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A universal strategy for proteomic studies of SUMO and other ubiquitin-like modifiers

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SUMMARY

Post-translational modification by the conjugation of small ubiquitin-like modifiers is an essential mechanism to affect protein function. Currently, only a limited number of substrates are known for most of these modifiers, thus limiting our knowledge of their role and relevance for cellular physiology. Here, we report the development of a universal strategy for proteomic studies of ubiquitin-like modifiers. This strategy involves the development of stable transfected cell lines expressing a double-tagged modifier under the control of a tightly negatively regulated promoter, the induction of the expression and conjugation of the tagged modifier to cellular proteins, the tandem affinity purification of the pool of proteins covalently modified by the tagged-modifier, and the identification of the modified proteins by liquid chromatography and mass spectrometry. By applying this methodology to the proteomic analysis of SUMO-1 and SUMO-3 we determined that SUMO-1 and SUMO-3 are stable proteins exhibiting half-lives of over 20 h, demonstrated that sumoylation with both SUMO-1 and SUMO-3 is greatly stimulated by MG-132 and heat shock treatment, demonstrated the preferential usage of either SUMO-1 or SUMO-3 for some known SUMO substrates, and identified 122 putative SUMO substrates of which only 27 appeared to be modified by both SUMO-1 and SUMO-3. This limited overlapping in the subset of proteins modified by SUMO-1 and SUMO-3 supports that the SUMO paralogues are likely to be functionally distinct. Three of the novel putative SUMO substrates identified, namely the polypyrimidine tract-binding protein-associated splicing factor PSF, the structural microtubular component alpha-Tubulin, and the GTP-binding nuclear protein Ran, were confirmed as authentic SUMO substrates. The application of this universal strategy to the identification of the pool of cellular substrates modified by other ubiquitin-like modifiers will dramatically increase our knowledge of the biological role of the different ubiquitin-like conjugations systems in the cell.

INTRODUCTION

The post-translational modification of proteins provides the cell with the ability to mount a rapid response to external changes and stimuli. The best-characterized types of post-translational modifications have been those involving the conjugation of small chemical groups to the target protein, such as phosphorylation and acetylation. However, during the last few years the post-translational modification of proteins by the covalent conjugation of small proteins has gained relevance as a very important mechanism to affect protein function. This is best exemplified by the conjugation of poly-ubiquitin chains to a target protein, leading to the proteasomal degradation of the modified protein. Currently there are

11 known small protein modifiers namely ubiquitin, ISG15, AUT7, APG12, NEDD8, the SUMO proteins (SUMO-1, -2, & -3), HUB1, FAT10, URM1, MNSF, and Ufm1, all of which are related to the prototypical member (ubiquitin) and are therefore considered to be ubiquitin-like proteins (1, 2). Conjugation with these modifiers exerts a wide variety of effects on the target protein, including changes in protein conformation, activity, protein-protein interactions, and cellular localization. This diversity of effects is associated with the large and chemically varied surface provided by these modifiers.

The best-characterized ubiquitin-like modifiers are ubiquitin itself and the SUMO proteins. SUMO was independently discovered by three groups during yeast 2-hybrid screens for partners to the promyelocytic leukemia (PML) protein (3), Rad51/Rad52 (4), and the Fas/APO-1 death domain (5). Because of its multiple discovery, the modifier initially had several early designations including Ubl1, PIC1, and sentrin. Sequence comparisons suggested that Ubl1/PIC1/sentrin was the mammalian homolog of the *Saccharomyces cerevisiae* *SMT3* gene, an essential gene in *S. cerevisiae* previously identified in a screen for suppressors of a yeast temperature-sensitive *MIF2* gene (6, 7). While the biological functions of this newly identified mammalian protein were unknown, it appeared to be a member of the ubiquitin family. These initial reports were rapidly followed by the discovery that the Ran GTPase-activating protein, RanGAP1, was covalently modified by conjugation of this same protein, now designated as SUMO (8, 9). A subsequent study determined that SUMO was conjugated to RanGAP1 via an isopeptide bond between the carboxyl group of SUMO glycine 97 and the ϵ -amino group of RanGAP1 lysine 526 (10), confirming that SUMO not only shared sequence relatedness to ubiquitin, but also was conjugated to substrates in a chemically analogous fashion. However, the SUMO conjugating enzyme, Ubc9, was shown to function only with SUMO and not with ubiquitin, demonstrating that these modification pathways are biochemically parallel yet distinct (11).

The pathway of SUMO conjugation exemplifies the conjugation pathway used for all the known ubiquitin-like protein modifiers. Briefly, SUMO is synthesized as an inactive molecule that must be cleaved in order to expose the di-glycine motif used for conjugation. This is accomplished by the action of a class of cysteine proteases, termed SUMO proteases. Upon cleavage, SUMO is activated in an ATP-dependent process by the dimeric structure formed by the SUMO-activating enzymes SAE1 and SAE2. SUMO is then transferred from SAE1/SAE2 to an internal cysteine residue in the SUMO conjugating enzyme Ubc9. Finally, Ubc9 conjugates SUMO to the ϵ -amino group in a lysine residue located in the target protein, forming an isopeptide linkage. This final step is enhanced by proteins known as SUMO-ligases which accelerate the transfer of SUMO to the target and are thought to provide specificity to the conjugation system by regulating the interaction between the target and the conjugating enzyme (12–14). Once a protein has been sumoylated, it can be desumoylated again by the action of the SUMO proteases. To date, 3 different types of SUMO ligases and 6 different SUMO proteases have been identified in mammals (14). Interestingly, even though the biochemical pathway of SUMO conjugation and deconjugation is well defined, the regulatory mechanisms that determine the specificity and extent of SUMO conjugation in the cell remain mostly unknown.

SUMO modification exerts a large variety of effects on its targets, altering their cellular localization, stability, ability to interact with other proteins, and activity, which can be either stimulated or repressed (14). For instance, many of the known SUMO substrates are transcription factors, and while for most of them SUMO modification decreases their transcriptional activation function (15–20), for others sumoylation augments their activity (21–23). A wide range of cellular processes are currently known to be affected or regulated by sumoylation, including chromosomal organization and function, DNA repair, nuclear transport, and signal transduction pathways. Obviously, the types of cellular processes

regulated by sumoylation are determined by the identity of the proteins targeted by SUMO conjugation. A broad identification of the spectrum of proteins modified by sumoylation is required to better define the range of cellular events regulated by sumoylation and is likely to provide significant clues about the mechanisms that provide specificity to the system. Similarly, defining the spectrum of proteins modified by any given ubiquitin-like modifier is essential to our understanding of the range of cellular processes affected by each ubiquitin-like modifier and the mechanisms that dictate their specificity.

Clearly, proteomics studies defining the range of proteins targeted by every ubiquitin-like modifier could provide great insights into the cellular role and regulation of each conjugation system. Discovery of entire proteomes is a very challenging task, but the identification and characterization of post-translational modifications on a proteomic scale is an even more difficult one, as for any given protein the amount of modified protein is only a small fraction of the total cellular pool, and a single protein may be modified at multiple sites. The compartmentalization or subfractionation of proteomes makes the analysis of the sample and the interpretation of the data more practical (24, 25). Recently, several groups have performed proteomics studies aimed at defining the range of cellular proteins targeted by sumoylation (26–30), following the lead established by an earlier proteomics study on ubiquitin conjugation (24). The most successful studies providing the most extensive lists of novel substrates for SUMO (27, 29) were performed with the yeast *S.cerevisiae*, as this system can be easily scaled-up, providing virtually unlimited amounts of starting material, and can be easily manipulated to replace the endogenous SUMO gene with one coding for tagged versions of the modifier. The studies performed with mammalian cell lines have yielded a much more limited spectrum of novel potential SUMO targets (26, 28, 30), due in part to the difficulties inherent to the production of sufficient quantities of starting material. However, both the yeast and mammalian studies were limited by the apparent low specificity provided by the use of single stage affinity purification methods for the enrichment of the sumoylated proteins. Single-stage affinity purifications, primarily those based on interactions between charged chemical groups and specific amino acids in the target proteins, but also including those based on protein-protein interactions such as antigen-antibody based affinity purifications, are known to produce relatively high backgrounds of spurious interacting proteins (31, 32).

In this paper we present a strategy for enriching and identifying SUMO modified proteins in mammalian cell lines that is applicable to the identification of the pool of proteins modified by any other ubiquitin-like modifier and therefore represents a universal strategy for proteomics studies of ubiquitin-like modifiers. The overall strategy involves the development of a stable transfected cell line expressing a double-tagged SUMO under a tightly negatively regulated promoter, followed by the induction of the expression and conjugation of the tagged modifier to cellular proteins, the use of a tandem affinity purification (TAP) method for the specific enrichment of the modified proteins, and the identification of the enriched proteins by liquid chromatography matrix-assisted laser desorption ionization tandem mass spectrometry (LC-MALDI-MS/MS). The application of this strategy allowed us to evaluate several basic aspects of SUMO biology (such as its half-life, the effects of SUMO over-expression on the cell cycle, and the changes in overall sumoylation induced by different environmental stresses), allowed us to compare the array of substrates modified by SUMO-1 and SUMO-3, and led to the identification of 122 putative SUMO substrates, some of which had been previously defined as genuine SUMO targets. Three of the novel potential SUMO substrates identified, namely the polypyrimidine tract-binding protein-associated splicing factor PSF, the GTP-binding nuclear protein Ran, and the structural microtubular component alpha-Tubulin, were confirmed as *bona fide* SUMO substrates by immunoblotting or *in vitro* sumoylation reactions, further supporting a role for SUMO in transcriptional regulation, RNA processing, nuclear transport, and

maintenance of chromosomal stability, and suggesting a novel role for SUMO in the regulation of cellular microtubular structures. The application of this proteomics approach to the identification of the pool of cellular substrates modified by other ubiquitin-like proteins could dramatically increase our knowledge of the physiology and regulation of the ubiquitin-like conjugation systems in the cell.

EXPERIMENTAL PROCEDURES

Development of stable transfected cell lines and flow cytometry

All cells lines used in these studies were grown in complete medium containing 1× Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), in a humidified incubator at 37°C, 5% CO₂, unless otherwise indicated. To develop stable transfected cell lines expressing tagged SUMO proteins, the genes encoding the human SUMO-1 and SUMO-3 genes (accession numbers NP003343 and AAH08420, respectively) were inserted into the pBAC-2cp vector (Novagen, Inc., Madison, WI), thereby adding a sequence coding for a hexa-histidine tag, a thrombin recognition sequence, the 15 amino acid residue S-tag, and an enterokinase recognition site to the 5' end of the genes. The tagged genes were PCR amplified and cloned into the pcDNA5/FRT/TO vector (Invitrogen Corp., Carlsbad, CA), which contains a Flp recombination target (FRT) sequence. The derivative plasmids obtained were co-transfected with the Flp recombinase expression plasmid pOG44, using LipofectAMINE 2000, into the FlpIn T-REx HEK293 (F293) cell line (all from Invitrogen Corp.), a HEK293-derivative containing a single integrated FRT site. Cells maintaining an integrated copy of the transfected plasmid were selected in medium containing 10 µg/ml of Blasticidin and 100 µg/ml of Hygromycin. Upon selection, the isogenic cell populations were amplified and maintained in antibiotic-containing medium, tested for β-galactosidase activity (lost upon gene insertion at the FRT site), and several aliquots were frozen in liquid nitrogen for long-term storage. Expression of the His-S-SUMO proteins was induced by adding tetracycline (Tet) to the culture medium at a final concentration of 1 µg/ml. For cell cycle distribution analyses the cell were cultured with or without Tet, changing the medium every 24 h, for a total of 72 h. The cells were trypsinized, washed with 1× PBS, fixed in 70% ethanol for 30 min, and stained for 10 min at 37°C with a solution containing 50 µg/ml of propidium iodide, 4 mM sodium citrate, 150 mM NaCl, 0.1% Triton X-100, and 30 U/ml RNase I, pH 7.8. Upon staining, the cells were maintained in the dark on ice and analyzed in a FACSCalibur flow cytometer using Cell Quest software (Becton Dickinson, San Jose, Calif.).

Protein and transcript stability analyses

For pulse-chase experiments, the F293-SUMO cell lines were plated at 3×10^6 cells per flask in 25 cm² flasks, Tet induced for 24 h, starved for 1 h, pulse labeled with 200 µCi of Trans-³⁵S label (MP Biomedicals, Irvine, CA) for 1 h, washed and chased in unlabeled complete medium, and collected at different times post-chase. Both the starvation and pulse labeling were performed in Met⁽⁻⁾, Cys⁽⁻⁾ 1× DMEM supplemented with Tet. The cells were collected and processed for protein purification as described in "tandem affinity purification" below. The purified samples were resolved by SDS-PAGE and the band corresponding to free-SUMO was quantified by phosphordensitometry. The half-life of each protein was defined as the time at which half the initial counts were present in the purified free SUMO, as calculated from the values obtained above. For studies aimed at measuring transcript stability, the F293-SUMO cell lines were plated at 4×10^6 cells per dish in 10 cm Petri dishes and induced with Tet. Twenty four hours post induction, Actinomycin D (ActD) was added to the medium at 5 µg/ml, and the cells were collected at different times post ActD addition. RNAs were purified using the RNAqueous®-Midi kit (Ambion, Inc., Austin, TX) as described by the manufacturer, and the His-S-SUMO transcripts were detected by

RT-PCR using primers targeting the sequence coding for the His-S-tag, thereby avoiding cross-detection of the endogenous SUMO transcripts, and allowing the direct comparison of the His-S-SUMO-1 and His-S-SUMO-3 transcripts. For northern blot analyses, the RNAs purified from the samples described above were run on a formaldehyde-agarose gel, transferred by capillary action using a TurboBlotter™ device (Schleicher & Schuell BioScience, Inc., Keene, NH) to a GeneScreen Plus® membrane (PerkinElmer Life Sciences, Inc., Boston, MA), and hybridized to a probe complementary to the His-S-tag, thus allowing the direct comparison of the tagged SUMO transcripts as indicated above for the RT-PCR analyses.

Tandem affinity purification (TAP)

For tandem affinity purifications, the F293-SUMO cell lines or the parental F293 cell line were plated at 1×10^7 cells in 175 cm² flasks and Tet induced 24 h after plating. To allow optimal His-S-SUMO expression and conjugation, the cells were maintained in the presence of Tet for 72 h, replacing the medium every 24 h. Eight hours before collection, the proteasomal inhibitor MG132 was added to the medium at a final concentration of 5 μ M, and 1 h before collection the cells were incubated at 41°C, 5% CO₂. At the time of collection, the cells were washed in 1× PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4), lysed in 1× Denaturing Buffer A (8 M Urea, 100 mM NaH₂PO₄, 10 mM Tris, 10 mM β -mercaptoethanol, pH 8.0) supplemented with 0.2 % Triton X-100 and 20 mM N-ethylmaleimide (NEM), and the resulting extracts were either stored at -70°C or processed immediately for TAP. The extracts were sequentially passed through 21, 23, and 27½ gauge needles, sonicated, and cleared by centrifugation at 15,000 g for 10 min at 4°C. The clarified extracts were incubated with His-Select™ Nickel Affinity Gel (SIGMA-Aldrich Co., St. Louis, MO) in a circular rocker for 16 h at 4°C. After incubation, the resulting suspension was poured through an empty column and the beads were washed with 50 bead volumes of 1× Denaturing Buffer A, and 20 bead volumes of 1× Denaturing Buffer B (1 M Urea, 100 mM NaH₂PO₄, 10 mM Tris, pH 6.3). The bound proteins were eluted with 5 bead volumes of 1× Denaturing Buffer C (1 M Urea, 100 mM NaH₂PO₄, 10 mM Tris, pH 3.9), and the eluate was neutralized with an equal volume of 1× Neutralizing Buffer (100 mM NaH₂PO₄, 190 mM Tris, pH 8.8). The neutralized eluate was incubated with S-Protein Agarose (Novagen, Inc.) in a circular rocker for 16 h at 4°C, washed with 90 bead volumes of 1× PBS, and the bound TAP purified proteins were either eluted with 2 volumes of 4× SDS-PAGE sample buffer (100 mM Tris, 20% glycerol, 8% SDS, 0.02% bromophenol blue, 4% β -mercaptoethanol), or by digestion with EKMax™ Enterokinase (Invitrogen Corp.) in 1× Enterokinase Reaction Buffer (50 mM Tris pH 8.0, 1 mM CaCl₂) for 16 h at 37°C.

Immunoblotting

For immunoblot analyses, proteins were resolved by SDS-PAGE and transferred to Immobilon™ membranes (Millipore Corp., Billerica, MA). The blotted membranes were blocked in 1× PBlotto (1× PBS, 0.05% Tween 20, 3% non fat milk) for 30 min at room temperature, incubated for 14 h at 4°C with the primary antibody diluted in 1× PBlotto, washed 3 times with 1× TPBS (1× PBS, 0.05% Tween 20), and incubated for 1 h at room temperature with the appropriate HRP-conjugated secondary antibody (from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:10,000 in 1× PBlotto. Immunoblots were developed by chemiluminescence using either the Western Lightning™ chemiluminescence reagent (Perkin Elmer™ Life Sciences, Inc.) or the SuperSignal® West Femto maximum sensitivity substrate (PIERCE Chemical Co., Rockford, IL). Rabbit polyclonal antibody #12783 to SUMO-1 was produced in house using affinity purified His-tagged SUMO-1 as immunogen. Rabbit polyclonal antibody to PSF was kindly provided by Dr. Herbert H. Samuels (New York University Medical Center) and Dr. Philip W. Tucker (University of

Texas at Austin). All other polyclonal and monoclonal antibodies used in this study were from commercial suppliers, including S-Protein HRP conjugate (Novagen, Inc.), anti-RanGAP-1 monoclonal antibody clone 19c7 (Zymed Laboratories, Inc., San Francisco, CA), anti-PML rabbit serum (Santa Cruz Biotechnology, Inc.), anti-HSF1 Ab-4 rat monoclonal antibody (NeoMarkers/Lab Vision Corp., Fremont, CA), anti-p53 clone PAB240 (Zymed Laboratories, Inc.), anti-alpha-Tubulin monoclonal antibody B-7 (Santa Cruz Biotechnology, Inc.), anti-Ran monoclonal antibody ARAN-1 (SIGMA-Aldrich, Co.), and anti-GST polyclonal goat serum (Amersham Biosciences Corp., Piscataway, NJ). All monoclonal and polyclonal antibodies were used either at a 1:5,000 or a 1:10,000 dilution.

***In vitro* sumoylation assays**

In vitro sumoylation assays were performed as previously reported (33). Briefly, 1 µg of purified target protein was incubated with or without 1 µg of SAE1/SAE2, 200 ng of Ubc9, and the indicated amounts of SUMO1, in a buffer containing 50 mM Tris pH 8.0, 5 mM MgCl₂, 5 mM ATP, and 0.5 mM DTT, in a final volume of 25 µl. The reactions were carried at 30°C for 90 min, stopped by the addition of 4× SDS-PAGE sample buffer, boiled for 3 min, and processed for immunoblotting as described above.

LC-MALDI-MS/MS

Enterokinase-eluted TAP purified proteins were digested with sequencing grade trypsin (Promega Corp., Madison, WI) at a 100:1 protein:trypsin ratio and 37°C for 4 hours. To maximize cleavage efficiency, the proteins were denatured for 20 min at 85°C, cooled down to 37°C, and incubated with another aliquot of trypsin overnight, as previously reported (34). The resulting solution (~0.5 ml) was concentrated in a speed-vac and the pellet was resuspended in 20 µl of 2% acetonitrile, 0.1% trifluoroacetic acid. 10 µl of sample were injected onto a 150 µm × 10 cm column (Vydac) using an LC-Packings autosampler and pumps (LC-Packings, Sunnyvale, CA). A gradient of 90 min from 2% to 60% acetonitrile was used to elute the peptides from the column at 1 µl/min. 5 mg/ml of α-Cyano-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) were mixed with the eluant through a “T” junction (Upchurch Scientific, Oak Harbor, WA) at 1.8 µl/min. The resulting mixture was spotted directly onto a MALDI plate using the LC-Packing Probot. Spots were obtained every 6 seconds. 624 spots were obtained per plate, and typically two plates were obtained per injection. The spots were analyzed using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA). Initially each spot was analyzed in reflectron mode. The resulting spectra were analyzed and the spots with the highest intensity level for each mass obtained were used to acquire tandem MS data. The tandem MS data was analyzed using GPS explorer (Applied Biosystems) and an in-house version of the MASCOT (www.matrixscience.com) search engine. Identified proteins from either the F293-SUMO-1 or the F293-SUMO-3 cell lines were only considered if they had a minimum of one peptide with an individual score greater than 44. Proteins with only 1 or 2 peptides, with at least one having a score greater than 44, were confirmed by *de novo* sequencing. For the control sample, the selection criterion was reduced to any protein identified, regardless of score. Any protein found in the control sample and the F293-SUMO cell lines was removed from the list of identified proteins.

RESULTS

Previous studies by ours (35, 36) and several other groups have revealed a relatively long list of viral proteins modified by SUMO [reviewed in (37)]. While such studies have suggested different roles for the sumoylation of the viral proteins, a more thorough understanding of the interactions between viruses and the host sumoylation system requires monitoring the overall changes in sumoylation occurring during infection. To this end, we

decided to develop a strategy for the broad identification of the spectrum of proteins modified by sumoylation. Furthermore, as at least one of the other ubiquitin-like modifiers [the Interferon-Stimulated Gene ISG-15 modification system (38)] is known to be up-regulated by viral infection, we sought to create a general strategy applicable to proteomic studies of other ubiquitin-like modifiers as well.

For most proteins targeted by the sumoylation system, the apparent amount of sumoylated protein at any given time appears to represent only a small fraction of the total cellular pool (14). Therefore, the first requirement for the proteomic evaluation of the pool of cellular proteins modified by SUMO is to enrich the sumoylated proteins while excluding and minimizing the amount of unmodified proteins. This is best achieved by the use of tandem affinity purification approaches. To make the strategy applicable to the identification of the pool of cellular proteins targeted by any ubiquitin-like modifier, a commercially available tandem affinity purification tag was added to the N-terminal region of SUMO by placing the genes for SUMO-1 and SUMO-3 in the pBAC-2cp vector (Novagen, Inc). This procedure introduced a sequence coding for a (His)₆~S-peptide tandem affinity purification tag and an enterokinase recognition site upstream from the SUMO genes (Fig. 1a). To prevent any undesirable effect due to the over-expression of SUMO, and to avoid the limitations associated to transient transfection approaches, stable transfected cell lines were developed using an inducible expression system for the controlled over-expression of the tagged SUMO. To this end, the tagged genes were cloned into a mammalian expression plasmid containing a Tetracyclin-regulated operator and an Flp recombination target (FRT) sequence, and the resulting plasmids were transfected into a 293 human embryonic kidney cell line derivative containing a single integrated FRT site. The polyclonal populations of cells that maintained an integrated copy of the plasmids were antibiotic-selected to produce two isogenic cell lines dubbed F293-SUMO-1 and F293-SUMO-3. The expression of the (His)₆~S-peptide~SUMO protein (hereafter designated His-S-SUMO) in these cell lines was negatively regulated by the constitutively expressed Tet repressor gene (TetR), and turned on by the addition of tetracycline (Tet) to the culture medium (Fig. 1b, **lanes 1–2 and 7–8**). At 24 h post Tet induction, His-S-SUMO-1 and His-S-SUMO-3 were readily detected, mostly in the unconjugated form (Fig. 1b, **lanes 2 and 8**), and sustained Tet induction led to a gradual increase in the accumulation of the conjugated forms up to 72 h post-induction (Fig. 1b, **lanes 2–6 and 8–12**), although a significant amount of SUMO remained in the unconjugated form. Flow cytometry experiments performed to measure the cellular DNA content at different times post-induction (up to 72 h) indicated that continuous expression of His-S-SUMO had a minimal effect on the cell cycle distribution of the cells, although both the induced and uninduced F293-SUMO cell lines exhibited a slight increase in the G1 population, accompanied by a decrease in the G2 population (Fig. 1c). Although intriguing, these slight differences do not seem directly associated with SUMO overexpression as Tet induction did not trigger further changes in cell cycle distribution, and His-S-SUMO expression in the absence of Tet was minimal. Therefore, SUMO over-expression did not seem to induce an overall increase in total sumoylation or to have gross deleterious or advantageous effects on cellular growth.

Interestingly, even though the tagged modifiers were expressed from the same promoter and in the same locus in the two cell lines developed, His-S-SUMO-3 consistently accumulated to higher levels than His-S-SUMO-1 (Fig. 1b), paralleling differences previously reported between SUMO-1 and SUMO-3 in untransfected cells (39). Therefore, the cause of this difference was sought experimentally. The half-life of each His-S-SUMO was measured in pulse-chase experiments performed by purifying SUMO from metabolically labeled cells collected at different times post-chase, and was determined to be over 20 h, indicating that SUMO-1 and SUMO-3 are very stable proteins (Fig. 2a). Then, we measured the stability of the transcripts by RT-PCR using primers complementary to sequences located in the His-S

tag, therefore enabling us to use the same set of primers for the detection of the His-S-SUMO-1 and the His-S-SUMO-3 transcripts, and avoiding interferences due to the endogenous SUMO transcripts. The cells were Tet induced for 20 h, treated with Actinomycin D (ActD), and collected at different times post ActD treatment. The RT-PCR analysis indicated that the transcripts for His-S-SUMO-1 and His-S-SUMO-3 were both fairly stable as the intensity of the products remained constant up to 8 h post ActD treatment. However, the His-S-SUMO-3 transcripts seemed more abundant than the His-S-SUMO-1 transcripts (Fig. 2b). This difference in abundance was confirmed by northern blots analysis of the samples collected at different times post ActD treatment, using a probe complementary to the tag (Fig. 2b). Therefore, the difference in the levels of His-S-SUMO protein expressed by the two F293-SUMO cell lines reflects differences in the accumulation of their respective transcripts, although the molecular basis for this remains unknown.

The next step was to standardize a procedure to consistently stimulate the conjugation of the over-expressed His-S-SUMOs. A previous report indicated that protein-damaging stimuli induce SUMO-2/3 conjugation (39). Therefore, we tested several different stress-inducers for their ability to increase the incorporation of the His-S-SUMO proteins into high molecular weight forms indicative of SUMO conjugation. Among several different conditions tested, an 8 h treatment with the proteasomal inhibitor MG-132, combined with a 1 h exposure at 41°C before harvesting led to the most substantial increase in the levels of expression and conjugation of His-S-SUMO-1 and His-S-SUMO-3 (Fig. 3a). This indicated that, unlike previously reported in Cos 7 cells (39), in the F293-SUMO cell lines stress-induced SUMO conjugation is not a property exclusive of SUMO-3 but instead is shared by SUMO-1. For all subsequent experiments, the F293-SUMO cell lines and the parental F293 cell line used as a control were induced using these conditions to ensure maximal conjugation of the His-S-SUMO.

Next, a tandem affinity purification (TAP) protocol was developed to purify the pool of sumoylated cellular proteins. TAPs minimize the background of co-purifying contaminant proteins (31) and therefore can potentially enhance the identification of low abundance proteins by mass spectrometry approaches. The covalent nature of the linkage between ubiquitin-like proteins and their targets, and the nature of the affinity tags selected, allowed the use of strong denaturing conditions during the initial stages of purification. Such conditions are expected to inactivate the de-conjugating enzymes, ensure proper solubilization of the modified targets [many of which are known to be trapped in insoluble nuclear domains (40–42)], and disassemble protein complexes thereby preventing the co-purification of interacting proteins. Therefore, the induced cells were collected directly in a buffer containing 8 M urea and 0.2% Triton X-100. Interestingly, preliminary trials indicated that some desumoylation occurred during the affinity purification of the sumoylated proteins, even after the use of the strongly denaturing conditions indicated above. Therefore, NEM was incorporated as an essential component of the buffer used during cell lysis and collection. The resulting cell lysate was clarified and incubated with an immobilized metal affinity chromatography (IMAC) matrix, the IMAC beads were washed extensively, and the bound proteins were eluted by the use of a low pH buffer. For the second affinity purification stage, different buffer conditions were tested as the interaction between the S-peptide and the S-protein is affected by pH, salt, and urea (43–46). The use of a low urea, low salt, and high pH buffer proved to yield the best recovery of tagged proteins, and was therefore incorporated into the TAP protocol. Application of the resulting TAP protocol (described in detail in materials and methods) led to a substantial enrichment of sumoylated proteins from the cell lines expressing the His-S-SUMOs, and a low background of untagged proteins from the parental cell line, as verified by SDS-PAGE and immunoblot analyses of aliquots taken at different stages of the purification process using anti-SUMO antibodies (Fig. 3, a and b), and Coomassie blue staining (Fig. 3c).

To further validate the TAP protocol, we tested by immunoblotting for the presence of several known SUMO targets in the affinity purified samples. The SUMO targets RanGAP-1 (47, 8, 10, 9), PML (48, 42, 49), p53 (50, 51), and HSF-1 (21) were all successfully detected in TAP-purified samples, and their altered migration, indicative of sumoylation, further validated the TAP developed (Fig. 4, a–d). For RanGAP-1, a single band suggestive of a single SUMO-conjugation event per molecule was obtained, and the apparent amount of sumoylated protein recovered from the F293-SUMO-1 cells was significantly higher than the one recovered from the F293-SUMO-3 cells (Fig. 4a). As RanGAP-1 is preferentially modified at a single site by SUMO-1 (39), these observations strongly indicated that although the cells had been stimulated for over-sumoylation, the overall specificity of the sumoylation reaction had not been compromised in the F293-SUMO cell lines. Interestingly, PML and HSF-1 appeared preferentially modified by His-S-SUMO-3 (Fig. 4, b and c, **respectively**), whereas p53 appeared preferentially modified by His-S-SUMO-1 (Fig. 4d).

Next, a protocol for the proteomic analysis of the TAP-purified proteins was developed. Direct protein identification from SDS-PAGE gel bands was attempted with very limited success, probably due to the presence of large numbers of proteins in very small quantities in each band. Therefore, an alternative method was employed to allow the identification of the purified proteins. First, the proteins that remained bound to the S-Protein beads after the TAP procedure were eluted off the beads by digestion with enterokinase. This digestion step added an additional degree of specificity to the purification, as only those proteins containing the enterokinase recognition sequence (which is contained within the His-S-tag) should be susceptible to enterokinase cleavage and release from the beads. Next, the enterokinase-released proteins were digested with trypsin and the resulting peptides were resolved by HPLC and eluted directly onto MALDI plates. Finally, the samples spotted on the MALDI plates were analyzed by MALDI MS, first in reflectron mode and then the spots with the highest intensity level for each mass obtained were used to acquire tandem MS data. This approach allowed the execution of repeated analyses on any given spot whenever it was considered necessary. In total, we performed two independent TAP experiments for every cell line used. To maximize the total number of proteins identified and increase the confidence of such identifications, the raw data captured in each experiment were combined and analyzed together. Altogether, the use of the TAP method herein developed in conjunction with LC-MALDI-MS/MS analyses of the proteins released by enterokinase digestion (exemplified in Figure 5, a and b) resulted in the identification of 122 putative sumoylated proteins, including 4 proteins previously suggested to be sumoylated but for which no further validation as authentic SUMO substrates exists [namely actin (28, 29), ataxin (28, 52), the transcription intermediary factor 1-beta (TIF1- β) (26), and Tubulin (29)], and 3 well-characterized SUMO targets (namely DNA Topoisomerase I, Histone H2A, and TFIIA)(Table 1). None of the proteins identified by LC-MALDI-MS/MS analysis of the TAP purified proteins from the F293 parental cell line (negative control) showed a best ion score above the threshold used for positive identification with the F293-SUMO TAP purified samples. However, 3 proteins identified in the F293-SUMO TAP purified samples were also identified in the negative control (although with a low ion score) and were therefore excluded from the list of putative sumoylated proteins (Table 2). Among those was enterokinase, the enzyme used to release the TAP purified proteins from the S-Protein Agarose beads, which was identified in the F293 parental, F293-SUMO-1, and F293-SUMO-3 TAP samples, as expected. Further details on the proteomic data obtained for every TAP purified sample are presented in the Supplemental Tables 1, 2, and 3. The proteomic data obtained is summarized in Figure 5c. Most of the proteins identified were either transcription factors, nucleic acid binding proteins, or cellular structural components. Out of the total number of proteins identified, 62 were found exclusively in the F293-SUMO-1 cell line, 34 were found exclusively in the F293-SUMO-3 cell line, and the

remaining 27 were found in both cell lines, therefore suggesting limited overlapping in the array of substrates modified by SUMO-1 and SUMO-3. Interestingly, none of the SUMO targets used to validate the TAP approach by immunoblotting were identified by LC-MALDI-MS/MS. This suggests that even though immunoblotting of TAP-purified samples is impractical for large-scale identification of sumoylated proteins, it is perhaps the most sensitive way to determine if a given protein is sumoylated *in vivo*.

Lastly, immunoblot analyses of TAP purified samples were performed to confirm the sumoylation of three selected novel putative SUMO targets identified by LC-MALDI-MS/MS: the polypyrimidine tract-binding protein-associated splicing factor or PSF, the structural microtubular component α -Tubulin, and the GTP-binding nuclear protein Ran. PSF was excluded from the final list of putative SUMO targets due to its presence in the TAP sample purified from the parental cell line (Table 2). However, since the highest peptide scores and the number of peptides identified in the parental and the F293-SUMO-3 cell lines were substantially different, we considered it important to verify if PSF was an authentic SUMO substrate. The other two targets, Ran and α -Tubulin, were selected because of their biological significance and the availability of specific antibodies. To verify the specificity of the purification used for testing the novel putative sumoylated targets, an immunoblot analysis was performed using anti-RanGAP-1 antibodies, as this protein had been previously established as an optimal positive control for the selective purification of sumoylated targets. Similar to our previous analysis, only the SUMO modified forms of RanGAP-1 were detected in the TAP purified samples (Fig. 6a, **lanes 5 and 6**), and no RanGAP-1 was detected in the TAP purification performed with the parental F293 cell line (Fig. 6a, **lane 4**), therefore indicating that the TAP purification worked successfully. The immunoblot analysis performed with the anti-PSF polyclonal antibody detected the unmodified form of PSF in the TAP purified samples obtained from the parental and the SUMO-1 and SUMO-3 derivative cell lines. However, in the TAP purified sample obtained from the F293-SUMO-3 cell line, two additional distinct bands exhibiting apparent molecular weights consistent with the addition of 1 or 2 chains of SUMO to PSF were also detected (Fig. 6b, **lane 6**). Such bands were not detected in the TAP samples from the parental or the F293-SUMO-1 cell lines (Fig. 6b, **lanes 4 and 5**). This finding suggests that PSF is an authentic SUMO target and that it is preferentially modified with SUMO-3. A similar analysis was performed using antibodies directed against α -Tubulin. The anti- α -Tubulin monoclonal antibody produced a complex profile in the total cell extracts, but reacted primarily with a 50 kDa protein, corresponding to the expected molecular weight for α -Tubulin (Fig. 6c, **lanes 1–3**). Interestingly, the only band detected in the purified samples was a 70 kDa band detected in the F293-SUMO-3 purified sample (Fig. 6c, **lane 6**). The altered migration of this band is within the range expected for a SUMO-modification associated shift, and therefore strongly supports the hypothesis that α -Tubulin is also a *bona fide* SUMO target. Lastly, a similar immunoblot performed with an anti-Ran monoclonal antibody detected Ran in the TAP purified sample from the F293-SUMO-3 cell line (Fig. 6d, **lane 6**), but did not detect any Ran on the TAP samples from the parental or the SUMO-1 derivative cell lines (Fig. 6d, **lanes 4 and 5**). However, high molecular weight forms of Ran were detected exclusively in the total cell extracts and not in the TAP purified samples, and only upon prolonged exposure of the immunoblot to the film. As the immunoblot analysis failed to provide conclusive evidence of the sumoylation of Ran, we performed an *in vitro* sumoylation experiment using affinity-purified bacterially-expressed Ran, and purified sumoylation components, also from bacterial expression systems. The use of recombinant proteins expressed in bacteria guaranteed that any potentially sumoylated Ran product had to be produced during the *in vitro* sumoylation reaction and could not be due to spurious cross-reactivities with other cellular proteins. In the presence of a full set of sumoylation components, Ran was readily sumoylated, and the apparent concentration of the sumoylated form of Ran increased proportionally to the amount of free SUMO-1 used in the

sumoylation reaction, therefore demonstrating that Ran is also an actual SUMO target (Fig. 6e). Altogether, the above demonstration that the tested proteins are legitimate SUMO targets strongly supports the hypothesis that the majority of the new sumoylation substrates identified by the proteomic analysis presented are also authentic SUMO targets.

DISCUSSION

This report presents the development and testing of a strategy for the assessment and identification of the pool of sumoylated proteins in a mammalian cell line. The overall strategy (summarized in Figure 7) is clearly applicable to the proteomic analysis of the pool of proteins modified by any other ubiquitin-like modifier, and therefore represents a universal strategy for proteomic studies of ubiquitin-like modifiers. This methodology constitutes a major improvement over the use of transient transfection as a way to deliver the tagged modifier into the cells (26), single step affinity purifications as a way to purify the pool of modified proteins (26–30), and SDS-PAGE followed by in-gel digestion and peptide mass fingerprinting as a way to identify novel SUMO targets (26). The application of this method allowed us to study some of the biological characteristics of SUMO (i.e. protein stability, effects of SUMO over-expression on cell cycle, and stimulatory treatments for conjugation), indicated the preferential conjugation of SUMO-1 or SUMO-3 to some known SUMO targets, and provided an extensive list of potential SUMO-1 and SUMO-3 sumoylation substrates in mammalian cell lines, three of which, namely PSF, Ran, and α -Tubulin, were confirmed as *bona fide* SUMO substrates. Furthermore, this method will allow the evaluation of changes in the pool of modified proteins throughout the cell cycle, during cellular differentiation, and among different cell lines, for SUMO and all other ubiquitin-like modifiers.

The stable transfected cell lines developed for this study expressed, in addition to the endogenous SUMOs, a His-S-tagged version of SUMO-1 or SUMO-3. While the size of the tag used is significant in comparison to the size of SUMO (58 amino acid residues for the His-S-tag versus 97 or 93 amino acid residues for SUMO-1 or SUMO-3, respectively), and large tags may decrease the efficiency of conjugation (27), our data indicated that under the stress conditions employed (heat shock and MG-132 treatment) the tagged SUMOs were efficiently conjugated to cellular targets. In fact, contrary to our expectations, heat-shocking the cells in coordination with exposure to MG-132 significantly increased the conjugation with both His-S-SUMO-1 and His-S-SUMO-3, and not exclusively with His-S-SUMO-3. This suggests that SUMO conjugation as a whole, and not only with SUMO2/3 as previously indicated (39), may be included among the cellular responses against stress. The increased conjugation observed under such conditions does not seem to alter the specificity of the sumoylation system because, although the overall sumoylation is substantially increased, the sumoylation profile observed for the SUMO substrates tested appeared mostly unchanged as compared to studies by other groups and by direct comparison with the parental cell line. Therefore, the increased conjugation achieved by heat shock and MG-132 treatment likely resulted in an overall increase in the fraction of sumoylated versus unsumoylated forms for most SUMO targets, and not in an overall change in the subset of sumoylated cellular proteins. While the molecular basis for the stimulatory effect mediated by MG-132 is unknown, it suggests a connection between proteasomal degradation, ubiquitination, and sumoylation. This connection is unlikely to involve proteasomal degradation of SUMO itself, as SUMO was shown to have a long life in the cell. The relatively long life of SUMO suggests that SUMO conjugation is more likely to be regulated at the conjugation and de-conjugation stages (by controlling the abundance and/or the activity of the SUMO ligases and isopeptidases) rather than by regulating the overall abundance of SUMO in the cell, as in the absence of rapid SUMO turn-over it would be hard to quickly decrease the abundance of SUMO in the cell. In fact, in the absence of a

stimulatory signal for conjugation most of the tagged SUMO remained unconjugated even when it was over-expressed, further stressing the relevance of factors affecting the conjugation and de-conjugation stages. Therefore, we postulate that MG-132 may act by stabilizing SUMO-E3 ligases required for the efficient conjugation of SUMO to a wide array of cellular substrates, while the heat shock may up-regulate those SUMO-E3 ligases and perhaps down-regulate some of the SUMO isopeptidases.

The observed differences in transcript abundance between His-S-SUMO-1 and His-S-SUMO-3 are particularly difficult to explain in view of the fact that both were under the control of the same promoter region, had the same upstream and downstream regulatory sequences, and had the same chromosomal location. However, such differences correlated well with the observed apparent abundance of each SUMO in the cell lines developed. It remains to be investigated if similar differences in transcript abundance exist between the endogenous SUMO-1 and SUMO-3 transcripts. Obviously, although little attention has been given to the mechanisms regulating SUMO expression, detailed knowledge of such mechanisms is essential to our overall understanding of sumoylation and its physiological roles in the cell. Interestingly, upon heat shock and MG-132 induction the differences observed in the expression of His-S-SUMO-1 and His-S-SUMO-3 disappeared. In fact, more potential SUMO substrates were identified in the F293-SUMO-1 samples than in the F293-SUMO-3 samples, and only approximately 23% of all the proteins identified were found in both groups of samples. This provides further support to the observation that a certain degree of specificity allowing the preferential modification of specific substrates with each SUMO modifier remains even when the SUMO modifiers are overexpressed and overconjugated. This specificity also supports the hypotheses that the SUMO paralogues are functionally different therefore providing different properties to the target protein, and that the selection of a SUMO paralogue for the modification of a given target is likely to be specifically regulated.

Proteomic studies face two interrelated issues: 1) lack of specificity, leading to the identification of proteins fortuitously purified due to the nature of the contaminant protein and the purification strategy employed; and, 2) lack of sensitivity, leading to a failure to identify some of the proteins present in the sample under analysis. The purification method presented here for the enrichment of proteins modified by ubiquitin-like modifiers substantially decreases the likelihood that any given protein identified by the proteomics analysis may be a spurious contaminant, as it involves two affinity purification steps, each based on a different type of intermolecular interaction, plus the final sequence-specific release of the purified proteins by enterokinase. In fact, in sharp contrast with other previous SUMO-proteomics studies (53, 28), very few proteins were identified in our negative control (consisting of the TAP purified sample obtained from the parental cell line, presented in Table 2 and Supplemental Table 3), leading to the exclusion of a small subset of proteins from the final list of putative SUMO substrates. Even more, one of the excluded proteins, PSF, was shown to be an authentic SUMO substrate by immunoblotting analyses. However, albeit minimal, spurious co-purifications are still probable, so the possibility remains that some of the novel putative SUMO targets identified may not be *bona fide* SUMO substrates. None of the proteins identified by immunoblotting during the validation of the TAP protocol were identified by LC-MALDI-MS/MS analysis of the TAP purified proteins under the stringent identification criteria applied for protein identification. This indicates that the list of putative SUMO substrates presented in Table 1 still represents only a limited fraction of all authentic SUMO substrates. Furthermore, it also indicates that the approach used to identify the purified proteins could be modified to increase its sensitivity. Out of all the steps involved in the TAP described, the one that is likely to result in the largest losses is the enterokinase treatment, as it is hard to experimentally assess the recovery achieved and optimize the conditions employed in this step. An attractive

experimental alternative to the use of enterokinase for the release of the modified proteins from the S-beads is the use of peptidases specific for the Ubiquitin-like modifier under study. This would potentially lead to a further increase in the specificity of the method, decrease the signal contributed by the modifier (as the modifier would remain bound to the beads), and consequently increase the sensitivity of the method for the proteomic detection of the cellular proteins targeted by the ubiquitin-like modifier. This alternative is currently under evaluation in our laboratories by the use of the SUMO-specific Ulp1 protease.

Out of the putative SUMO substrates presented herein, three novel SUMO substrates were confirmed as *bona fide* SUMO targets: α -Tubulin, PSF, and Ran. α -Tubulin was identified as a putative SUMO substrate in a previous proteomic study (29), but it was not formally validated as a genuine SUMO target. α -Tubulin, in conjunction with β -Tubulin, forms the structural unit of cellular microtubules. Mammalian microtubules originate in the centrosome and in coordination with kinesin and dyneins form the molecular motors responsible for the intracellular transport of vesicles and organelles and the migration of chromosomes during mitosis and meiosis. Tubulins are subjected to several types of post-translational modifications, including detyrosination, acetylation, phosphorylation, palmitoylation, polyglutamylation, and polyglycylation. Some of these post-translational modifications affect the interaction between microtubules and motor proteins (54). While Tubulin sumoylation may play a similar role, it is tempting to speculate that sumoylation may confer to this cytoskeletal component the ability to concentrate or sequester other sumoylated proteins at specific intracellular locations, acting as “nucleation” sites to increase the local concentration of specific proteins, therefore enhancing the formation of multimeric protein machines. Interestingly, although only the sumoylation of α -Tubulin was confirmed, β -Tubulin was also identified as a putative SUMO-1 and SUMO-3 target. Furthermore, Actin, the other major cytoskeletal component and the structural unit of microfilaments, was identified as a putative SUMO substrate here as well as in a previous SUMO proteomic study (28). Should all of these cytoskeleton components be authentic SUMO substrates, sumoylation could be a major regulator of cellular architecture and transport. Alternatively, sumoylation of these cytoskeleton structural units could be restricted to specific stages of the cell cycle and therefore could be relevant only for a limited set of processes, such as chromosomal segregation. Clearly, further studies are required to explore these alternatives.

PSF and Ran, the other two novel SUMO substrates that were validated in this study, were not identified as potential SUMO targets in any of the previous SUMO proteomic studies. PSF was initially characterized as a factor that co-purified with the Polypyrimidine Tract-Binding protein (PTB) and appeared essential for pre-mRNA splicing, hence it was named PTB-associated Splicing Factor (or PSF). PSF exhibits a varied cellular distribution that includes the nucleolus, the nuclear membrane, the nucleoplasm, and a novel nuclear domain termed paraspeckles. Functionally, PSF appears to be a multifunctional protein with DNA and RNA binding properties, and has been implicated in a variety of cellular processes including pre-mRNA splicing, intranuclear retention of promiscuously edited RNAs, transcriptional repression, and enhancement of the helicase activity of DNA Topoisomerase I (55). Sumoylation could clearly regulate any of the activities suggested above for PSF. Ran is perhaps the most intriguing novel substrate presented. Unlike PSF and Tubulin, Ran lacks a predicted consensus sumoylation site on its sequence. However, a recent report indicated that the frequency at which SUMO is added to Lys residues located in non-consensus sequences is much higher than previously recognized (56). Therefore, Ran sumoylation is likely to involve a non-canonical target sequence which, if defined, could reveal structural features common to other SUMO targets. Ran plays 3 major roles in the cell, acting as a regulator of nuclear transport, spindle assembly, and post-mitotic nuclear envelope assembly. In all of these roles, a Ran-GTP gradient is used to direct spatially regulated

processes in reference to chromosomal localization. The Ran-GTP gradient is established via the localization of the guanine nucleotide exchange factor RCC1, the GTPase activating protein RanGAP-1, and the Ran-binding proteins RanBP1, 2, and 3 (57, 58). Sumoylation is already known to play a role in these processes as only the sumoylated form of RanGAP-1 binds to RanBP2 (47, 8, 9), and RanGAP-1 remains bound to RanBP2 throughout mitosis (59). Furthermore, sumoylation appears to regulate the nuclear traffic of several of the known SUMO substrates, as recently reviewed (60, 61). Ran sumoylation may provide yet another mechanism to control the cellular events regulated by Ran, therefore increasing the relevance of SUMO for those cellular events. It would be interesting to determine if the proportion of sumoylated Ran increases at specific stages during the cell cycle, particularly during mitosis, as this may indicate if Ran sumoylation is equally relevant to all of the specific roles attributed to Ran. Altogether, the 3 novel SUMO substrates herein presented open new and exciting areas of SUMO research that will require extensive exploration.

Sumoylation is likely to be a rather transient modification as the fraction of sumoylated versus unsumoylated forms for any given protein is very small. This makes the proteomics evaluation of the total pool of sumoylated proteins intrinsically difficult. The same is probably true for all other ubiquitin-like modifiers. The proteomics approach herein presented allows for the rapid and highly specific enrichment of the pool of proteins modified by a given Ubiquitin-like modifier, and, combined with state-of-the-art MS techniques, it allows for the rapid identification of the cellular targets modified by the Ubiquitin-like modifier under study, as supported by the proteomics data presented for SUMO-1 and SUMO-3. The application of this proteomics approach to the identification of the pool of cellular substrates modified by other ubiquitin-like proteins could dramatically increase our knowledge of the physiology and regulation of the ubiquitin-like conjugation systems in the cell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ActD	Actinomycin D
FRT	Flp recombination target
F293	A human embryonic kidney 293 (HEK293) derivative cell line containing a single FRT sequence and expressing the Tet repressor gene
His-S-SUMO	N-terminal fusion of a (His) ₆ ~S-peptide and SUMO
IMAC	Immobilized metal affinity chromatography
LC-MALDI-MS/MS	Liquid chromatography matrix-assisted laser desorption ionization tandem mass spectrometry
NEM	N-ethylmaleimide
TAP	Tandem affinity purification

Tet

Tetracyclin

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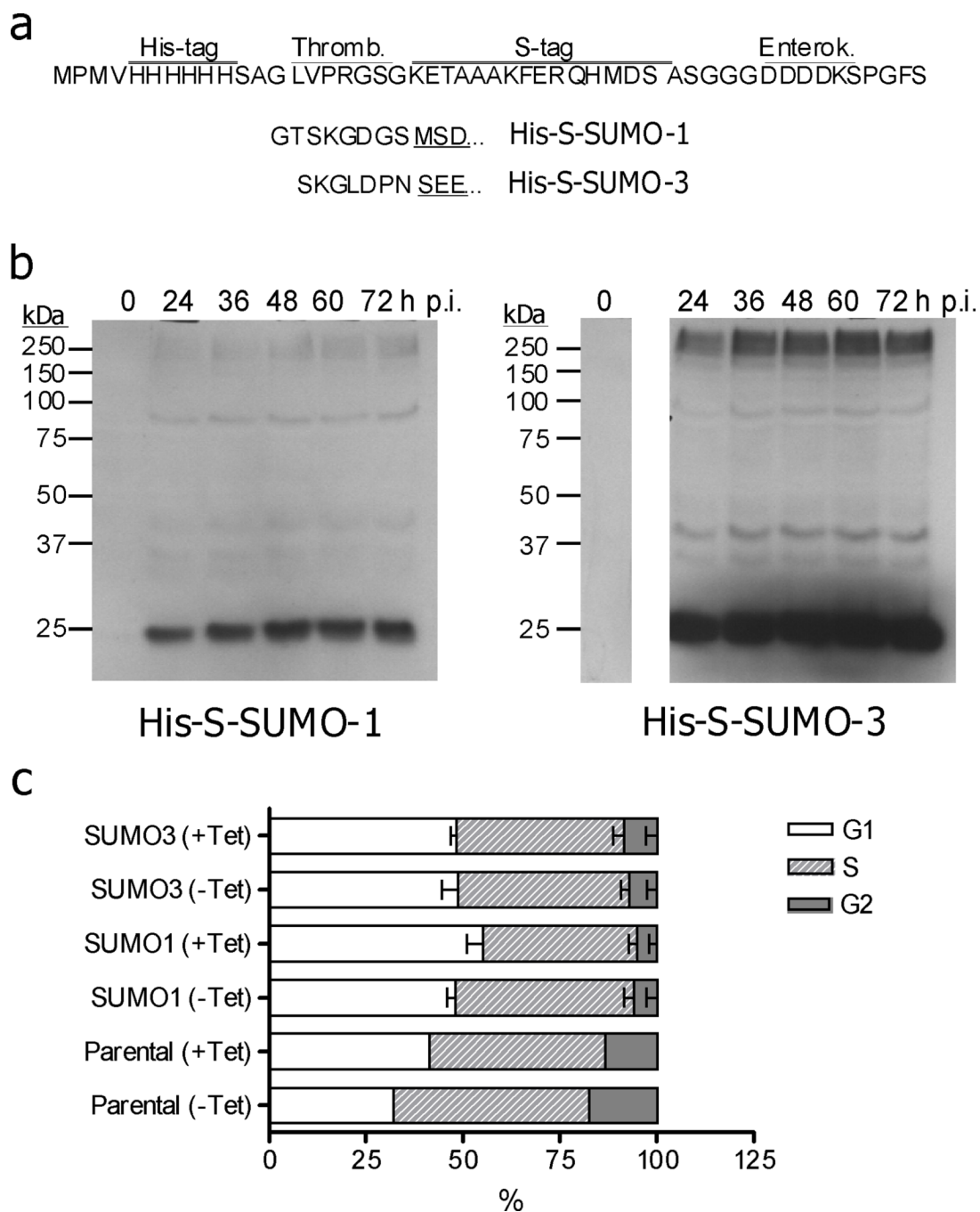


Figure 1.

Characterization of stable transfected cell lines expressing His-S-tagged human SUMO-1 or SUMO-3. **a.** N-terminal sequences of the His-S-tagged human SUMO-1 and SUMO-3. Sequences specific to the His-S-SUMO-1 and the His-S-SUMO-3 are indicated and follow the sequence on top. The first 3 amino acid residues of each SUMO are underlined. Enterok: Enterokinase site. Thromb: Thrombine site. **b.** Time course of expression and conjugation of His-S-SUMO. Cells collected at different times post Tet induction were lysed and resolved by 10% SDS-PAGE, blotted, and probed with S-Protein HRP conjugate. Numbers on top indicate time post induction (p.i.) at which the cells were collected. Arrowheads and

brackets indicate the position of free and conjugated SUMO, respectively. **c.** Cell cycle distribution of the parental and derivative cell lines used in this study after 72 h of exposure to medium supplemented with (+TET) or without (-TET) Tetracycline, as determined by flow cytometry. The data presented corresponds to the distribution observed in 3 independent experiments.

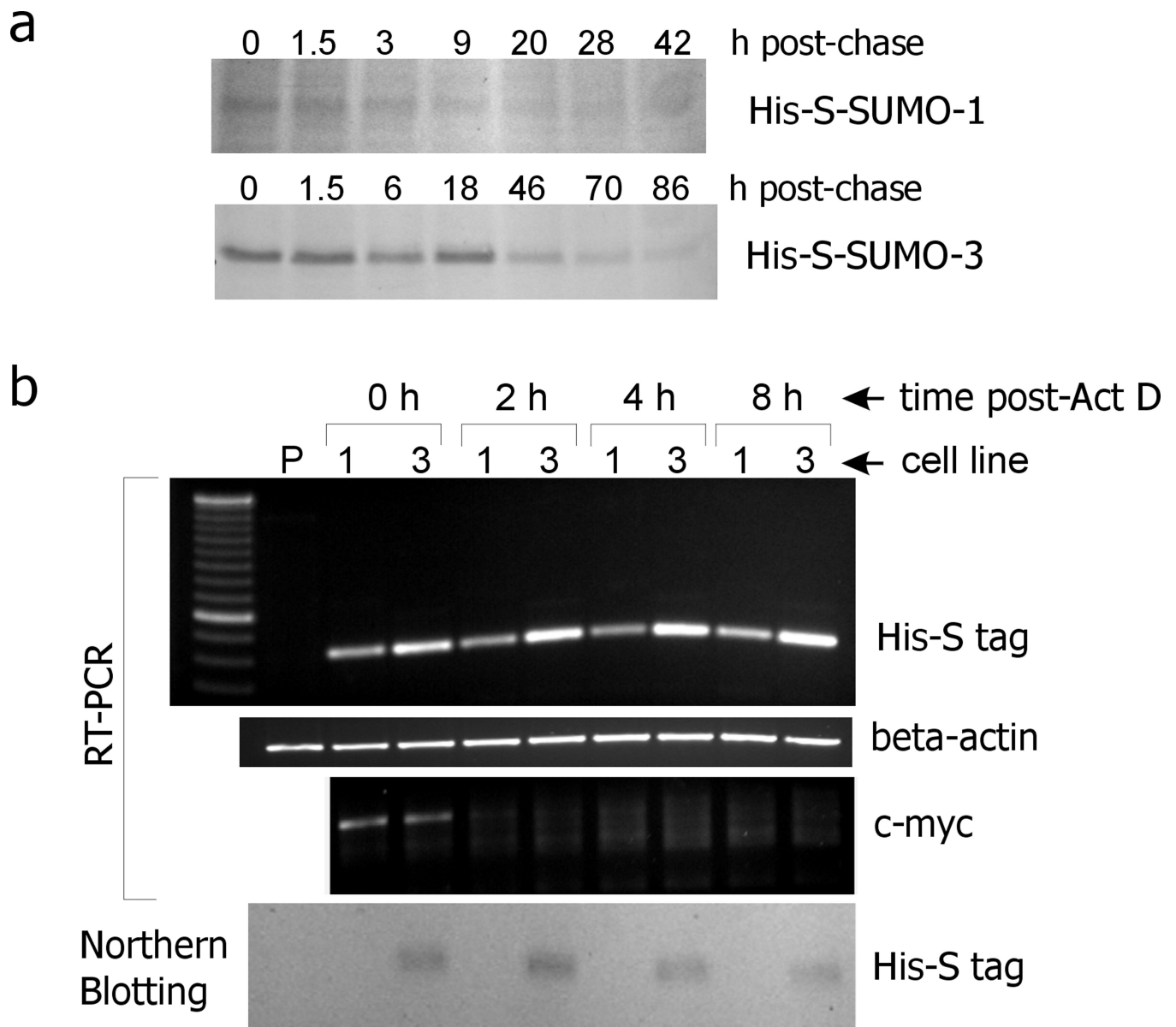
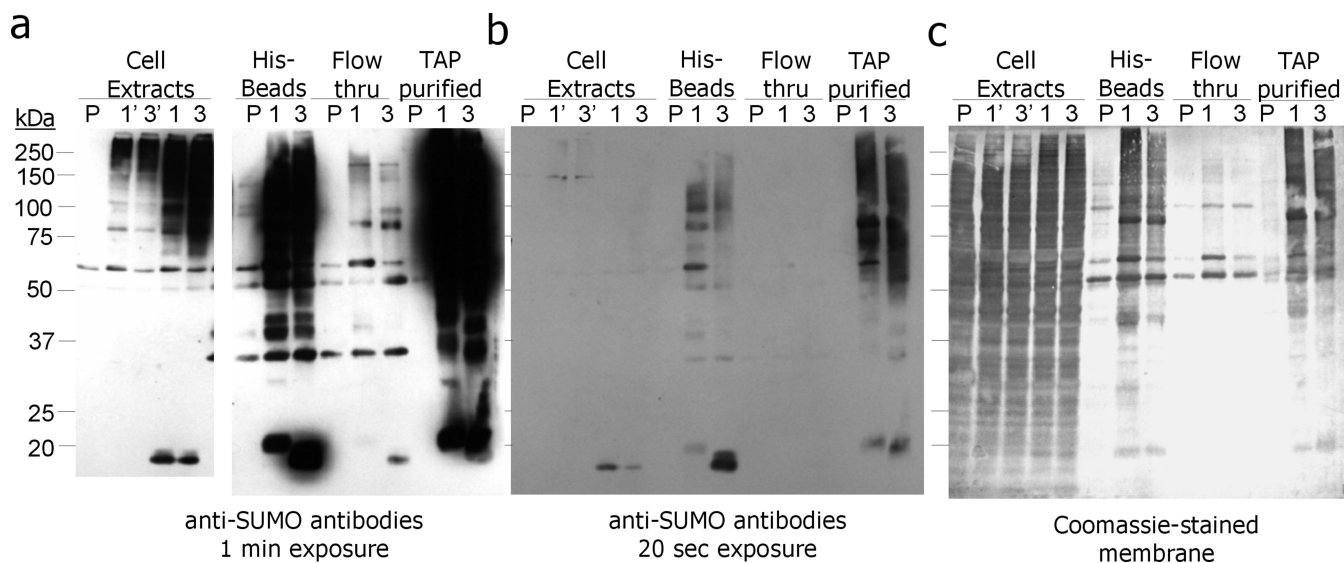
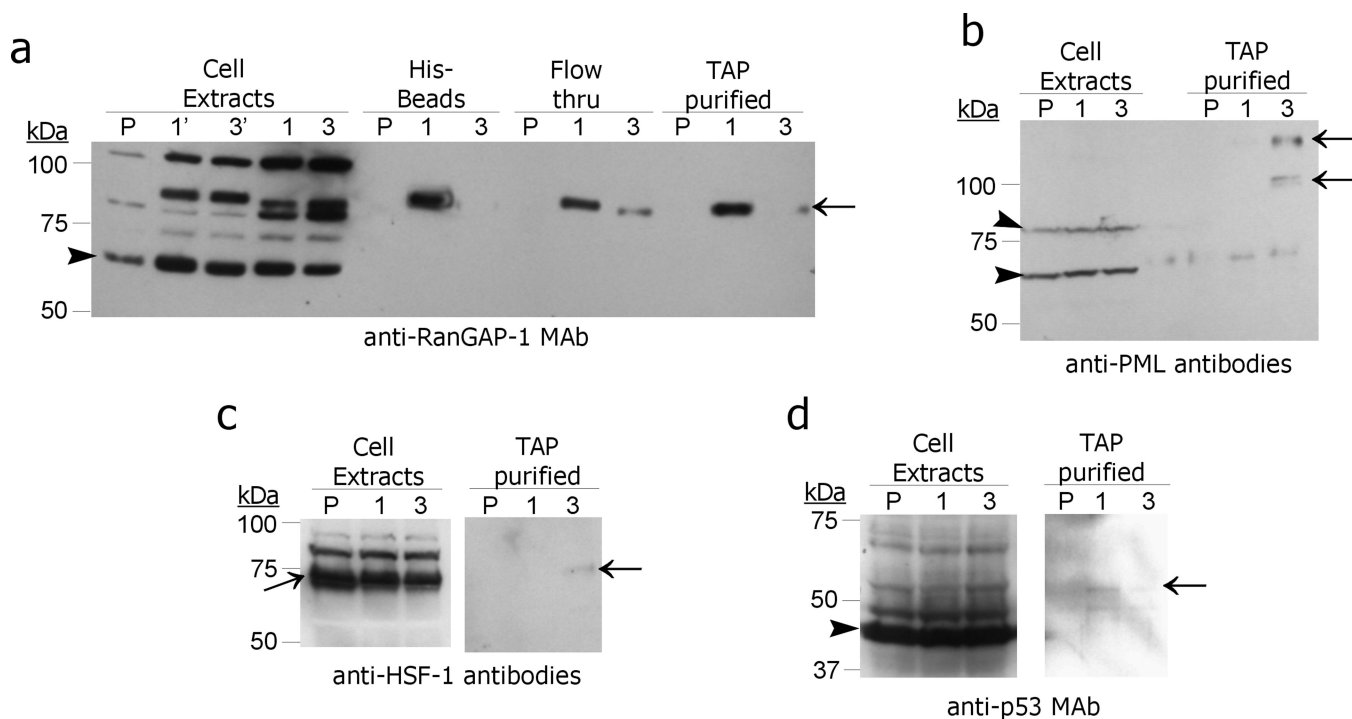


Figure 2. Stability of the His-S-SUMO proteins and transcripts expressed by the F293-SUMO cells. **a.** Pulse chase analyses to assess the stability of His-S-SUMO-1 and His-S-SUMO-3. Metabolically labeled unconjugated SUMO was tandem affinity purified from cells collected at different times post chase as described in **Methods**. The samples were resolved by SDS-PAGE and analysed by autoradiography and phosphordensitometry. **b.** Stability and abundance of the His-S-SUMO-1 and His-S-SUMO-3 transcripts. Tet-induced cells were treated with Actinomycin D (Act D) and collected at different times post Act D treatment. Total RNA was isolated and the relative amounts of His-S-SUMO mRNA were evaluated by RT-PCR using primers complementary to the His-S tag. β -Actin was used as a loading control and c-myc was used as an unstable transcript control. The total RNA was also analysed by northern blotting using a probe complementary to the His-S tag. The identity of the cell lines from which the mRNA was isolated is indicated above the gel images. 1: F293-SUMO-1 cell line. 3: F293-SUMO-3 cell line. P: Parental F293 cell line.

**Figure 3.**

Induction of SUMO conjugation and validation of the tandem affinity purification by immunoblotting with anti-SUMO antibodies. **a–c**. Samples collected at different stages during the tandem affinity purification were resolved by 10% SDS-PAGE, blotted, and probed with anti-SUMO-1 rabbit polyclonal antibodies (**a & b**), or stained by Coomassie blue (**c**). Notice that **b** represents a shorter exposure of the image presented in **a**. Cell Extracts: unfractionated cell extracts. His-Beads: Samples eluted from the HIS-Select™ Nickel Affinity Gel (SIGMA-Aldrich Co.). Flow thru: unbound fraction after incubation with the S-Protein agarose. TAP purified: proteins bound to the S-Protein agarose at the end of the TAP procedure, eluted by treatment with the SDS-PAGE sample buffer. P: Parental F293 cell line. 1' & 3': Tet induced unstimulated F293-SUMO-1 and F293-SUMO-3 cell lines, respectively. 1 & 3: Tet induced heat shocked and MG-132 stimulated F293-SUMO-1 and F293-SUMO-3 cell lines, respectively.

**Figure 4.**

Known SUMO targets are sumoylated in the Tet induced F293-SUMO cell lines and are purified by tandem affinity purification. To validate the TAP method, immunoblot analyses of TAP purified samples were performed using antibodies directed against known SUMO targets including RanGAP-1 (**a**), PML (**b**), HSF-1 (**c**), and p53 (**d**). To exemplify the purification profile of a known SUMO substrate, samples taken at different stages during the TAP procedure were used in the RanGAP-1 immunoblot. Cell Extracts: unfractionated cell extracts. His-Beads: Samples eluted from the HIS-Select™ Nickel Affinity Gel (SIGMA-Aldrich Co.). Flow thru: unbound fraction after incubation with the S-Protein agarose. TAP purified: proteins bound to the S-Protein agarose at the end of the TAP procedure, eluted by treatment with the SDS-PAGE sample buffer. P: Parental F293 cell line. 1' & 3': Tet induced unstimulated F293-SUMO-1 and F293-SUMO-3 cell lines, respectively. 1 & 3: Tet induced heat shocked and MG-132 stimulated F293-SUMO-1 and F293-SUMO-3 cell lines, respectively. Arrowheads and arrows indicate the position of the unmodified and sumoylated proteins, respectively.

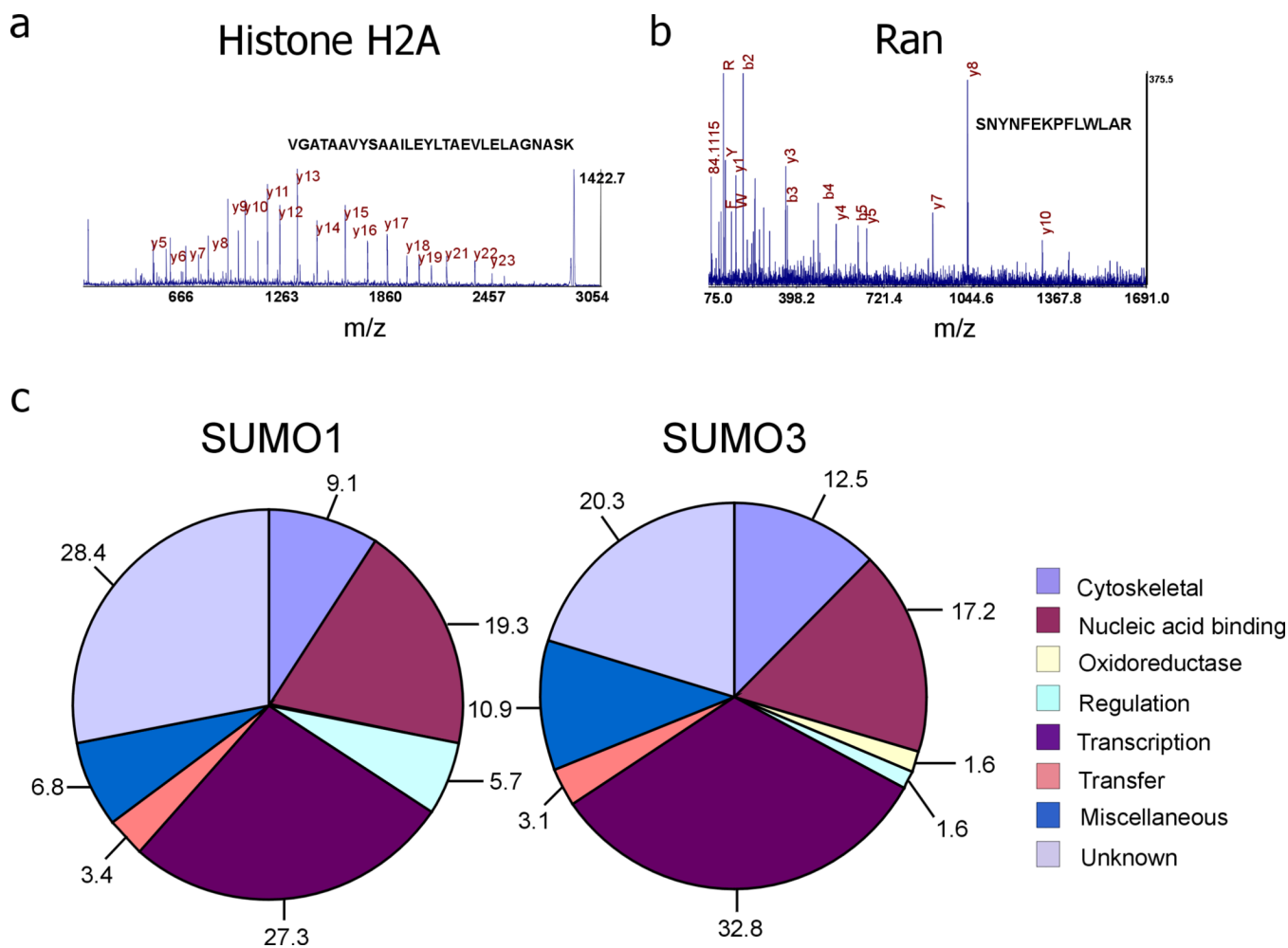


Figure 5. Proteomic analysis of the TAP purified samples obtained from the parental and derivative F293-SUMO cell lines. **a & b.** Fragmentation patterns of 2 of the proteins identified in the LC-MALDI-MS/MS proteomic analysis of the TAP purified proteins. **a.** Histone H2A. **b.** Ran (GTP-binding nuclear protein Ran). **c.** Pie chart summarizing the functional classification of the proteins identified by the proteomic analysis of the TAP purified proteins.

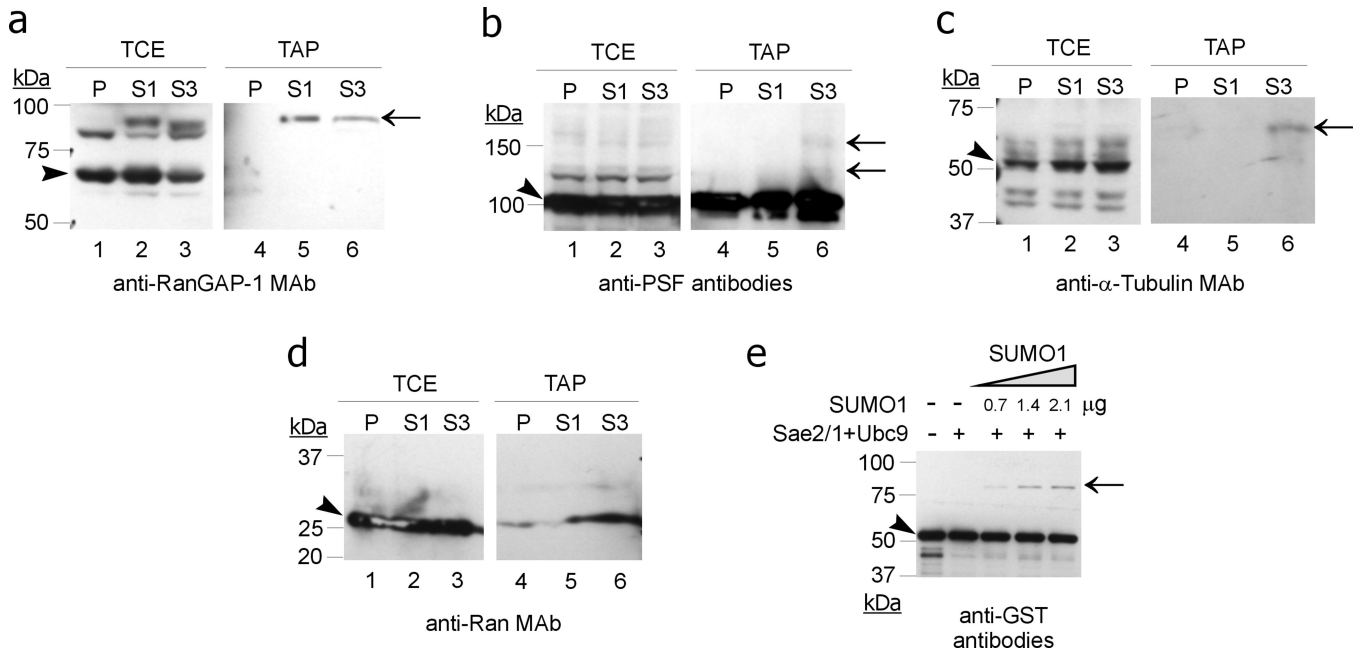


Figure 6.

PSF, α -Tubulin, and Ran are authentic SUMO substrates. **a–d.** To confirm the SUMO modification of PSF, α -Tubulin, and Ran, TAP purified proteins were resolved in a 5–15% gradient SDS-PAGE gel, transferred to Immobilon™ membranes, and immunoblotted with antibodies directed against the specific proteins tested. **a.** RanGAP-1 (positive control used to verify the specificity achieved in the TAP used to validate the novel SUMO substrates). **b.** PSF. **c.** Tubulin. **d.** Ran. **e.** *In vitro* sumoylation of GST-Ran. Bacterially-expressed affinity-purified GST-Ran was sumoylated *in vitro* using affinity purified SUMO-activating enzymes (Sae2/1), Ubc9, and increasing amounts of SUMO-1. The *in vitro* sumoylation reactions were denatured with sample buffer, boiled, resolved in a 10% SDS-PAGE gel, transferred to an Immobilon membrane, and immunoblotted with anti-GST antibodies. No significant sumoylation was observed in a GST-only control (data not shown), as previously reported by others (62). TCE: unfractionated cell extracts. TAP: TAP purified proteins bound to the S-Protein agarose at the end of the TAP procedure, eluted by treatment with SDS-PAGE sample buffer. P: Parental F293 cell line. S1 and S3: Tet induced heat shocked and MG-132 stimulated F293-SUMO-1 and F293-SUMO-3 cell lines, respectively. Arrowheads and arrows indicate the position of the unmodified and sumoylated proteins, respectively.

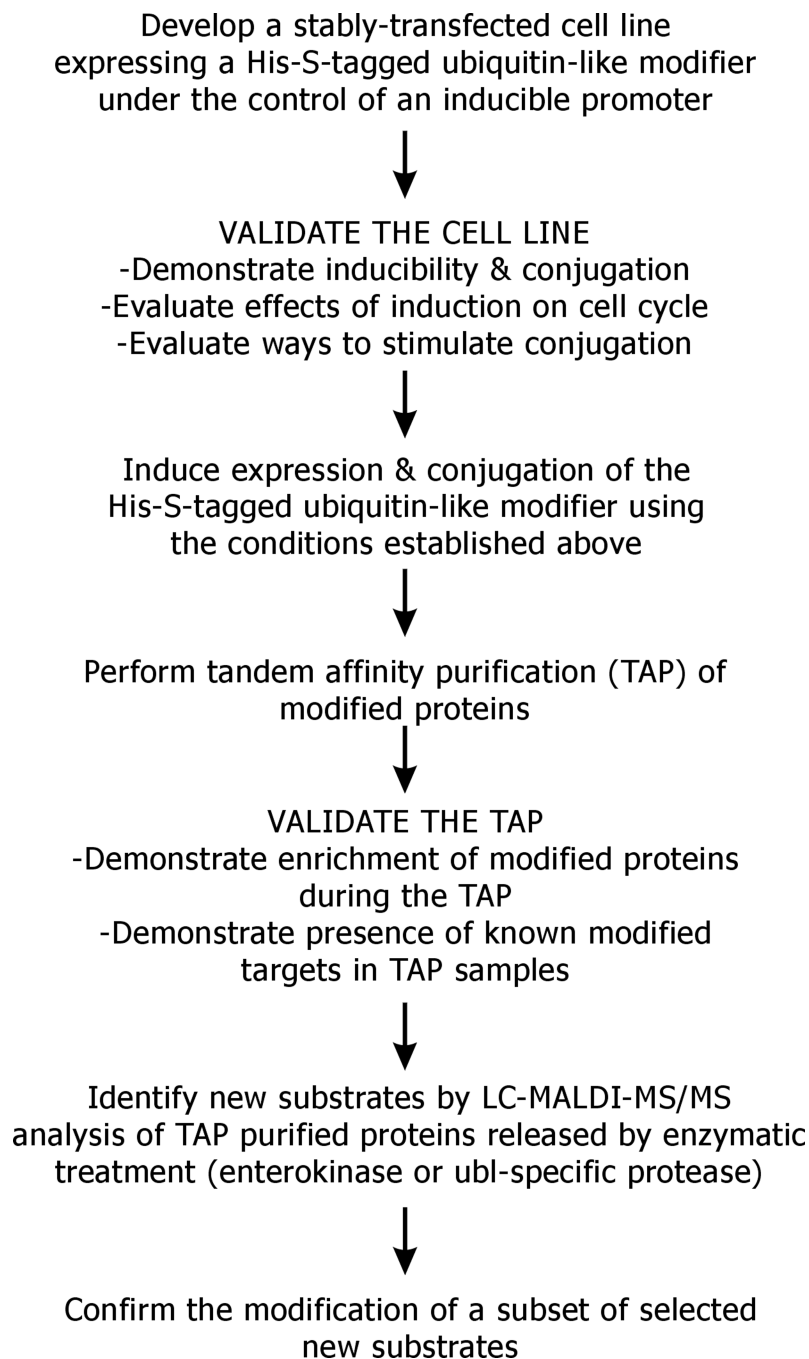


Figure 7. Flow chart representation of the generic approach for proteomic studies of ubiquitin-like modifiers herein described.

Table 1

Putative SUMO-1 and SUMO-3 substrates identified by LC-MALDI-MS/MS analysis of TAP purified proteins.

Protein Name	Accession Number	SUMO-1	SUMO-3	Molecular Function
Actin, alpha cardiac	spt P04270	5 / 105	5 / 104	Cytoskeletal protein->Actin family
Actinin, alpha 2 (Fragment)	trm Q86TI8	6 / 95	5 / 53	Cytoskeletal protein->Actin family
Actinin alpha	gb AAC17470.1	18 / 95	9 / 53	Cytoskeletal protein->Actin family
Alpha-2-macroglobulin receptor-associated protein precursor (Alpha-2-MRAP)	spt P30533	3 / 102		Select regulatory molecule
Ataxin 2 related protein isoform A	rf NP_009176.2	5 / 47		
Bone marrow zinc finger protein 2	trm Q8IZC8	2 / 45	2 / 63	Transcription factor->Zinc finger transcription factor
Calcium homeostasis endoplasmic reticulum protein (CHERP)	trm Q8WU30		4 / 58	
cAMP responsive element binding protein 5	rf NP_004895.1	2 / 56		Transcription factor->Other transcription factor
Citrate transporter protein - human	pir G01789	1 / 51		Transfer/carrier protein->Mitochondrial carrier protein
DNA topoisomerase I	gb AAB60380.1	12 / 73	5 / 55	Select regulatory molecule; Nucleic acid binding
ERPROT 213-21	trm O00302	4 / 65		
Fatty acid synthase	rf NP_004095.3	3 / 75		Synthase and synthetase->Transferase
FB19 protein	trm O00405	13 / 66	11 / 50	
Fibrillarin	gb AAA52453.1	3 / 67	3 / 91	Nucleic acid binding->Ribonucleoprotein
FMRP interacting protein, 82kD	trm Q7Z417		5 / 52	
Glucose-6-Phosphate Isomerase (GPI)	spt P06744	7 / 89	5 / 81	Isomerase->Other isomerase
heat shock 90kDa protein 1, alpha	rf NP_005339.1		3 / 51	Chaperone->Hsp 90 family chaperone
heat shock 90kDa protein 1, beta	rf NP_031381.2	7 / 47		Chaperone->Hsp 90 family chaperone
Heterogeneous nuclear ribonucleoprotein L	trm Q9H3P3	7 / 95		Nucleic acid binding->Ribonucleoprotein
Histone H2A.o (H2A/o) (H2A.2) (H2a-615)	spt P20670	2 / 51		Nucleic acid binding->Histone
Histone H2A.z (H2A/z)	spt P17317	4 / 178	4 / 58	Nucleic acid binding->Histone
Homeodomain protein DLX-2	gb AAA19663.1		1 / 47	Transcription factor->Homeobox transcription factor
Hypothetical protein	trm Q7Z722	1 / 68		Transcription factor->Zinc finger
Hypothetical protein	trm Q7Z791	3 / 62		Transferase->Transketolase
Hypothetical protein	trm Q8IYX0	2 / 70		Transcription factor->Zinc finger
Hypothetical protein	trm Q9NSV0	3 / 61		
Hypothetical protein	trm Q9NSV0		1 / 45	

Protein Name	Accession Number	SUMO-1	SUMO-3	Molecular Function
Hypothetical protein (Fragment)	trm Q8N395	1 / 71		
Hypothetical protein (Fragment)	trm Q9BTD2	8 / 105	7 / 104	Cytoskeletal protein->Actin family
Hypothetical protein (Fragment)	trm Q9Y3V8	3 / 78		Nucleic acid binding->Helicase->RNA helicase
Hypothetical protein DKFZp434H0127.1 - human (fragment)	pir T42688	1 / 52		Molecular function unclassified
Hypothetical protein FLJ10903	trm Q9NV63	2 / 51		Nucleic acid binding->Histone
Hypothetical protein FLJ11012	trm Q9NV06	2 / 66		Miscellaneous function->Other miscellaneous function
Hypothetical protein FLJ12489	trm Q9H9X4		1 / 50	
Hypothetical protein FLJ23109	trm Q9H5S6	2 / 48		
Hypothetical protein FLJ36350	trm Q8N211	3 / 70	3 / 69	Transcription factor->Zinc finger
Hypothetical protein KIAA1321 (Fragment)	trm Q9P2M5	6 / 86		
Hypothetical protein KIAA1401 (Fragment)	trm Q9P2E6	3 / 47		Molecular function unclassified
Hypothetical protein KIAA1805	trm Q96ME7	4 / 76		
KIAA1969 protein	dbj BAB85555.1		4 / 69	Transcription factor->Zinc finger
Lactate dehydrogenase B	rf NP_002291.1		1 / 48	Oxidoreductase->Dehydrogenase
M4 protein deletion mutant	trm Q9Y492		7 / 69	Nucleic acid binding->Ribonucleoprotein
Mitochondrial 60S ribosomal protein L27 (L27mt) (HSPC250)	spt Q9P0M9	2 / 45		Molecular function unclassified
Multiple myeloma transforming gene 2	trm Q8IZH3	3 / 69		Molecular function unclassified
NFAR (Nuclear Factor Associated with dsRNA) or ILF3	gb AAK07425.1	3 / 136	2 / 92	Nucleic acid binding->Other RNA-binding protein
Non-POU-domain-containing, octamer-binding	trm Q9BQC5		18 / 172	Transcription factor; Nucleic acid binding
Nucleophosmin (NPM) (Nucleolar phosphoprotein B23) (Numatrin) (Nucleolar protein NO38)	spt Q96DC4		1 / 50	Chaperone->Other chaperones
PHD-like zinc finger protein	trm Q8IWS0	2 / 64		
Poly(ADP-ribose) synthetase	gb AAB59447.1	9 / 54		Transferase->Glycosyltransferase
Polypyrimidine tract-binding protein 1 (Heterogeneous nuclear ribonucleoprotein I), isoform 2	trm Q9BUQ0		4 / 63	Nucleic acid binding->mRNA processing factor
Polypyrimidine tract-binding protein 1 (Heterogeneous nuclear ribonucleoprotein I), isoform 1	spt P26599	3 / 79		Nucleic acid binding->mRNA processing factor
Poly [ADP-ribose] polymerase-1 (PARP-1) (ADPRT)	sp P09874		7 / 63	Transferase->Glycosyltransferase
Probable ATP-dependent RNA helicase p47 (HLA-B associated transcript- 1)	spt Q13838	2 / 48		Nucleic acid binding->Helicase->RNA helicase

Protein Name	Accession Number	SUMO-1	SUMO-3	Molecular Function
Pyrroline-5-carboxylate synthase	gb AAD00169.1	5 / 48		Kinase; Synthase and synthetase; Oxidoreductase
Ran (GTP-binding nuclear protein Ran)	spt P62826	1 / 81		Select regulatory molecule->G-protein->Small GTPase
Ribosomal protein L27a	dbj BAA25837.1	2 / 84	2 / 76	
Ribosomal protein L18a (60S subunit)	spt Q02543		2 / 89	Nucleic acid binding->Ribosomal protein
Ribosomal protein S18 (KE-3) (40S subunit)	spt P25232	2 / 53		Nucleic acid binding->Ribosomal protein
RNA helicase-related protein	trm Q86XP3		6 / 95	Nucleic acid binding->Helicase->RNA helicase
RNA helicase-related protein (SF3b125 DEAD-box protein)	trm Q96BK1	6 / 78		Nucleic acid binding->Helicase->RNA helicase
Rotamer Strain As A Determinant Of Protein Structural Specificity	pdb 1C3T_A	1 / 48		Molecular function unclassified
RPL8 protein	gb AAH00047.2	1 / 72	2 / 60	Nucleic acid binding->Ribosomal protein
Scaffold attachment factor B	rf NP_002958.2		3 / 73	Molecular function unclassified
Scaffold attachment factor B2	spt Q14151		4 / 73	Molecular function unclassified
SFPQ protein	gb AAH04534.2	21 / 160		Transcription factor; Nucleic acid binding
Similar to 60S ribosomal protein L15	rf XP_166446.1	1 / 71		Nucleic acid binding->Ribosomal protein
Similar to cytochrome c oxidase subunit IV isoform 1	trm Q86WV2	1 / 49		
Similar to cytoplasmic beta-actin	rf XP_293924.1	2 / 105	3 / 104	Cytoskeletal protein->Actin family
Similar to Hypothetical zinc finger protein KIAA1473	rf XP_047554.5	4 / 70		Transcription factor->Zinc finger
Similar to Hypothetical zinc finger protein KIAA1473	rf XP_294565.2	3 / 70		Transcription factor->Zinc finger
Similar to Hypothetical zinc finger protein KIAA1473	rf XP_294565.2		2 / 69	Transcription factor->Zinc finger
Similar to KIAA0326	rf XP_034819.2	3 / 58		Transcription factor->Zinc finger
Similar to KIAA0542 protein	rf XP_038520.4	2 / 53		Nucleic acid binding->Ribosomal protein
Similar to KRAB zinc finger protein	rf XP_056426.2	4 / 70		Transcription factor->Zinc finger
Similar to pote protein	rf XP_351497.1	7 / 105	4 / 104	Molecular function unclassified
Similar to ribosomal protein L18a	rf XP_293412.1	3 / 66	2 / 89	Nucleic acid binding->Ribosomal protein
Similar to RP2 protein, testosterone-regulated - ricefield mouse (Mus caroli)	rf XP_352259.1	2 / 52		Molecular function unclassified
Similar to SMT3 suppressor of mif two 3 homolog 2	rf XP_168354.2		2 / 104	Miscellaneous function->Other miscellaneous function
Similar to stratifin	trm Q96DH0	2 / 59		Select regulatory molecule->Kinase modulator

Protein Name	Accession Number	SUMO-1	SUMO-3	Molecular Function
Similar to transketolase (Wernicke-Korsakoff syndrome) (Fragment)	trm Q8TBA3		1 / 69	Transferase->Transketolase
Similar to tubulin alpha 2	trm Q8WU19	2 / 60		Cytoskeletal protein->Microtubule family
Similar to Zinc finger protein 208	rf XP_088081.2		2 / 69	Transcription factor->Zinc finger
Similar to Zinc finger protein 208	rf XP_092087.4		2 / 69	Transcription factor->Zinc finger
Similar to zinc finger protein 208	trm Q8IYN0	6 / 70		Transcription factor->Zinc finger
Similar to zinc finger protein 257	trm Q8NE34	3 / 70		Transcription factor->Zinc finger
Similar to zinc finger protein 91 (HPF7, HTF10)	rf XP_065116.5	4 / 70		Transcription factor->Zinc finger
Similar to zinc finger protein 91 (HPF7, HTF10)	rf XP_065124.5	2 / 70		Transcription factor->Zinc finger
Similar to zinc finger protein 91 (HPF7, HTF10)	rf XP_171973.3	3 / 70		Molecular function unclassified
Similar to zinc finger protein 91 (HPF7, HTF10)	rf XP_352255.1		2 / 69	Transcription factor->Zinc finger
Similar to Zinc finger protein 93 (Zinc finger protein HTF34)	rf XP_292832.3	3 / 70	2 / 69	Transcription factor->Zinc finger
Similar to zinc finger type transcription factor MZF-3	rf XP_351686.1		2 / 47	Transcription factor->Zinc finger
Similar to ZNF43 protein	rf XP_292838.2		2 / 69	Transcription factor->Zinc finger
SMT3 suppressor of mif two 3 homolog 1	rf NP_008867.1		3 / 104	Miscellaneous function->Other miscellaneous function
Solute carrier family 39 (zinc transporter), member 7	rf NP_008910.1	1 / 65		Molecular function unclassified
Spliceosome-associated protein SAP 62 - human	pir A47655	3 / 75	2 / 64	
Splicing factor 3B subunit 4 (Spliceosome associated protein 49) (SAP 49) (SF3b50)	spt Q15427	5 / 112	6 / 136	Nucleic acid binding->mRNA processing factor
Splicing factor homolog - human	pir S41768	21 / 176		Transcription factor; Nucleic acid binding
Stromal cell-derived factor 2 precursor	rf NP_008854.2	2 / 55		Molecular function unclassified
Small Ubiquitin-Related Modifier SUMO-1	spt P63165	1 / 50		Miscellaneous function->Other miscellaneous function
Testis specific ZFP91	trm Q96JP4	3 / 45		Transcription factor->Zinc finger
TRAF6-binding zinc finger protein	trm Q8TD23		2 / 69	Transcription factor->Zinc finger
Transcription factor ZFM1	gb AAB03514.1	5 / 141		
Transcription initiation factor IIA alpha and beta chains (TFIIA p35 and p19 subunits) (TFIIA-42)	spt P52655	2 / 71	2 / 77	
Transcription intermediary factor 1-beta (TIF1-beta; KAP-1)	gb AAB37341.1		2 / 48	Transcription factor->Transcription cofactor
Transcriptional repressor protein Yin and Yang 1 (YY1)	gb AAA59926.1	7 / 112	7 / 116	Transcription factor->Zinc finger transcription factor
Tubulin alpha 1	emb CAA25855.1		2 / 71	Cytoskeletal protein->Microtubule family
Tubulin alpha-6 chain (Alpha-tubulin 6)	spt Q9BQE3	2 / 60		Cytoskeletal protein->Microtubule family

Protein Name	Accession Number	SUMO-1	SUMO-3	Molecular Function
Tubulin beta-1 chain	spt P07437	6 / 90	5 / 51	Cytoskeletal protein->Microtubule family
Ubiquitin activating enzyme E1	emb CAA40296.1		4 / 53	Ligase->Other ligase
Ubiquitin-52 amino acid fusion protein	trm Q9UPK7	1 / 48		Molecular function unclassified
Unknown (protein for MGC:10722)	gb AAH06248.1		2 / 58	Molecular function unclassified
Unnamed protein product	dbj BAC87465.1		3 / 69	Transcription factor->Zinc finger
YWHAZ protein (Fragment)	trm Q86V33	2 / 59		Select regulatory molecule->Kinase modulator
ZIC2 protein	gb AAC96325.1	3 / 52		Transcription factor->Zinc finger
Zinc finger protein	emb CAB70967.1	4 / 82	3 / 69	Transcription factor->Zinc finger
Zinc finger protein 121 (Zinc finger protein 20) (Fragment)	spt P58317	1 / 57		Molecular function unclassified
Zinc finger protein 141	spt Q15928	2 / 70	2 / 69	Transcription factor->Zinc finger
Zinc finger protein 15 (Zinc finger protein KOX8) (Fragment)	spt P17019	2 / 70	2 / 69	Transcription factor->Zinc finger
Zinc finger protein 431	spt Q8TF32	6 / 70		Transcription factor->Zinc finger
Zinc finger protein 91 (Zinc finger protein HTF10) (HPF7)	spt Q05481	5 / 70	4 / 69	Transcription factor->Zinc finger
Zinc finger protein ZFD25	spt Q9UII5	3 / 70		Transcription factor->Zinc finger
ZNF207 protein	gb AAH08023.1		1 / 92	

*The fraction numbers in the SUMO-1 and SUMO-3 columns indicate # of identified peptides / highest peptide score

Table 2

Proteins identified in the negative control (F293 parental cell line) and in the F293-SUMO cell lines by LC-MALDI-MS/MS analysis of TAP purified proteins

Protein Name	Accession Number	SUMO-1	SUMO-3	Parental	Molecular Function
Enteropeptidase precursor (EC 3.4.21.9) (Enterokinase)	spt P98073	1 / 63			Protease->Serine protease
Enteropeptidase precursor (EC 3.4.21.9) (Enterokinase)	spt P98073		1 / 68		Protease->Serine protease
Enterokinase	dbj BAA95557.1			1 / 35	Protease->Serine protease
SF1-Bo isoform	emb CAA70019.1		5 / 125		
SF1-Bo isoform	emb CAA70019.1			1 / 28	
Splicing factor proline/glutamine rich (Polypyrimidine tract binding protein associated)	trm Q86VG2		16 / 157		Transcription factor; Nucleic acid binding
Splicing factor proline/glutamine rich (Polypyrimidine tract binding protein associated)	trm Q86VG2			1 / 21	Transcription factor; Nucleic acid binding

*The fraction numbers in the SUMO-1, SUMO-3, and Parental columns indicate # of identified peptides / highest peptide score