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SUMO proteomics to decipher the SUMO-modified proteome regulated by various diseases

Wei Yang and Wulf Paschen*

Molecular Neurobiology Laboratory, Department of Anesthesiology, Duke University Medical Center, Durham, NC, USA

Abstract

Small ubiquitin-like modifier (SUMO1–3) conjugation is a posttranslational protein modification whereby SUMOs are conjugated to lysine residues of target proteins. SUMO conjugation can alter the activity, stability, and function of target proteins, and thereby modulate almost all major cellular pathways. Many diseases are associated with SUMO conjugation, including heart failure, arthritis, cancer, degenerative diseases, and brain ischemia/stroke. It is, therefore, of major interest to characterize the SUMO-modified proteome regulated by these disorders. SUMO proteomics analysis is hampered by low levels of SUMOylated proteins. Several strategies have, therefore, been developed to enrich SUMOylated proteins from cell/tissue extracts. These include proteomics analysis on cells expressing epitope-tagged SUMO isoforms, use of monoclonal SUMO antibodies for immunoprecipitation and epitope-specific peptides for elution, and affinity purification with peptides containing SUMO interaction motifs to specifically enrich polySUMOylated proteins. Recently, two mouse models were generated and characterized that express tagged SUMO isoforms, and allow purification of SUMOylated proteins from complex organ extracts. Ultimately, these new analytical tools will help to decipher the SUMO-modified proteome regulated by various human diseases, and thereby, identify new targets for preventive and therapeutic purposes.

Keywords

Biomarker; Biomedicine; Disorders; SUMO; Therapeutic target

1 Introduction

The stability, activity, and functions of proteins are regulated in a complex way by PTMs, including phosphorylation, ubiquitylation, and modification by ubiquitin-like modifiers. Posttranslational protein modifications enable cells to rapidly adjust to changes in environmental conditions, and thereby to better withstand various kinds of stresses that would otherwise harm cellular functions. Ubiquitin and ubiquitin-like modifiers are

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Correspondence: Professor Wei Yang, Multidisciplinary Neuroprotection Laboratories, Department of Anesthesiology, Duke University Medical Center, 130 Sands Building, Research Drive, PO Box 3094, Durham, NC 27710, USA, wei.yang@duke.edu, Fax: +1-919-684-6692. *Additional corresponding author: Professor Wulf Paschen, wulf.paschen@duke.edu.

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predominantly conjugated to lysine residues of target proteins [1]. Ubiquitin-like modifiers include interferon-stimulated gene 15 (ISG15), neuronal precursor cell expressed developmental downregulated 8 (NEDD8), ubiquitin-fold modifier 1 (UFM1), Fub1 ubiquitin-like protein (FUB1), and the group of small ubiquitin-like modifiers (SUMO1–3). While ubiquitin was identified and sequenced in 1975 [2, 3], more than 20 years passed before SUMO1 was discovered and characterized. SUMO1 is a ubiquitin-like protein that plays a pivotal role in targeting the Ras-related nuclear protein GTPase-activating protein (RanGAP1) to the nuclear pore complex [4, 5]. Characterization of SUMO2 and SUMO3 followed soon thereafter [6–8].

Similar to the ubiquitylation pathway, the SUMO conjugation (SUMOylation) pathway requires activating (E1), conjugating (E2), and ligating (E3) enzymes to conjugate SUMOs to lysine residues of target proteins (Fig. 1). However, SUMOylation and ubiquitylation are distinct with respect to the number of E1, E2, and E3 enzymes expressed in cells, implying different levels of specificity. While several E2 and more than 600 E3 enzymes regulate ubiquitin conjugation, only one heterodimer E1 enzyme (SAE1/SAE2), one E2 enzyme (ubiquitin conjugating enzyme 9 (Ubc9)), and a few E3 enzymes regulate SUMO conjugation of the many target proteins identified so far. We do not yet know how SUMOylation specificity is achieved, considering the small number of SUMOylation enzymes compared to ubiquitylation enzymes.

SUMOylation facilitates formation of protein complexes by physical interaction of SUMO-conjugated proteins with SUMO-interacting motifs (SIMs) in target proteins [9–11]. SUMOylation plays key roles in nuclear processes involving large protein complexes such as DNA damage repair [12]. It has been proposed that in such large protein complexes, individual interactions of SUMOylated proteins with SIMs in target proteins may be redundant, and that specificity is achieved by preassembled protein groups interacting with the SUMOylation machinery [13].

The SUMOylation pathway is primarily controlled by the activities of Ubc9 and SUMO-specific proteases (SENPs; Fig. 1). Indeed, global SUMOylation is markedly increased in Ubc9 transgenic mice and SENP1 mutant embryos [14, 15]. In mammals, six SENPs have been identified—SENP1–3 and SENP5–7. SENPs have two distinct functions (Fig. 1): to process SUMO precursors via C-terminal cleavage to expose the GG motif required for conjugation (maturation step, endopeptidase activity), and to remove SUMO from lysine residues of target proteins (de-conjugation, isopeptidase activity). Recently, new classes of SUMO proteases have been identified [16, 17]. DeSUMOylating isopeptidase 1 (DeSI-1) recognizes different sets of substrates than SENPs [16], while ubiquitin-specific protease-like 1 (USPL1) is a SUMO isopeptidase with functions in Cajal body biology [17].

SUMO2 and SUMO3 are highly homologous as they differ in only three amino acids. They are distinct from SUMO1, however, sharing only about 50% homology. SUMO2 and SUMO3 are often referred to as SUMO2/3, because they cannot be distinguished by available antibodies. It is yet not clear whether SUMO1, SUMO2, and SUMO3 are functionally distinct.

Quantitative proteomics analysis on stably transfected cells expressing His₆-SUMO1 or His₆-SUMO2 revealed both distinct and overlapping sets of SUMO1 and SUMO2 target proteins, suggesting redundant and nonredundant functions [18]. Results from studies on SUMO mutant mice indicate high redundancy of SUMOylation by SUMO1–3, despite preferential conjugation. Mouse embryos deficient in *Ubc9* die at the early postimplantation stage, supporting the pivotal role of SUMOylation in embryogenesis [19]. On the other hand, *Sumo1*^{−/−} mice are viable, and SUMO1 functions can be compensated for by SUMO2 and SUMO3 [20, 21]. Thus, the interesting question is whether SUMO2/3 functions can be compensated for by SUMO1. Surprisingly, however, *Sumo2* deficiency is lethal to embryos, while *Sumo3*^{−/−} mice are viable, and do not show any overt phenotype [22]. A plausible explanation for these unexpected findings is that SUMO1–3 isoforms are functionally redundant, and that SUMOs are preferentially conjugated to distinct protein targets controlled by their availability and expression levels. For example, the nuclear pore complex protein RanGAP1 can be modified by both SUMO1 and SUMO2 in vitro. SUMO1 is, however, preferentially conjugated to RanGAP1 in vivo, although expression levels of SUMO1 and SUMO2 account to about 15 and 70% of total SUMO1–3 expression, respectively [22]. In *Sumo1* null mutant mice, SUMO2/3 is conjugated to RanGAP1 [20].

SUMO conjugation has distinct unpredictable functional consequences for target proteins. SUMOylation and other PTMs compete for some target proteins. Examples are the transcriptional activator myocyte-specific enhancer factor 2A (MEF2A) and the nuclear factor κ B (NF- κ B) regulatory inhibitor- α (I κ B- α). MEF2A is a transcription factor highly expressed in brains and is involved in synapse formation. MEF2A activity is controlled by a dephosphorylation-dependent switch from SUMOylation to acetylation at lysine K403 [23]. I κ B- α is an example of competition between SUMOylation and ubiquitin conjugation at lysine K21 [24]. SUMOylation of I κ B- α increases its stability resulting in inhibition of NF- κ B activation [24].

A prominent feature of SUMOylation is facilitation of protein–protein interactions. This has been studied in detail, particularly as it relates to the DNA double-strand break repair system [25]. Indeed, the SUMOylation machinery is a key component of the DNA double-strand repair process [26, 27]. DNA damage activates a wave of SUMOylation of several repair proteins at multiple sites. It has been proposed that interaction between SUMOylated proteins and partner proteins containing SIMs functions as glue that potentiates physical interactions, thereby accelerating the repair process [25].

SUMO2 and SUMO3 have an internal SUMOylation site at K11, and can, therefore, form polySUMO chains [28]. The internal SUMOylation site is missing for SUMO1. SUMO1 can still be conjugated to SUMO2 or SUMO3, but this process terminates further chain growth [29]. SUMO2/3 chains accumulate in cells exposed to the proteasome inhibitor MG132, and this accumulation is suppressed by blocking protein synthesis, suggesting a role in protein quality control [30]. PolySUMO-modified proteins serve as a substrate for SUMO-targeted ubiquitin ligases (STUbLs), linking the SUMOylation and ubiquitylation pathways [31, 32]. STUbLs play a prominent role in genome stability [31], and SUMOylation-dependent ubiquitin conjugation is massively activated after brain ischemia, and in cells exposed to ischemia-like conditions [33, 34]. Notably, activation of ubiquitin conjugation induced by

ischemia-like conditions is almost completely suppressed in cells in which expression of both SUMO2 and SUMO3 is silenced [34]. These observations point to a prominent role for SUMO2/3-conjugation-dependent ubiquitin conjugation in transient ischemia.

2 SUMOylation and disease

The SUMOylation pathway contributes to many cellular processes that are essential for cell functions. These include gene expression and genome stability, DNA damage repair, RNA processing, and quality control of newly synthesized proteins. It is, therefore, not surprising that SUMO conjugation plays key roles in many human diseases such as cancer, heart disease, degenerative diseases, and brain ischemia/stroke. Thus, characterization of the SUMO-modified proteome regulated by these disorders is of tremendous clinical interest because it will identify novel targets that may lead to prevention and treatment.

2.1 Cancer

The potential importance of the SUMO conjugation pathway as a target for treating tumors has been appreciated [12, 35–41]. Notably, many groups have reported higher expression levels of components of the SUMO conjugation pathway, and data suggest that activated SUMOylation supports tumor growth. For example, the SUMO-activating enzyme SAE1/SAE2 is required for myelocytomatosis oncogene (Myc) dependent tumors in mice, and low SAE1/SAE2 levels correlate with longer metastasis-free survival of patients with Myc-dependent breast cancers [42]. Further, Ubc9 expression levels are high in lung cancer, primary colon and prostate cancer, and astrocytic brain tumors [43–45]. Importantly, in astrocytic brain tumors, Ubc9 levels and SUMO1- and SUMO2/3-conjugated protein levels are high in glioblastoma multiforme brain tumors that carry a very poor prognosis [45]. Furthermore, SUMO ligase PIAS1 (protein inhibitor of STAT-1) levels are high in prostate cancer, and support cell proliferation [46]. On the other hand, several groups have reported an association between SENP-induced de-SUMOylation and cancer growth [47–51]. Researchers have concluded that SENP overexpression may disrupt SUMO homeostasis, and thereby promotes cancer development and progression [52]. Notably, genetic variations of the recently identified SUMO protease USPL1 is associated with risk of breast tumors [53].

2.2 Heart disease

Results from experimental studies suggest that the SUMOylation pathway must be in balance for the heart to function properly. Overexpression of SENP2, and expression of a SUMOylation-deficient mutant of the cardiac-specific homeobox protein (Nkx2.5) lead to congenital heart defects and cardiac dysfunctions in mice [54, 55]. The regulatory effect of SUMOylation is lost in lamin A mutations that are associated with heart failure and familial cardiomyopathies [56,57]. Furthermore, SUMO1 conjugation of the sarcoplasmic/ER Ca^{2+} ATPase 2a (SERCA2a) controls its activity and stability, and is critically important for heart function [58]. SUMOylated SERCA2a levels are markedly reduced in failing hearts. This pathological process is reversed in mice with heart failure, by virus-mediated SUMO1 gene delivery [58]. Together, these observations demonstrate the critical role of SUMOylation in normal heart functioning.

2.3 Degenerative diseases

The potential role for the SUMOylation pathway in the pathological processes associated with degenerative diseases has been reported [59–63]. Notably, many of the proteins that play key roles in neurodegenerative disorders are targets for SUMO conjugation. These include huntingtin with expanded polyglutamin repeat associated with Huntington's disease [64]; tau, DJ1, and α -synuclein, associated with Parkinson's disease [65, 66]; superoxide dismutase 1 (SOD1), associated with amyotrophic lateral sclerosis [67]; and ataxin-1, a polyglutamin repeat protein, associated with spinocerebellar ataxia type 1 [68]. The contribution of the SUMOylation pathway in degenerative disorders is still not fully understood, and may vary in different diseases. A recent study showed that SUMOylation-deficient α -synuclein has a strong propensity for aggregation, both in cultured cells and in substantia nigra neurons in vivo [69]. Importantly, α -synuclein aggregation in vitro is significantly delayed when only a small fraction is SUMOylated [69]. Together, these findings confirm a role for SUMOylation in degenerative diseases, and highlight the need for proteomics analysis to compare the SUMO-modified proteome in the physiological and disease states.

2.4 Brain ischemia/stroke

In the brain, only a small fraction of SUMO2/3 is conjugated to target proteins. Transient cerebral ischemia induces a dramatic increase in levels and nuclear accumulation of SUMO2/3-conjugated proteins [70, 71]. Results from many in vitro and in vivo studies support the notion that the postischemic activation of SUMO conjugation is a protective stress response that shields neurons from damage triggered by transient ischemia [14, 72–74]. It is, therefore, of major clinical interest to characterize the SUMO-modified proteome regulated by transient cerebral ischemia, and thereby identify the putative protective proteins/pathways associated with SUMO conjugation in postischemic brains. Notably, cerebral ischemia is the only pathological state of significant clinical interest that has been investigated using SUMO proteomics analysis to identify novel potential targets for preventive and therapeutic purposes [33, 34]. Indeed, the glucocorticoid receptor (GR) was identified as a key SUMO target in postischemic brains, and postischemic GR SUMOylation is associated with nuclear accumulation of this transcription factor [33]. GR activation contributes to ischemia-induced cell death [75], and SUMOylation results in suppression of GR activity [76]. Together, these observations suggest that GR SUMOylation is a protective stress response, shielding neurons from ischemia-induced damage, and they underscore the tremendous potential of SUMO proteomics analysis.

A transient episode of brain ischemia is associated with many pediatric and adult cardiovascular surgeries involving the use of cardiopulmonary bypass. Moderate to deep hypothermia is, therefore, used to protect organs from ischemia-induced damage. Moderate to deep hypothermia results in a massive increase in levels and nuclear accumulation of SUMO2/3-conjugated proteins in neurons, which is thought to be a mechanism underlying hypothermia-induced protection [77, 78]. Identifying the proteins that are SUMO-conjugated during hypothermia could help to design new therapeutic strategies and screen for small molecules to activate this process [79], and thereby avoid the adverse effects associated with hypothermia.

3 SUMO proteomics analysis

SUMO proteomics analyses have been carried out on extracts from plants [80–83], yeast [9, 84–86], the pathogens *Trypanosoma cruzi* [87], *Taxoplasma gondii* [88], and *Candida albicans* [89], *Drosophila* and *Caenorhabditis elegans* [90,91], established cell lines including B35, HEK293, K562, and HeLa [18, 30, 34, 92–105], and mouse brains [33, 106]. SUMO proteomics analysis is challenging because SUMOylated protein levels are low. Several approaches have been reported to enrich SUMOylated proteins for proteomics analysis, as will be discussed below. A combination of different approaches may be required to improve the outcome of the analysis, depending on the final goal of the study.

Two basically different strategies have been reported to enrich SUMOylated proteins from cell/tissue extracts for MS analysis (Fig. 2), purification of endogenous SUMO-conjugated proteins (Fig. 2A and B), and purification of exogenously expressed SUMOs using genetically modified cells expressing tagged SUMO isoforms (Fig. 2C and D). Purification of endogenous polySUMOylated proteins was reported by Ronald Hay and his group [107]. This purification approach takes advantage of the SIMs present on the RING-finger 4 ubiquitin ligase (RNF4), which recognize and bind polySUMO chains [32]. Using this strategy, more than 300 putative polySUMOylated proteins were identified in heat-stressed cells [107]. Recently, another approach was described to purify endogenous SUMOylated proteins using monoclonal antibodies for immunoprecipitation and epitope-specific peptides for elution of SUMO1- or SUMO2/3-conjugated proteins [108]. Using this approach and SUMO1- and SUMO2/3-specific antibodies, the authors performed proteomics analysis to compare, for the first time, the endogenous SUMO1- and SUMO2/3-modified proteome in mammalian cells. The antibody approach was also used to confirm SUMOylated proteins in liver extracts [108]. However, whether these two strategies can be used for purification and identification of SUMOylated proteins by proteomics analysis on tissue extracts must still be verified.

The most frequently used strategy for purification and identification of SUMOylated proteins by proteomics analysis involves genetically modified cells/organisms expressing tagged SUMO isoforms. In the first study designed to identify SUMO2-conjugated proteins, a stable cell line expressing His₆-SUMO2 was established and SUMO2-conjugated proteins were pulled down with Ni²⁺-NTA beads [94]. SUMOylated proteins were then eluted with imidazole buffer, and purified proteins were identified by MS analysis. Later, many SUMO proteomics studies used genetically modified organisms/cells expressing SUMO isoforms with several tags, allowing two-step affinity purification to further increase specificity [9, 30, 85, 87, 90, 92, 93, 95].

In many instances, SUMO proteomics requires a stringent analysis to verify that proteins identified by MS are indeed authentic SUMO substrates, and were SUMOylated under the experimental conditions studied. This analysis can be performed at a low or high level of confidence. A low level of confidence is achieved by incubating the protein of interest in a test tube with E1, E2, SUMO, and ATP, and using Western blot analysis to verify that slower migrating bands are present, indicating SUMOylated forms of the protein of interest [98], or by expressing E1, E2, SUMO, and the protein of interest in *Escherichia coli* [81]. A

more direct way of verification with a higher level of confidence is by Western blot analysis using antibody specific for the protein of interest, and immunoprecipitation samples [106]. To increase specificity, eluates are exposed to SUMO protease to de-SUMOylate the target proteins resulting in disappearance of the slower migrating bands on Western blots and appearance of the nonconjugated protein [33, 34]. The likelihood of identification of authentic SUMO target proteins is much higher when a quantitative approach is used, e.g. the SILAC technique [18, 30, 34, 93, 95, 97, 98, 102, 104], or when the approach used allows identification of SUMOylation sites (see below).

To conduct functional studies of SUMO target proteins, it is of great interest to characterize SUMOylation sites in order to mutate those sites and determine how SUMO conjugation modulates the function of the protein under investigation. In the ubiquitin field, a new MS-based strategy has recently been described to identify endogenous ubiquitylated proteins and ubiquitylation sites of target proteins [109–111]. This approach has been successfully used to characterize the ubiquitin-modified proteome under various experimental conditions [112–117]. The approach takes advantage of the fact that after trypsin digestion of ubiquitylated proteins, the C-terminal two glycine residues of ubiquitin conjugated to lysine residues in target proteins are left intact. This K- ϵ -GG remnant motif is recognized by specific antibodies used for immunoprecipitation, resulting in highly specific enrichment of ubiquitylated peptides. The K- ϵ -GG remnant causes a mass shift of 114.04 Da, enabling precise localization of ubiquitylation sites. However, this strategy is not suitable for precise localization of SUMOylation sites of target proteins, because large branched side-chain remnants of SUMOs conjugated to lysine of target proteins are generated upon protease digestion. This generates complex MS spectra that are difficult to interpret using standard database searching methods. To overcome these limitations, several approaches have been described including strategies to help interpret complex MS spectra [103, 118, 119], modified protease digestion protocols [120–122], and to mutate SUMO's C-terminal amino acids so that protease treatment generates a short SUMO remnant on lysine of target proteins [80,99,101,103,105,123,124]. Notably, more than 1000 SUMOylated lysine residues were identified using the latter strategy with cells expressing SUMO2^{T90K} and Lys-C cleavage that generates K- ϵ -GG remnants on SUMOylated, but not on ubiquitylated, target proteins [105].

Most of the reported SUMO proteomics studies have been performed on plants, small organisms, or mammalian cell cultures. Several studies focused on characterizing the SUMO-modified proteome regulated by heat shock, oxidative stress, or DNA damage [80–83, 89, 93, 95, 99, 100]. Others have characterized the SUMO-modified proteome degraded via the ubiquitin proteasome system, by performing SUMO proteomics analysis on cells exposed to the proteasome inhibitor MG132 [30, 92, 98, 102]. Notably, these studies identified an extensive crosstalk between the SUMOylation and ubiquitylation pathways in cells exposed to MG132 [102], and provided strong evidence that SUMOylation-activated ubiquitin conjugation plays a key role in the quality control of newly synthesized proteins [30]. This crosstalk also plays a key role in maintaining genome stability [31, 125], and is activated in cells exposed to ischemia-like conditions [34].

As discussed above, characterization of the SUMO-modified proteome regulated by human diseases is crucial to the development of novel preventive and therapeutic strategies. Identifying SUMOylated proteins in tissue samples by proteomics analysis poses tremendous challenges, and thus, it is not surprising that the use of SUMO proteomics to uncover the disease-regulated SUMO-modified proteome is still in its infancy.

Recently, a His₆-HA-SUMO1 knock-in mouse model was generated and characterized [106]. Notably, this was the first study to characterize the SUMO1-modified proteome in brains. Yang and his colleagues conducted the only study to date that has characterized the SUMO-modified proteome regulated by a disease [33]. This study of transient cerebral ischemia was based on a novel SUMO transgenic mouse model with expression of His₆-SUMO1, HA-SUMO2, and FLAG-SUMO3 specifically in the forebrain. Notably, all three exogenously expressed SUMO isoforms were functionally active, and responded to transient brain ischemia by nuclear accumulation, as do endogenous SUMOs. Proteomics analyses on nuclear fractions isolated from wild-type animals and SUMO transgenic animals subjected to sham and transient forebrain ischemia surgery revealed the postischemic SUMO3-modified proteome [33]. Importantly, several processes and pathways with putative neuroprotective functions were identified. These include GR signaling, SUMOylation-dependent ubiquitinylation, and RNA processing [33]. Thus, SUMO transgenic mouse models are promising new tools for future studies to decipher the SUMO-modified proteome regulated by human diseases for which mouse models are established.

Today, three well established strategies allow SUMO proteomics analysis on samples from organs/tissues in pathological states—affinity purification with SIM peptides (Fig. 2B) [107], immunoprecipitation with SUMO monoclonal antibodies (Fig. 2A) [108], and use of transgenic mice expressing tagged SUMO isoforms (Fig. 2C) [33, 106]. Using SIM peptides or monoclonal antibodies for immunoprecipitation allows for the enrichment of endogenous polySUMOylated or SUMOylated proteins, respectively. However, these strategies have not yet been verified for proteomics analysis on tissue extracts, and the SIM peptide approach will enrich only proteins with polySUMO chains, but not monoSUMOylated proteins.

On the first glance, the antibody approach seems clearly superior to the SUMO transgenic mouse model approach, because only the antibody approach allows purification and identification of endogenous SUMOylated proteins. In addition, the SUMO1 and SUMO2/3 antibodies can be produced in large quantities at relatively low costs. Finally, only this strategy is suitable to analyze samples derived from patients suffering from various diseases. The suitability of SUMO proteomics to analyze postmortem specimens has not yet been verified. Notably, the postmortem delay can be considered a period of global ischemia that may change the SUMOylation status. Indeed, a short period of only 10 min of brain ischemia is sufficient to induce a massive de-SUMOylation of SUMO1- and SUMO2/3-conjugated proteins [33]. This suggests that even a short postmortem delay is expected to result in a marked change in the SUMOylation status. Therefore, a safe way to avoid de-SUMOylation is to use only surgical specimens snap-frozen after excision, as has been done for analysis of human brain tumor samples [45].

An important weakness of the antibody approach is that it does not allow the SUMO2-modified proteome to be distinguished from the SUMO3-modified proteome, as SUMO2 and SUMO3 are highly homologous and can, therefore, not be separated using available antibodies. The high homology suggests that both isoforms are functionally identical, but this has not yet been confirmed [126, 127].

The use of SUMO transgenic animals for proteomics studies is new. SUMO proteomics on cell culture samples is very challenging due to the low levels of SUMOylated proteins, and advancing from cell cultures to tissue samples adds a level of complexity that requires modified protocols. Tissue cells are embedded in a network of extracellular matrix proteins and fibers, and cellular proteins account for only a fraction of total proteins. However, the SUMO transgenic mice generated so far and the protocols developed for enriching SUMOylated proteins from tissue samples impressively illustrate the potential of this approach for future studies [33,106]. It is, therefore, foreseeable that in the near future, additional SUMO transgenic mouse models will be developed. For example, transgenic mice expressing mutant SUMOs so that Lys-C digestion generates K- ϵ -GG remnants on SUMOylated target proteins, as has been successfully done in cultured cells [101,103,105], would be useful to identify SUMOylation sites from tissue samples using the anti- K- ϵ -GG approach.

A major concern when performing SUMO proteomics on genetically modified organisms is that the tagged SUMO-modified proteome may not be representative of the proteome modified by endogenous SUMOs. The Brose group used a knock-in strategy, with endogenous SUMO1 replaced by His₆-HA-SUMO1 so that expression of tagged SUMO1 is driven by the endogenous SUMO1 promoter, thus avoiding overexpression artifacts [106]. The novel SUMO transgenic mouse model with expression of His₆-SUMO1, HA-SUMO2, and FLAG-SUMO3 was, therefore, rigorously tested and characterized under control and stress conditions to analyze potential overexpression artifacts [33]. Notably, exogenously expressed SUMOs were functionally active, and did not disturb the endogenous SUMOylation machinery. Most importantly, however, stressing these animals by a transient cerebral ischemia induced the same increase in levels and nuclear accumulation of exogenous HA-SUMO2- and endogenous SUMO2/3-conjugated proteins in brains of SUMO1–3 transgenic mice [33]. These results convincingly demonstrate that SUMO transgenic animals are useful tools for SUMO proteomics to decipher the SUMO-modified proteome associated with human diseases.

SUMO transgenic mice require considerable time and effort to generate and maintain. This is particularly true for generating conditional SUMO transgenic mice [33]. However, once generated, these animals are tremendously useful for SUMO proteomics studies on any tissue/cell type for which Cre mice are available. For example, the conditional SUMO transgenic mice used for the brain ischemia proteomics study are double transgenic mice with expression of tagged SUMO1–3 in most cells of the forebrain using Emx1-Cre mice [33]. Brain ischemia is a pathological state that impairs the functions of neuronal, glial, and endothelial cells [128–130]. Therefore, mice with cell type specific expression of tagged SUMO isoforms make it possible to characterize the cell-specific pattern of the SUMO-modified proteome regulated by transient cerebral ischemia in future studies.

The design of novel SUMO transgenic mouse models expressing tagged SUMO isoforms requires careful considerations to determine which of the available tags is most suitable for the planned proteomics analysis studies. For SUMO proteomics on cells exogenously expressing tagged SUMO isoforms, His₆, HA, and FLAG are the most frequently and successfully used tags. The situation is, however, different for proteomics analyses on tissue extracts from mice expressing tagged SUMO isoforms. Importantly, the HA and FLAG tags are suitable for enriching SUMOylated proteins from tissue extracts [33, 106]. The His₆ tag, in contrast, is less suitable for enrichment of SUMO-conjugated proteins from tissue extracts because of unspecific coprecipitation of proteins containing stretches of histidine residues. This is particularly true for enrichment of His₆-tagged SUMOs from brain extracts using Ni²⁺-NTA chromatography [131]. Therefore, the His₆ tag is not recommended for future studies to generate transgenic mice expressing tagged SUMO isoforms.

The recently developed and comprehensively characterized mouse models expressing tagged SUMO isoforms have successfully been used, for the first time, to characterize the SUMO-modified proteome in control and postischemic brains by proteomics analysis [33, 106]. These new models are, therefore, valuable experimental tools to advance SUMO research from cell cultures to animal models of human diseases. Ultimately, the novel mouse models will help to characterize the SUMO-modified proteome regulated by the diseases under investigation and thereby identify new targets for disease prevention and therapeutic intervention.

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Abbreviations

GR	glucocorticoid receptor
IκB-α	NF-κB regulatory inhibitor-alpha
MEF2A	myocyte-specific enhancer factor 2A
Myc	myelocytomatosis oncogene
NF-κB	nuclear factor kappa B
Nkx2.5	cardiac-specific homeobox protein
PIAS1	protein inhibitor of STAT 1 (SUMO ligase)
RanGAP1	Ras-related nuclear protein GTPase-activating protein
RNF4	RING-finger 4 ubiquitin ligase
SAE1/SAE2	heterodimer SUMO-activating enzyme (E1)
SENp	SUMO-specific protease

SERCA2a	sarcoplasmic/ER Ca ²⁺ ATPase-2a
SIM	SUMO-interacting motif
SOD1	superoxide dismutase 1
STubL	SUMO-targeted ubiquitin ligase
SUMO	small ubiquitin-like modifier
Ubc9	ubiquitin conjugating enzyme 9

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Figure 1.

Scheme of the SUMO conjugation pathway. SUMOs are synthesized as precursors from which a small C-terminal peptide is cleaved by SUMO proteases (endopeptidase activity; red arrow) to expose the diglycine (GG) motif required for conjugation (maturation step). SUMO conjugation requires three consecutive enzymatic reactions catalyzed by the activating enzyme SAE1/SAE2 (E1), conjugating enzyme Ubc9 (E2), and ligating enzymes (E3), resulting in covalent conjugation to lysine (K) residues in target proteins. SUMOs are removed from lysine residues of target proteins by SUMO proteases (deconjugation; isopeptidase activity; red arrow).

Figure 2. Scheme of strategies to enrich SUMOylated proteins from cell/tissue extracts for MS analysis. (A, B) Strategies allowing purification of endogenous SUMO-conjugated proteins. (A) SUMOylated proteins are immunoprecipitated by SUMO monoclonal antibodies and protein A/G beads. Elution is achieved using epitope-specific peptides. (B) PolySUMOylated proteins are affinity purified with peptides containing the SUMO-interacting motifs (SIMs) present on the RING-finger 4 ubiquitin ligase (tandem SIM). (C) Strategy for purification of exogenously expressed epitope-tagged SUMO isoforms. (D) Strategy for purification of exogenously expressed SUMO isoforms with C-terminal mutations that enable protease treatment to generate a short SUMO remnant on lysine (red K) of target proteins. Immunoprecipitation is then achieved with anti-K- ϵ -GG antibody.