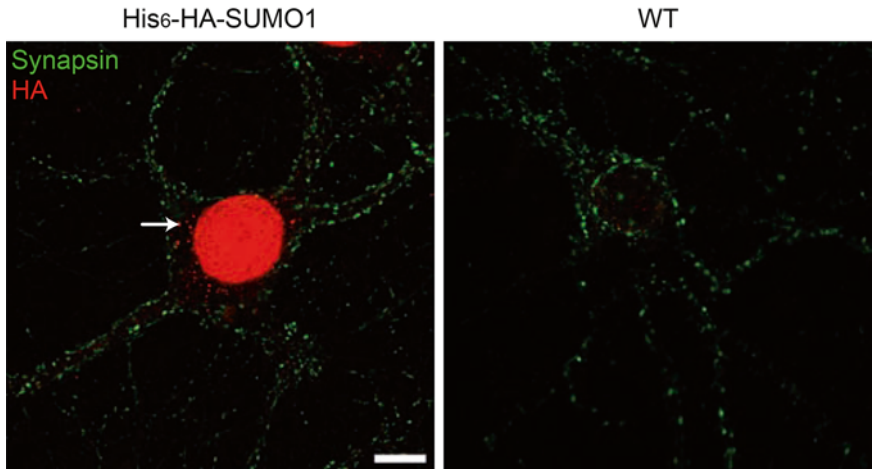

Fig. 4 Localization of His_6-HA-SUMO1 conjugates in the cytosol and nucleus of His_6-HA-SUMO1 KI neurons. Images of KI (*left*) and WT control (*right panel*) primary hippocampal neurons are shown. Neurons were stained using antibodies to HA (*red*) and Synapsin 1 (*green*; presynaptic terminals). Scale bar, 10 μm. The white arrow indicates that extra nuclear His_6 -HA-SUMO1 conjugates are not localized at synapses

4 Notes

1. For higher enrichment of His_6-HA-SUMO1 targets, it is recommended to use a chromatography-based procedure instead of a batch adsorption protocol.
2. A two-step purification (nickel-nitrilotriacetic acid (Ni-NTA) combined with anti-HA affinity purification) successfully enriches His_6-HA-SUMO1 substrates for Western blot analysis as compared to WT but does not yield enough material for routine proteomics analysis, independently of whether the Ni-NTA chromatography is performed before or after anti-HA affinity chromatography. The reason for this is the loss of proteins when changing between biological and denaturing buffers. Additionally, single Ni-NTA chromatography to enrich His_6-HA-SUMO1 substrates from His_6-HA-SUMO1 KI and WT leads to major nonspecific binding [16]. Therefore, we recommend performing the Ni-NTA chromatography as a second purification step. A detailed description of our Ni-NTA chromatography protocol from mouse brain is described elsewhere [25].
3. When a mass spectrometric comparative analysis of WT and His_6-HA-SUMO1 KI material is planned, a fresh batch of beads should be used.
4. Isopentane freezing of mouse brains did not result in proper staining of RanGAP1 at the nuclear pore complex.
5. We do not recommend using digitonine for the permeabilization of neurons, as in our hands it does not prevent the strong labeling of the nuclear envelope and leads to poor labeling of neuronal synapses with antibodies to synaptic markers.

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SUMO Methods and Protocols

Manuel S. Rodriguez

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In the copyright page (p. iv) of the Front Matter, the sentence

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