

Defining the SUMO-modified Proteome by Multiple Approaches in *Saccharomyces cerevisiae**[§]

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SUMO, or Smt3 in *Saccharomyces cerevisiae*, is a ubiquitin-like protein that is post-translationally attached to multiple proteins *in vivo*. Many of these substrate modifications are cell cycle-regulated, and SUMO conjugation is essential for viability in most eukaryotes. However, only a limited number of SUMO-modified proteins have been definitively identified to date, and this has hampered study of the mechanisms by which SUMO ligation regulates specific cellular pathways. Here we use a combination of yeast two-hybrid screening, a high copy suppressor selection with a SUMO isopeptidase mutant, and tandem mass spectrometry to define a large set of proteins (>150) that can be modified by SUMO in budding yeast. These three approaches yielded overlapping sets of proteins with the most extensive set by far being those identified by mass spectrometry. The two-hybrid data also yielded a potential SUMO-binding motif. Functional categories of SUMO-modified proteins include SUMO conjugation system enzymes, chromatin- and gene silencing-related factors, DNA repair and genome stability proteins, stress-related proteins, transcription factors, proteins involved in translation and RNA metabolism, and a variety of metabolic enzymes. The results point to a surprisingly broad array of cellular processes regulated by SUMO conjugation and provide a starting point for detailed studies of how SUMO ligation contributes to these different regulatory mechanisms.

Many types of post-translational protein modifications alter protein function, and in some cases the modifying group is itself a protein. The prototypical example of this is ubiquitin, a small, highly conserved polypeptide that is reversibly linked to many

different proteins (probably thousands) in the cell (1). Polypeptides distinct from but related to ubiquitin, called ubiquitin-like proteins or UbIs, can also be ligated to proteins (2, 3). Ligation to each Ubl has unique mechanistic and functional consequences. SUMO (Smt3 in the yeast *Saccharomyces cerevisiae*) is a divergent Ubl that has crucial roles in many organisms (4, 5). Vertebrates have four SUMO variants, SUMO1–SUMO4, whereas yeasts have only one. Only human SUMO1, and not the other SUMO variants, can substitute for the essential yeast protein (6).

Activation and conjugation reactions involving SUMO have much in common with those of ubiquitin (reviewed in Refs. 2, 5, and 7). Attachment of substrates to Smt3/SUMO depends on a heterodimeric SUMO-activating enzyme (E1),¹ called Uba2-Aos1 in yeast, and a SUMO-conjugating enzyme (E2), Ubc9. Both enzymes form transient thioester bonds with the C terminus of SUMO. Substrate recognition factors (E3s) that stimulate the transfer of SUMO from E2 to substrate have also been identified. SUMO, like ubiquitin, is always synthesized in precursor form, requiring enzymatic removal of a C-terminal peptide. Specialized proteases, called Ubl-specific proteases or Ulp1s, are responsible for these SUMO processing reactions and for reversing the post-translational attachment of SUMO to proteins.

Unlike ubiquitin ligation, many sumoylation sites in proteins match a short consensus sequence, hKXE, where h represents a hydrophobic residue, X is any residue, and lysine (K) is the site of SUMO attachment (5). This consensus can be rationalized by structural data showing the direct binding of the Ubc9 E2 to the conserved elements of this sequence (8, 9). This E2-substrate interaction is unlikely to be sufficient for specific substrate discrimination in the cell, so E3 factors are expected to be important for achieving the requisite *in vivo* specificity.

The SUMO system has been implicated in multiple physiological pathways (3–5). *SMT3*, as well as the genes encoding the SUMO E1, E2, and Ulp1 protease, are all essential for viability in budding yeast, and cells with a conditional allele of either *UBC9* or *ULP1* are defective in cell cycle progression. The first identified target of SUMO conjugation was vertebrate RanGAP1, a protein required for nucleocytoplasmic trafficking. Modification by SUMO is required for RanGAP1 localization to the nuclear pore complex. Among the many (>50) mammalian targets for SUMO now known are RanBP2, another nuclear pore complex component; PML, a nuclear protein that is altered in certain leukemias; Sp100, an autoantigen in primary biliary cirrhosis;

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¹ The abbreviations used are: E1, SUMO-activating enzyme; E2, SUMO-conjugating enzyme; E3, SUMO-protein ligase; ORF, open reading frame; MS, mass spectrometry; GBD, Gal4 DNA-binding domain; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

TABLE I
Yeast strains

| Strain | Genotype |
|---------|---|
| MHY501 | α <i>his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52</i> |
| MHY1380 | α <i>his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 ulp2Δ::HIS3</i> |
| MHY1538 | α <i>his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 smt3Δ::HIS3 ulp1Δ::HIS3[pVT102U-SMT3gg YCp50-ULP1]</i> |
| MHY2809 | α <i>his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 smt3Δ::HIS3 ulp1Δ::HIS3[YRTAG310-H6-SMT3gg]</i> |
| MHY2811 | α <i>his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 smt3Δ::HIS3 ulpΔ::HIS3[YRTAG310-HFT-SMT3gg]</i> |
| PJ69-4A | <i>a trp1-901 leu2-3,112 ura3-52 his3-Δ200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i> |

the p53 tumor suppressor; the Sp3 transcription factor; and the NF- κ B inhibitor I κ B. Even this partial list of *in vivo* targets makes it clear that the SUMO system must exert a major influence on mammalian growth and regulation. Misregulation of the system is likely to contribute to tumorigenesis, abnormal inflammatory responses, and autoimmune defects.

In yeast, a much more limited number of SUMO-linked substrates has been characterized. The first was a subset of the septins, which are proteins essential for cytokinesis; however, eliminating septin sumoylation has no detectable phenotypic effect (10, 11). Yeast topoisomerase II is also sumoylated *in vivo*, and this modification, while not required for the essential function of the enzyme, may contribute to the cohesive properties of the centromere (12). SUMO is also ligated to the DNA replication/repair protein PCNA (Pol30), and sumoylation inhibits the DNA repair activity of PCNA (13, 14). Although all of these substrate proteins are essential for growth, in no case is their sumoylation required for viability. Therefore, the protein or proteins that must be sumoylated for cell division in yeast remain to be identified. Additional proteins involved in chromosome cohesion, Pds5 and Ysc4, have also recently been reported to be transiently sumoylated in yeast, but the sites of sumoylation were not mapped (15, 16).

Determination of the precise functions of specific SUMO-protein modifications in *S. cerevisiae* should generally be more facile than in other eukaryotes. However, a much more comprehensive list of sumoylated proteins in this organism must first be obtained. Toward this end, we have taken several independent genetic and proteomic approaches. Our data reveal a surprisingly large and functionally diverse set of SUMO system substrates. Interestingly, the different methods of substrate identification have yielded overlapping but distinct groups of proteins, underscoring the value of using multiple approaches to establish the SUMO proteome.

EXPERIMENTAL PROCEDURES

Yeast and Bacterial Methods—Rich (YPD) and minimal (SD) media were prepared as described (17). Standard methods were used for genetic analysis of yeast (17). The *Escherichia coli* strains used in this study were JM101, MC1061, BL21(DE3), and DH10B, and bacterial methods and media were as described (18).

Construction of Yeast Strains—Yeast strains used are listed in Table I. The strain background for all experiments other than the two-hybrid screen was MHY501 (19). To make the MHY2811 strain for purification of HFT-SUMO-protein conjugates, MHY1538 was transformed with YRTAG310-HFT-SMT3GG, and transformants were plated on 5-fluorouracil to select for cells that had lost the two original *URA3*-marked plasmids. Other strains listed were generated by standard molecular genetic methods. The library of yeast strains with genes fused to the TAP tag was obtained from Open Biosystems (Huntsville, AL).

Plasmid Constructions—The plasmid pGBD-U-C1-SMT3 for the two-hybrid screen was constructed by ligating a BamHI, SalI-cut PCR product that contained the entire *SMT3* open reading frame (ORF) into pGBD-U-C1 (20). Derivatives of this plasmid that lacked either N-terminal or C-terminal coding sequences of *SMT3* were made by EcoRI digestion and religation of the plasmid to make pGBD-SMT3-C30 or by BglII digestion and religation to make pGBD-SMT3-N30, respectively. Plasmid pGBD-SMT3 Δ GG was made by PCR amplification of *SMT3* sequence encoding its first 96 residues and ligation into BamHI, SalI-cut pGBD-U-C1.

Plasmid pQE30-FLAG-TEV-SMT3GG was made by PCR amplifica-

tion of the *SMT3* ORF using a 5' primer that included a BamHI site and sequences encoding a FLAG epitope (DYKDDDDK) followed by the tobacco etch virus recognition sequence (TEV; ENLYFQ/G) and a 3' primer with sequences for *SMT3* codons 90–98 followed by a stop codon and a SalI recognition site. The PCR product was cut with BamHI and SalI and ligated to the identically digested pQE30 (Qiagen) His₆-tagging vector. A DNA fragment encoding His₆-FLAG-TEV-SMT-GG was then amplified by PCR, digested with SalI and SacI, and cloned into the yeast *CUP1*-driven expression vector YRTAG310 that had been cut with XhoI and SacI, creating YRTAG310-HFT-SMT3GG. *SMT3* and FLAG-TEV-tagged *SMT3* alleles encoding mature SUMO were also amplified by PCR and were cloned into pRS426GAL for galactose-inducible expression. Insert sequences were confirmed by DNA sequencing.

Two-hybrid Screen with SUMO Bait—Full-length *SMT3* (mature domain) was fused to the sequence encoding the Gal4 DNA-binding domain. This was used as bait for a two-hybrid screen of yeast plasmid libraries made of partial digested *S. cerevisiae* genomic sequences ligated to a sequence encoding the Gal4 activation domain (20). Purified DNA from the libraries was transformed into yeast strain PJ69-4A carrying the bait plasmid pGBD-U-C1-SMT3, and transformants were grown on triple dropout plates (SD-trp-leu-his) for 5 days at 30 °C. The PJ69-4A strain carries three reporter genes (*HIS3*, *ADE2*, and *lacZ*), each under the control of a different *GAL* promoter. Transformants that grew on the triple dropout plates (his⁺ and therefore able to activate *GAL-HIS3*) were rescreened on SD-trp-leu-ade dropout plates to test *ADE2* expression and on X-gal-containing plates to test *lacZ* expression. Plasmids were isolated from yeast transformants that were his⁺ ade⁺ and blue on X-gal. The plasmids were retransformed into the two-hybrid tester strain to confirm the two-hybrid signal, and the plasmid insert junctions were sequenced using the suggested upstream and downstream primers (20). We tested the positive clones for interactions with the SUMO deletion mutant pGBD-SMT3 Δ GG and in a tester strain that also carried *ULP1* on a 2- μ m plasmid.

Selection for High Copy Suppressors of *ulp2 Δ* —Selection of dosage suppressors of *ulp2 Δ* temperature-sensitive growth at 37 °C was done by transformation of the *ulp2 Δ* strain MHY1380 (21) with a YE_p24 (2 μ m, *URA3*)-based yeast genomic library. Transformants were plated on uracil dropout plates and incubated at 37 °C and, as a control, 30 °C. Approximately one in 10⁴ to 10⁵ ura⁺ transformants appeared as colonies on the 37 °C plates after 7 days. The ura⁺ thermoresistant colonies were restreaked at 37 °C to confirm the phenotype. Plasmid DNA was isolated from individual clones and transformed again into MHY1380 to test for the plasmid dependence of high temperature growth. Nine plasmid-dependent, temperature-resistant clones from ~10⁶ MHY1380 transformants were identified. Vector-insert junctions in these plasmids were determined by DNA sequencing and data base searching. For the four strongest suppressors, DNA subcloning and deletion was used to identify the genes responsible for the suppression.

Immunoblotting—Proteins were electrotransferred to polyvinylidene difluoride membranes using either a GENIE blotter at 20 V for 2 h at 4 °C or a Bio-Rad semidry blotter at 25 V for 30 min at room temperature. The membrane was blocked with 10% skim milk in TBST solution (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% (v/v) Tween 20) for 1 h at room temperature and washed three times with TBST for 10 min. Primary antibodies diluted 1:3,000 to 1:10,000 in TBST with 1% skim milk were incubated with membrane for 1–2 h. The membrane was washed with TBST and incubated for 1 h with secondary antibody diluted 1:4,000 in TBST. After washing with TBST, antibody binding was detected using ECL Western blotting reagents (Amersham Biosciences). SUMO conjugates were visualized with a purified rabbit polyclonal antibody raised against yeast SUMO (22).

Purification of SUMO-Protein Conjugates from Yeast—MHY2811 yeast cells were grown in 4 liters of YPD (no added copper) at 24 °C to an A₆₀₀ of 0.5–1.0. Approximately 7–14 g of cells (wet weight) were pelleted, washed twice with deionized water, and shock-frozen in liquid

nitrogen. Frozen cells were ground to a powder to facilitate later resuspension in lysis buffer and were stored at -80°C . Cells were resuspended in 3 volumes of extraction buffer (8 M guanidinium HCl, 100 mM sodium phosphate, pH 8) at room temperature. Cells were lysed at room temperature with three passes through a French press at 18,000 p.s.i. The lysate was cleared at $30,000 \times g$ for 30 min at room temperature, and the supernatant was carefully removed with a pipette from below the cloudy lipid layer on top.

The cleared lysate was bound to 2–4 ml of Talon beads for 60 min at room temperature. Protein-bound beads were then loaded into a column and washed three times with 10 volumes of wash buffer (8 M urea, 100 mM sodium phosphate, pH 8). Bound proteins were eluted with 300 mM imidazole in wash buffer, and 2-ml fractions were collected. Eluates were pooled, and the resulting 10–20-ml sample was diluted into 24 volumes of FLAG-binding buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40) with protease inhibitors, in which 0.1 volumes of anti-FLAG beads (Sigma) were already present. After binding in batch for 90 min at 4°C , the beads were transferred to a column, drained, and washed with 100–200 ml of FLAG wash buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Nonidet P-40) and then 10–20 ml of TEV cleavage buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, 1 mM dithiothreitol). The drained beads were resuspended in 1–2 ml of TEV cleavage buffer containing 100 units of recombinant TEV protease (Invitrogen). TEV cleavage was executed in the sealed column on a shaker at 16°C for at least 16 h. The column was drained and then washed several times with TEV cleavage buffer to collect all of the eluted protein. Samples of 2 ml were collected. Eluate 1 (E1) is the material from the original draining plus one column volume of wash, and E2 the following 2 column volumes of washings. The anti-FLAG beads were stripped with 0.1 M glycine at pH 3.5. Samples were precipitated with 10% trichloroacetic acid at -20°C for 2 h and spun down at $16,000 \times g$ at 4°C . Pellets were washed four times with ice-cold acetone to remove residual Nonidet P-40. Protein samples were checked by anti-SUMO immunoblot analysis and by Gel Code Blue (Pierce) staining.

Mass Spectrometry and Computational Methods—The final purified protein fractions were reduced with 1 mM dithiothreitol and then carboxyamidomethylated with 5 mM iodoacetamide. The samples were diluted with 50 mM ammonium bicarbonate, pH 8.5, and digested with 1:20 (v/v) Poroszyme-immobilized trypsin (Applied Biosystems, Streetsville, Canada) for 48 h at 30°C with rotation. The resulting peptide mixtures were solid phase-extracted with SPEC-Plus PT C18 cartridges (Ansys Diagnostics; Lake Forest, CA) according to the manufacturer's instructions. The resulting peptide mixtures were sequenced by shotgun tandem mass spectrometry using the multidimensional protein identification technology (MudPIT) of Yates and colleagues (23), as adapted by Kislinger *et al.* (24). Briefly, a 150- μm inner diameter fused silica capillary microcolumn (Polymicro Technologies; Phoenix, AZ) was pulled to a fine tip using a P-2000 laser puller (Sutter Instruments; Novato, CA) and packed to a 10-cm bed height with 5 μM Zorbax Eclipse XDB-C₁₈ resin (Agilent Technologies, Mississauga, Canada) followed by 6 cm of 5 μM Partisphere strong ion exchanger (Whatman). The sample was loaded manually onto the column using a pressure vessel and then connected in-line to a quaternary high pressure liquid chromatography pump and interfaced with an LCQ DECA XP quadrupole ion trap tandem mass spectrometer (Thermo Finnigan; San Jose, CA). The eluate was analyzed by electrospray ionization using a fully automated four-buffer, 15-step chromatographic cycle essentially as described (24). Individual eluting peptides were subject to automated, data-dependent collision-induced fragmentation.

The peptide spectra were searched against a locally maintained data base of yeast protein sequences obtained from the *Saccharomyces* Genome Database (around August 2001) using a distributed version of the SEQUEST algorithm running on a multiprocessor computer cluster (25) and filtered for high confidence protein matches (p value <0.05) using the STATQUEST statistical evaluation algorithm (24). The data sets were arranged into tabular format using the DTASelect and Contrast software programs (26) and subgrouped into select gene ontology functional annotation categories (27) using the FunSpec program (28).

RESULTS AND DISCUSSION

We have undertaken a series of approaches to identify sumoylated proteins in yeast. These different methods were expected to yield different but related kinds of information about SUMO-protein ligation and the roles of SUMO modification in the cell.

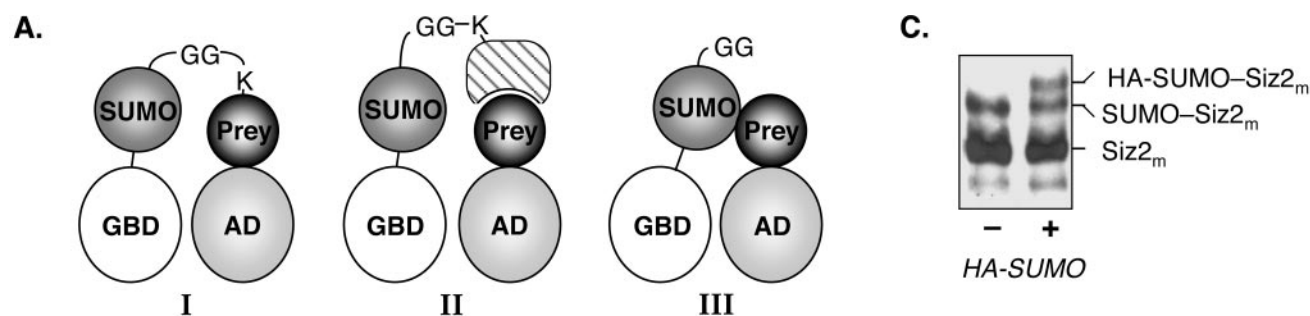
A "Covalent Interaction Trap" for SUMO-linked Proteins—A

protein that can be covalently coupled to other proteins offers an interesting type of bait in an otherwise standard two-hybrid screen for protein-protein interactions (Fig. 1A). We fused SUMO (Smt3) to the C terminus of the Gal4 DNA-binding domain (GBD), creating a fusion that can functionally replace normal SUMO (data not shown). Unlike most baits, a positive signal in the two-hybrid screen can be generated by covalent ligation of the bait, GBD-SUMO, to a library substrate protein fused to the Gal4 transcriptional activation domain (AD) (Fig. 1A, I). Alternatively, GBD-SUMO may get attached to an endogenous yeast protein that then can interact with the activation domain fusion (Fig. 1A, II). Finally and more conventionally, GBD-SUMO could interact noncovalently with an activation domain fusion (Fig. 1A, III). As will be described, it is possible to distinguish ligation-dependent association from noncovalent interactions. Thus, the screen could identify both SUMO-conjugated substrates and proteins that bind noncovalently to SUMO or SUMO-protein conjugates. The latter proteins may be SUMO pathway regulators or enzymes of the SUMO system.

We screened $\sim 5 \times 10^5$ yeast genomic clones and identified segments of 13 different proteins that gave strong two-hybrid interaction signals with three reporter genes, each under the control of a different *GAL* promoter (20) (Fig. 1B). To determine whether any of the two-hybrid interactions were likely to depend on SUMO ligation, we tested them against a GBD-SUMO Δ GG fusion, which lacks the terminal pair of glycines of SUMO and therefore cannot be attached to other proteins (29). Seven of the tested interactions appear to require SUMO ligation because the interaction signal was no longer seen with GBD-SUMO Δ GG (*minus signs* in Fig. 1B), as predicted for binding modes I and II in Fig. 1A. We also tested the sensitivity of these interactions to Ulp1 isopeptidase overexpression, since most SUMO-protein conjugates are depleted in cells overproducing this enzyme (22). Of the seven SUMO Δ GG-sensitive interactors, five were also sensitive to Ulp1 overproduction; some SUMO conjugates might be poor substrates for Ulp1 *in vivo*, which might account for the two exceptions (Hex3 and Ufd1). In contrast, all but one of the identified prey proteins that also bound SUMO Δ GG still interacted with SUMO in the presence of high Ulp1. *ULP1*^{HC}-sensitive, SUMO diglycine-dependent interactors represent strong candidates for SUMO-linked substrates or proteins that bind such substrates.

Among the 13 identified SUMO-interacting proteins are proteins involved in RNA metabolism, DNA repair, protein degradation, and gene silencing. Two of the proteins, Siz2/Nfi1 and Wss1, had been linked to the SUMO pathway in previous studies (11, 30). Siz2 is an SP-RING-bearing E3-like factor involved in protein sumoylation, although its substrates are not known. The function of Wss1 is unclear, but it is a weak, high copy suppressor of a temperature-sensitive *smt3* mutant. Interestingly, Siz2 and Nis1 have both been found to bind to septins, which are the most abundant sumoylated proteins in yeast. The septins localize to the bud neck during cell division, and another two-hybrid hit, Fir1, also localizes to this region.

Besides Siz2, two other proteins, Ris1 and Hex3, contain a RING motif, a signature sequence for E3 ligases. Therefore, these proteins might be SUMO E3-like enzymes as well. Both Siz2 and Hex3 require a conjugation-competent form of SUMO for detection of a two-hybrid interaction. RING E3s in the ubiquitin pathway frequently ubiquitinate themselves. Auto-sumoylation might be occurring with Siz2 and Hex3, but the minimal segments of these two proteins that interacted with GBD-SUMO in the two-hybrid screen lacked the catalytic RING motif, which would suggest that sumoylation was occurring *in trans*.



B.

| Protein | Independent Hits | GBD-SUMOΔGG fusions | Ulp1 ^{HC} | GBD-Ubc9 | Interaction boundaries | Putative functions / domains |
|---------|------------------|---------------------|--------------------|----------|------------------------|-----------------------------------|
| Fir1 | 8 | 8 | + | + | H654-P842 (876 aa) | mRNA 3' processing |
| Ris1 | 6 | 4 | + | + | G336-E599 (1619 aa) | gene silencing; ATPase; RING |
| Sap1 | 3 | 3 | + | + | S179-F264 (897 aa) | AAA+ ATPase |
| Nis1 | 3 | 3 | + | + | S355-end (407 aa) | Interacts with septins |
| Srs2* | 1 | 1 | +/- | - | S1010-end (1174 aa) | DNA helicase; DNA repair |
| Siz2 | 2 | 2 | - | - | A486-D722 (726 aa) | SUMO E3; SP-RING |
| Hex3 | 4 | 3 | - | + | R38-F443 (619 aa) | DNA repair; RING |
| Ufd1 | 5 | 4 | - | + | A290-end (361 aa) | ER protein degradation |
| Yen1 | 5 | 5 | - | - | S563-end (759 aa) | Possible DNA nuclease |
| Psa1 | 1 | 1 | - | - | D212-end† (361 aa) | GDP-mannose pyrophosphorylase |
| Kns1 | 1 | 1 | - | - | R12-T170 (737 aa) | Ser/Thr protein kinase homolog |
| Wss1 | 1 | 1 | ND | ND | G227-end (269 aa) | Weak suppressor of <i>smt3-ts</i> |
| YGL250w | 1 | 1 | - | - | V51-D238 (245 aa) | Uncharacterized ORF |

FIG. 1. **A variant yeast two-hybrid screen to identify sumoylated proteins.** *A*, three different ways by which a GBD fusion to SUMO can interact with a prey-Gal4 activation domain (AD) fusion to generate a two-hybrid signal. For *I* and *II*, but not *III*, the interaction should be lost if a conjugation-incompetent GBD-SUMOΔGG bait is used and should generally also be impaired if the broad specificity SUMO protease Ulp1 is overexpressed in the tester strain. *B*, summary of yeast proteins identified by two-hybrid screening with GBD-SUMO. A plus sign in any of the columns indicates that a two-hybrid interaction is seen, whereas a minus sign denotes the absence of a signal. The "interaction boundaries" were deduced from the overlap of different two-hybrid clones identified by the screen; each pair represents the maximum segment that is sufficient for interaction. The size of the full-length ORF is given in parentheses. *C*, verification of SUMO ligation to Siz2 by a mobility shift assay. The chromosomal *SIZ2* gene was tagged with a 13× Myc (*m*) epitope-expressing DNA segment. Shown is an anti-Myc immunoblot of extracts from cells either expressing or not expressing a hemagglutinin epitope-tagged version of the SUMO protein in addition to the untagged chromosomally expressed copy. *, weak background signal with GBD bait lacking SUMO. †, 3' junction not sequenced. ND, not determined.

We chose Siz2 to determine whether it is in fact sumoylated *in vivo* and to validate the use of the two-hybrid screen to identify substrates for SUMO ligation. Previously, we had developed a gel shift assay to demonstrate *in vivo* protein ubiquitination (31); an analogous strategy was used here to test sumoylation. When Myc-tagged Siz2, expressed from the normal chromosomal locus, was analyzed by anti-Myc immunoblotting, we detected a major band and a second, more slowly migrating, species, which potentially represented a sumoylated form of Siz2 (Fig. 1C, left lane). To test this, a hemagglutinin epitope-tagged version of SUMO was transformed into the same strain. If the upper Siz2 band were SUMO-modified, its mobility should be further reduced by the N-terminally extended SUMO. Indeed, a slower migrating species was detectable by anti-Myc blotting, and the original band above unmodified Siz2 was reduced in intensity (Fig. 1C, right lane). These data indicate that Siz2 could be monosumoylated by either endogenous SUMO or plasmid-encoded hemagglutinin-SUMO, leading to a mixture of the two higher mass species.

A Potential SUMO-binding Motif—From the two-hybrid analysis, four proteins (Fir1, Ris1, Sap1, and Nis1) still bound to a nonconjugatable form of SUMO and were unaffected by Ulp1 overexpression. These factors were therefore candidates for proteins that bound noncovalently to SUMO or SUMO conjugates. We used the minimal SUMO-binding domains

found in these proteins to search for potential conserved elements using BLOCKS (32). A consensus sequence for a conserved segment was derived (Fig. 2A). The consensus is characterized by a cluster of 3 or 4 aliphatic residues followed by a cluster of 3 or 4 negatively charged amino acids. We used ScanProsite (33) to identify additional occurrences of the consensus sequence in the yeast proteome. Only 11 proteins besides the four input sequences were found (Fig. 2B). One of them was in the N-terminal domain of Cdc48, a broadly conserved AAA ATPase with multiple functions. Interestingly, Cdc48 associates with Ufd1, which we identified in our two-hybrid screen as a protein that bound to SUMO but not SUMOΔGG (Fig. 1B). It is tempting to speculate that the interaction between Ufd1 and Cdc48 is modulated by sumoylation of the Ufd1 protein (also see below).

The consensus sequence we derived is related but not identical to a SUMO-binding motif (SBM), recently suggested from a combination of bioinformatics and NMR analyses with the SUMO-binding protein RanBP2 (34). The consensus sequence derived in this latter study, (V/I)X(V/I)(V/I), is related to the core hydrophobic region shown in Fig. 2A. The same researchers found that RanBP2 binds to a region of SUMO that includes a positively charged surface patch. The acidic stretch in our consensus sequence might contribute to the interaction with this patch. A SUMO interaction motif centered on two

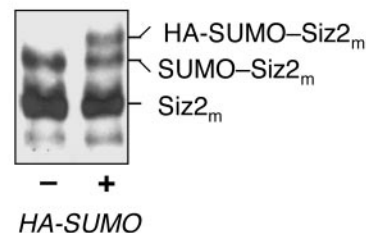


FIG. 2. Potential SUMO-binding site derived from the two-hybrid screen. A, consensus sequence derived from BLOCKS analysis of the four indicated proteins. The clusters of hydrophobic (*h*) and negatively charged (*n*) residues are indicated under the aligned block. B, 11 *S. cerevisiae* proteins, other than the four inputs, contain the consensus sequence for noncovalent SUMO binding.

| | | | |
|--------------------|---|------------|-----------------------------|
| A. | Fir1 | 753 | DGKMVEVILLDEDEDVGLK |
| | Nis1 | 385 | ETKSNPIIIPDSQDDSI LS |
| | Ris1 | 365 | EQKNSIIILSDEDESGAG |
| | Sap1 | 228 | KYISEPILIDLTDNEDDHD |
| | | | hhh nnn |
| Consensus : | | | |
| | K-x₃₋₅ - [I/V] - [I/L] - [I/L] -x₃ - [D/E/Q/N] - [D/E] - [D/E] | | |
| B. | | | |
| | Drs2, Cdc48, Cog4, Gfa1, Ubp1, Yap1801, Ela1, Syt1, Msc6, Psh1, Rup1 | | |

serines, the “SXS” motif, was proposed earlier by Minty *et al.* (35). Their aligned sequences bear an N-terminal cluster of hydrophobic residues and a C-terminal cluster of acidic amino acids, which is strikingly similar to the consensus we deduced. Mutagenesis by Minty *et al.* (35) suggested the importance of both clusters, as well as the two serines, for SUMO binding. The SXS element does not appear to bind directly to SUMO, based on NMR data (34), but it might be important for orienting the flanking clusters on the SUMO surface. More extensive binding and mutational studies will be needed to detail the necessary and sufficient features of the SBMs suggested by these different studies.

High Copy Suppressors of *ulp2Δ*—The preceding results show that two-hybrid screening can identify sumoylation substrates and potential SUMO-interacting factors as well as components of the SUMO ligation system. Isolating suppressors of *ulp* mutants is another potential way to identify sumoylated substrates; moreover, these suppressors would be linked to specific physiological pathways impacted by the SUMO system. Defects associated with loss of either Ulp1 or Ulp2 may be due to the failure to remove SUMO from specific substrates. If the relevant substrate were overproduced, enough of the unsumoylated protein might accumulate to overcome the SUMO protease defect, perhaps by titrating a binding partner of the substrate. We therefore isolated high copy suppressors of the temperature-sensitive growth of the *ulp2Δ* mutant using a YEp24-based yeast genomic library. Plasmids carrying four different genomic fragments were found that allowed growth of *ulp2Δ* cells at 37 °C (Fig. 3). Subcloning identified the gene responsible in each case. All except *SIZ2* were present as full-length ORFs in the original library isolate; full-length *SIZ2* was shown to suppress as well. Overexpression of several of these genes also partially suppressed the hypersensitivity of the *ulp2Δ* mutant to the microtubule-depolymerizing drug benomyl.

Interestingly, this screen netted two proteins that were found in our two-hybrid screen: Siz2 and YGL250w. As shown in Fig. 1, Siz2 is sumoylated *in vivo*. However, the original high copy *SIZ2* clone encoded only the first 494 residues of Siz2, which includes the catalytic SP-RING domain but overlaps by just 8 residues with the region inferred from the two-hybrid analysis to be important for SUMO modification. Overexpression of the Siz2 SUMO ligase might drive a growth-inhibitory SUMO-protein conjugate into a polysumoylated form that is no longer inhibitory, or it might titrate Ubc9 and thereby limit the inhibitory sumoylation. The two most potent *ulp2Δ* dosage suppressors were Siz2 and Top2, the essential topoisomerase II enzyme. Sumoylation of Top2 occurs in yeast as well (see below), where it correlates with changes in the cohesive properties of centromeres (12). Finally, we identified Ubp3, a deubiquitinating enzyme, as a weak high copy suppressor of *ulp2Δ*.

We could not detect desumoylating activity for Ubp3 (data not shown), so this is an unlikely explanation of its ability to suppress the loss of the Ulp2 enzyme. Ubp3 has been linked to the regulation of chromatin structure, so it is possible that high levels of Ubp3 activity change chromosome structure in a way that partially bypasses the requirement for dynamic sumoylation (*e.g.* of Top2 or Pds5).

In summary, high copy suppression of an Ulp2 SUMO isopeptidase mutant yielded both SUMO substrates and a SUMO ligase. Several of these proteins have been functionally linked to Ulp2 by other experiments, validating this approach as a means to identify SUMO-associated proteins (also see below).

Affinity Purification of SUMO-Protein Conjugates—A sensitive biochemical approach to the identification of SUMO-linked proteins has also been devised. It combines cell lysis under strongly denaturing conditions followed by a two-step affinity purification of SUMO-linked proteins and tandem mass spectrometric (MS/MS) identification of tryptic peptides derived from the purified mixture. To maximize the chances of identifying even low abundance SUMO conjugates, we used a strain in which the Ulp1 isopeptidase gene had been deleted, resulting in higher level accumulation of many conjugates. *ULP1* deletion is normally lethal, but if a high copy plasmid expressing a mature form of SUMO is supplied, growth is rescued, although the growth rate is reduced (22). The chromosomal copies of both *ULP1* and *SMT3* (encoding SUMO) were deleted, with viability maintained with a plasmid encoding either mature wild-type SUMO or HFT-SUMO (Fig. 4A). HFT-SUMO has a His₆ tag followed by a FLAG epitope and the recognition sequence for TEV protease. The placement of sequences at the N terminus of SUMO, including the HFT tag, reduced the efficiency of SUMO conjugation (Fig. 4B, compare *lane 1* with *lanes 4* and *5*; also see Fig. 5A), but the tagged SUMO supported full viability of an *smt3Δ* strain.

Cells were lysed with a French press in a buffer containing 6 M guanidinium hydrochloride. Use of this strong denaturant is expected to destroy any residual SUMO isopeptidase activity in the extract and to break apart noncovalent protein complexes, which should minimize the chance of copurifying non-sumoylated proteins that are in complexes with SUMO-modified polypeptides. Strongly denaturing conditions were maintained with 8 M urea during the first affinity purification step on a column that binds the His₆ tag. Protein eluted with 0.3 M imidazole from the first column was loaded onto an anti-FLAG affinity column. SUMO-conjugated proteins were gently eluted by incubation with TEV protease, which cleaves between the FLAG tag and SUMO in HFT-SUMO. Aliquots of the purified proteins were examined by both protein staining and anti-SUMO immunoblot analysis (Fig. 4C). The close correspondence between the banding patterns observed by these

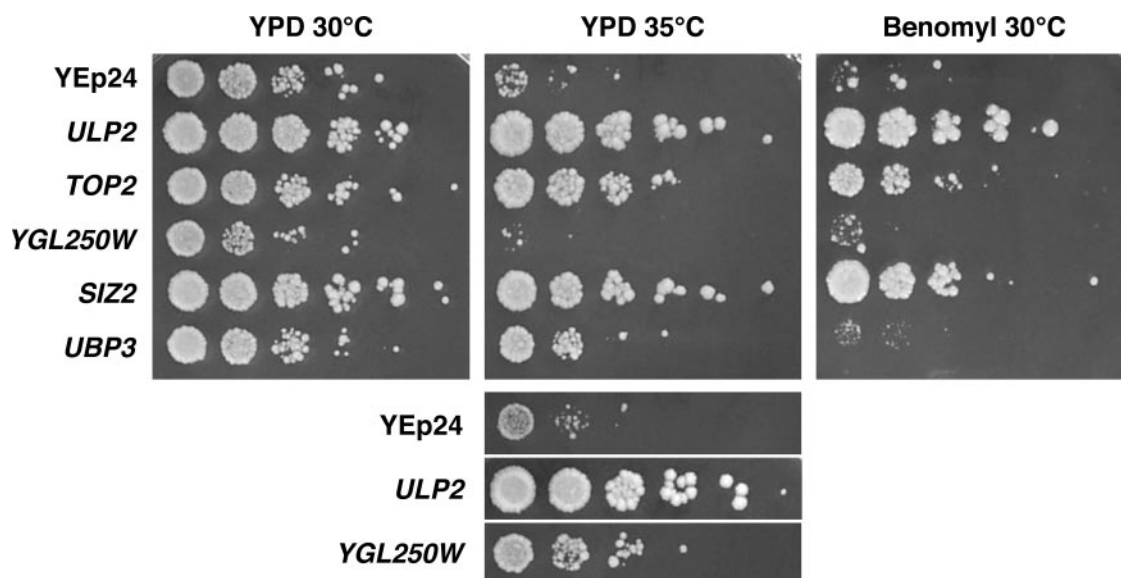


FIG. 3. Dosage suppressors of the *ulp2Δ* growth defect at high temperature. Shown are cells retransformed with the original library clones. 10-fold serial dilutions of MHY1380 cells (*ulp2Δ*) were spotted onto the indicated media and incubated for 4 days. *YGL250W* was a weak suppressor and in some experiments failed to suppress the *ulp2Δ* growth defect. The lower set of serial dilutions shows suppression by high copy *YGL250W* in another *ulp2Δ* strain (W303 background).

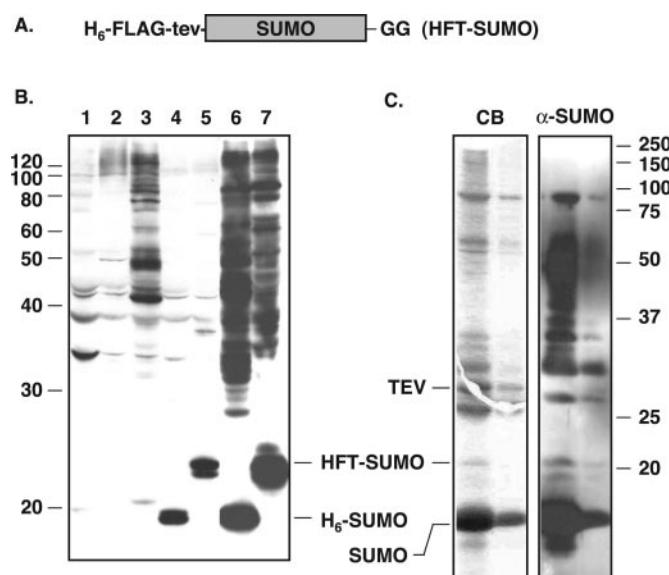


FIG. 4. Purification of bulk SUMO conjugates from yeast. A, schematic of the HFT-SUMO protein. At its N terminus, the protein has six histidines followed by a FLAG epitope and a cleavage site for TEV protease (TEV). B, anti-SUMO immunoblot. Equal amounts of whole cell lysate were loaded. Lane 1, wild type; lane 2, *ulp2Δ*; lane 3, *ulp1-ts*; lane 4, *smt3Δ/pH6-SUMO*; lane 5, *smt3Δ/pHFT-SUMO*; lane 6, *smt3Δ ulp1Δ/pH6-SUMO*; lane 7, *smt3Δ ulp1Δ/pHFT-SUMO*. C, comparison of proteins purified by two-step affinity purification from *ulp1Δ* cells carrying an HFT-SUMO plasmid (MHY2811). Gel Code Blue (CB) staining of the first two step elutions with TEV protease from the anti-FLAG resin is shown alongside an anti-SUMO immunoblot of the same fractions. Protein size markers are indicated (in kDa).

two methods indicated that SUMO conjugates were highly purified by the two-step purification protocol used.

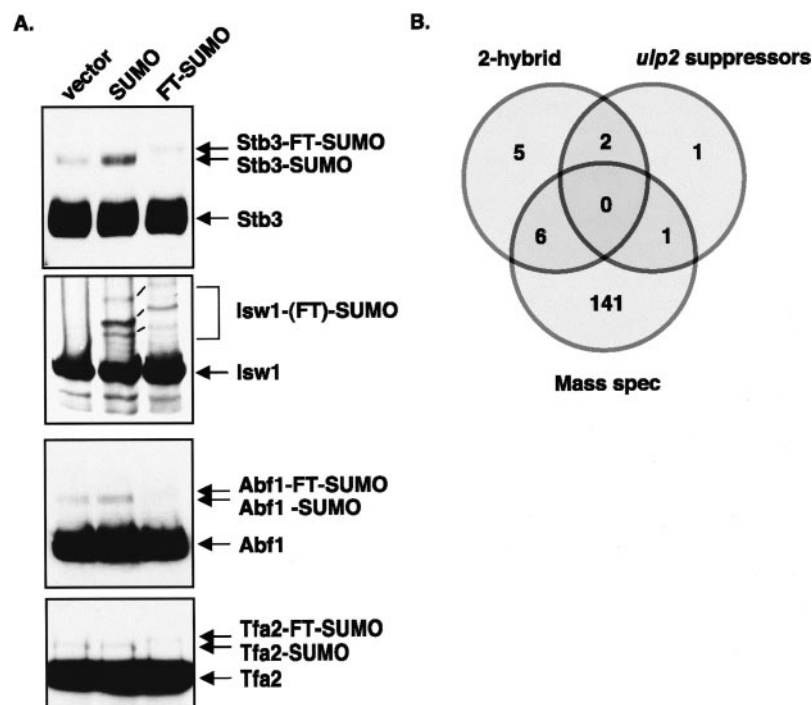
Mass Spectrometric Identification of SUMO Conjugates—To identify putative SUMO substrates, we used a highly sensitive and efficient proteomic shotgun profiling procedure based on gel-free high performance capillary scale liquid chromatography fractionation of tryptic protein digests coupled to real time electrospray ion trap tandem mass spectrometry (see “Experimental Procedures”). To maximize the capture of informative spectra from lower abundance target proteins and circumvent

preferential biased detection of the tagged SUMO variant itself, the peptide mixture was extensively resolved prior to MS/MS fragmentation analysis in order to avoid ion-ion interference leading to ion suppression and reduced dynamic range. The spectra were then sequence-matched against a data base of putative yeast gene products, and high confidence matches were predicted by statistical analysis of the resulting candidates. A total of 146 proteins were identified by at least two peptides; two more proteins were identified by a single peptide each (Supplemental Material). These proteins represent a very broad range of functional categories and intracellular locales.

To verify that the proteins identified were indeed SUMO-protein conjugates, we first determined whether any of these proteins co-purified as nonspecific contaminants. The purification procedure was repeated with a yeast strain identical to the one used above except that the SUMO construct lacked the HFT epitope tag. MS/MS analysis of proteins purified from this strain did not yield any yeast proteins, implying that all or almost all of the proteins identified in the original analysis (Supplemental Material) were SUMO-modified or were extremely tightly bound to a sumoylated protein.

A number of the sumoylated proteins we found by MS/MS were previously identified in other studies (e.g. three septins, Top2, and PCNA), further indicating that our method of purification and analysis allowed the identification of *bona fide* SUMO conjugates. We also directly analyzed a small subset of the proteins found by MS/MS for SUMO modification by an independent assay, specifically the gel shift assay illustrated in Fig. 1C. Yeast strains were used that had the chromosomal copy of a gene of interest tagged with a sequence encoding the TAP double epitope, which includes a protein A segment (36). These strains have wild-type *ULP1* and *SMT3* genes and do not overproduce the target proteins. Each TAP-tagged cell line was transformed with an empty vector, a *GAL-SUMO*, or a *GAL-FT-SUMO* plasmid. Immunoblotting with an antibody that recognizes the protein A tag revealed protein bands migrating more slowly than the primary unmodified polypeptide; these represented candidates for sumoylated species (Fig. 5A). If true, a supershift to an even slower migrating form should be observed in cells overexpressing an N-terminally extended SUMO derivative (FT-SUMO). This can be seen in Fig. 5A for

FIG. 5. A, confirmation of sumoylation of selected substrates by a gel shift assay. The *STB3*, *ISW1*, *TFA2*, and *ABF1* chromosomal loci were modified by a sequence encoding the tandem affinity purification (TAP) epitope (36). Cells were transformed with empty vector or vector expressing either untagged or FLAG-tev-tagged SUMO (*FT-SUMO*) from a *GAL1* promoter. Strains were grown overnight in raffinose and then induced for 5 h with 2% galactose before lysis. Proteins in lysates from these cells were examined by peroxidase-anti-peroxidase immunoblotting. For Tfa2-TAP the *GAL1* promoter was replaced with the *GPD* promoter, and cells were grown in dextrose containing medium. For Abf1-TAP analysis, the endogenous *ULP1* allele was replaced with *ulp1-333*, and cells were evaluated at 30 °C. B, Venn diagram showing overlap of proteins identified by two-hybrid screen with SUMO bait, high copy suppression of *ulp2Δ*, and MS/MS.



Stb3, a protein that regulates the Sin3 histone deacetylase, and for Isw1, an ATP-dependent chromatin-remodeling factor, and for Tfa2, the small subunit of the TFIIE complex, which is a general initiation factor for RNA polymerase II transcription. (As noted above (Fig. 4A), epitope tagging of SUMO reduced its conjugation to substrates and when overproduced appears to impair conjugation of endogenous SUMO.) These data confirm that multiple factors functioning in chromatin structure and remodeling, which figured prominently among the proteins we identified, are likely to be regulated by sumoylation. For Abf1, an essential DNA-binding protein involved in transcriptional regulation, the sumoylated form was also detected, but this required introduction of a *ulp1ts* mutation (22).

In Fig. 5B, a Venn diagram shows the overlap of proteins identified by yeast two-hybrid screening with a SUMO bait, high copy suppressor screening of *ulp2Δ* and MS/MS analysis of sumoylated proteins. These three approaches had different biases and yielded vastly different numbers of proteins, but several proteins were identified by more than one of the independent screens. Interestingly, although the MS/MS method was the most sensitive of the approaches used, several likely SUMO substrates were identified only by the two-hybrid screen, and at least one sumoylated protein, Siz2 (Fig. 1C), was identified in both the two-hybrid screen and the high copy suppressor analysis but not by MS/MS. These results confirm that MS/MS analysis of complex, affinity-purified mixtures of SUMO-linked proteins is capable of identifying specific substrates of the yeast SUMO system, but they also emphasize the utility of using alternative approaches.

We do not expect that all SUMO substrates have been identified. For example, two *S. cerevisiae* proteins reported to be modified by SUMO *in vivo*, Pds5 and Ysc4, were not detected in any of our screens. There are many reasons why certain proteins might have been missed. For the two genetic screens, some genes might either have been absent or were present in a very small number of bacterial clones in the DNA libraries used. Our MS/MS analysis was done in *ulp1Δ* cells. In these cells, some stages of the cell cycle are expected to be underrepresented, and certain proteins (*e.g.* Pds5) might only be sumoylated in one of these intervals (15). Other proteins are probably

TABLE II
Comparison of MS/MS studies on yeast protein sumoylation

| No. of proteins identified | Also found in current study |
|---------------------------------|-----------------------------|
| Zhou <i>et al.</i> (37) | |
| YPD | 11 (69%) |
| H ₂ O ₂ | 10 (43%) |
| Ethanol | 27 (55%) |
| Panse <i>et al.</i> (38) | |
| YPD | 29 (20%) |
| Wohlschlegel <i>et al.</i> (39) | |
| YPD | 36 (13%) |

only modified by SUMO under very specific conditions. Another difficulty with the MS/MS analysis of SUMO and SUMO-conjugate mixtures was the very large amount of SUMO present. Hundreds of peptides derived from SUMO were sequenced in each sample, and this limited our ability to detect rarer substrate peptides. Strategies to circumvent this limitation are being pursued.

Comparison of Studies on SUMO-conjugated Proteins in Yeast—Very recently, three other mass spectrometry studies have been published in which proteome scale evaluation of yeast SUMO-protein conjugates was attempted (37–39). A comparison of the overlap between proteins identified in our study and in these other reports is given in Table II. In all four studies, yeast were grown in rich (YPD) medium. Whereas we observed over two-thirds of the sumoylated proteins found by Zhou *et al.* (37) under these conditions (69%), much poorer concordance was seen with the other two studies (13 and 21%). Even when Zhou *et al.* (37) examined cells under specific stress conditions, which are expected to induce a distinct array of SUMO-linked proteins, the overlap with our observed modified proteins was still quite high (43 and 55% when compared with cells exposed to H₂O₂ and ethanol, respectively).

It is likely that the much greater overlap between sumoylated proteins identified in our study and that of Zhou *et al.* (37) compared with the other studies reflects the more extensive conjugate purification used in the former two studies. In both our procedure and that of Zhou *et al.* (37), two affinity purification steps were used *versus* the single-step purifications of

Panse *et al.* (38) and Wohlschlegel *et al.* (39). As shown in Fig. 4C, the pattern of bands seen by a general protein stain and by anti-SUMO immunoblotting was very similar for our purified protein preparation, indicating that the sumoylated proteins were highly purified. A similar analysis by Wohlschlegel *et al.* (39) showed little similarity between the protein stain and an anti-SUMO blot, suggesting that most of the eluted proteins in their preparation were not sumoylated.

In all but the Panse *et al.* (38) study, strongly denaturing conditions were used during cell lysis and purification. Therefore, it is unlikely that proteins that are not themselves sumoylated but might bind to sumoylated proteins would copurify. This is clearly illustrated with the five septins present in vegetatively growing cells. Only three are directly modified by SUMO (10). However, all five septins interact with one another to form the 10-nm filaments that form at the bud neck. All three studies employing strong denaturants identified only the three septins that are directly sumoylated, whereas Panse *et al.* (38), using less stringent isolation conditions, reported four septins in their MS/MS identifications.

Based on the above considerations, we believe that the number of proteins misidentified as sumoylated proteins in our study is very low. This does not mean, however, that the physiological function of every SUMO-modified protein we observed is strongly dependent on this modification. It is often the case that only a small fraction of a protein is actually coupled to SUMO *in vivo*. This might be a functionally significant fraction, but it might sometimes reflect a low level of nonspecific sumoylation that can be tolerated by the cell. Some of the identified proteins are highly abundant, and even if only a very small fraction were modified, the sumoylated forms might be detectable by MS/MS. A high fractional modification of a particular protein, however, does not necessarily mean that the sumoylation is physiologically important. The septins, which are heavily sumoylated in a cell cycle-dependent manner, do not require this modification for their essential function in cytokinesis (10, 11). Detailed genetic and biochemical studies with each sumoylated protein are required to determine the exact mechanistic consequences of a particular SUMO modification event.

Functional Implications—A wide variety of SUMO system substrates have been identified in our study. They fall into distinct functional clusters (Supplemental Material). Here we comment on some unexpected aspects. One intriguing feature is that a number of metabolic enzymes were identified. The majority of these are glycolytic enzymes. Specifically, all nine enzymes that sequentially convert fructose 1,6-bisphosphate to ethanol are found in our sumoylated protein mixture, with phosphoglycerate kinase (Pgk1) consistently the most heavily represented. The significance of this is uncertain, but coordinate regulation of the pathway by SUMO is suggested. Coordinate regulation of protein complexes, possibly at a particular stage in their biogenesis, is also suggested by the finding of 21 sumoylated ribosomal subunits, 12 from the large subunit and nine from the small one. Perhaps sumoylation facilitates ribosomal protein import into the nucleus or regulates the function of a specific subset of ribosomes. Ribosomes might also be transiently modified at a particular point in the cell cycle. The Rpl28 (L29) protein is polyubiquitinated primarily during S phase (40), and this subunit is also heavily represented in our SUMO-protein samples.

SUMO and many SUMO system enzymes are concentrated in the nucleus. Consistent with this, a large number of SUMO-modified proteins in our MS/MS analysis are known to be nuclear. Both general transcription factors (contributing to all three classes of RNA polymerases) and gene-specific transcription factors were among the targets identified as were a variety

of DNA repair proteins. In addition, we identified many factors that participate in chromatin remodeling, chromosome structural maintenance, gene silencing, and genome stability. That there were multiple proteins in each of these categories suggests that SUMO modification regulates chromosome transactions at many levels and by multiple mechanisms.

Intriguingly, several transmembrane proteins were also found. Most of these reside in the endoplasmic reticulum, but at least two, Ist1 and Pma1, are plasma membrane proteins. This indicates that SUMO modification is not limited to soluble proteins and, like ubiquitin, might potentially be used as a membrane trafficking signal. It is interesting that several proteins (Ris1 and Sap1) that we identified by MS/MS as proteins that are modified by SUMO were found by the two-hybrid study to interact with SUMO noncovalently. In the endosomal membrane-trafficking pathways regulated by ubiquitin, a number of endosomal proteins have a similar property; they are both modified by ubiquitin and bear ubiquitin-binding sites. This can create dynamic networks of interacting proteins that depend on whether a protein-ubiquitin interaction motif is bound by a ubiquitin conjugated to a site on the same protein or on another protein.

Such a mechanism might also be relevant for the AAA ATPase Cdc48 (called p97/VCP in mammals). This essential ATPase assembles into a homohexamer, and its activity is regulated by various adaptor proteins, such as the Ufd1 and Ubx proteins (41, 42). We identified Cdc48, Ufd1, and Ubx4 as sumoylated proteins in our MS/MS samples. As noted earlier, Cdc48 might also have a SUMO-binding motif (Fig. 2). We suggest that interactions between Cdc48 and its adaptor proteins might be regulated by SUMO modification in ways analogous to ubiquitin modulation of endosomal protein factors. Besides Cdc48, at least three other AAA/AAA+ ATPase-related proteins were identified as SUMO targets: Rfc1, Sap1, and Elg1. Sumoylation could therefore have a more general role in regulating multimeric ATPase complexes. This is also strongly suggested by our finding that components of several ATP-dependent chromatin remodeling complexes (Snf2, Isw1, and Rsc58) are all modified by SUMO *in vivo*. Determining the exact mechanistic contributions of sumoylation to these various ATP-dependent protein complexes will be an important area for further study.

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REFERENCES

- Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S. P. (2003) *Nat. Biotechnol.* **21**, 921–926
- Hochstrasser, M. (2000) *Nat. Cell Biol.* **2**, E153–E157
- Schwartz, D. C., and Hochstrasser, M. (2003) *Trends Biochem. Sci.* **28**, 321–328
- Seeler, J. S., and Dejean, A. (2003) *Nat. Rev. Mol. Cell Biol.* **4**, 690–699
- Johnson, E. S. (2004) *Annu. Rev. Biochem.* **73**, 355–382
- Johnson, P. R., and Hochstrasser, M. (1997) *Trends Cell Biol.* **7**, 408–413
- Melchior, F. (2000) *Annu. Rev. Cell Dev. Biol.* **16**, 591–626
- Bernier-Villamor, V., Sampson, D. A., Matunis, M. J., and Lima, C. D. (2002) *Cell* **108**, 345–356
- Hochstrasser, M. (2002) *Mol. Cell* **9**, 453–454
- Johnson, E. S., and Blobel, G. (1999) *J. Cell Biol.* **147**, 981–994
- Johnson, E. S., and Gupta, A. A. (2001) *Cell* **106**, 735–744
- Bachant, J., Alcasabas, A., Blat, Y., Kleckner, N., and Elledge, S. J. (2002) *Mol. Cell* **9**, 1169–1182
- Hoegge, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. (2002) *Nature* **419**, 135–141
- Stelter, P., and Ulrich, H. D. (2003) *Nature* **425**, 188–191
- Stead, K., Aguilar, C., Hartman, T., Drexel, N., Meluh, P., and Guacci, V. (2003) *J. Cell Biol.* **163**, 729–741
- D'Amours, D., Stegmeier, F., and Amon, A. (2004) *Cell* **117**, 455–469
- Sherman, F., Fink, G. R., and Hicks, J. B. (1986) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds.) (1989) *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., New York

19. Chen, P., Johnson, P., Sommer, T., Jentsch, S., and Hochstrasser, M. (1993) *Cell* **74**, 357–369
20. James, P., Halladay, J., and Craig, E. (1996) *Genetics* **144**, 1425–1436
21. Li, S. J., and Hochstrasser, M. (2000) *Mol. Cell. Biol.* **20**, 2367–2377
22. Li, S.-J., and Hochstrasser, M. (1999) *Nature* **398**, 246–251
23. Washburn, M. P., Wolters, D., and Yates, J. R., III (2001) *Nat. Biotechnol.* **19**, 242–247
24. Kislinger, T., Rahman, K., Radulovic, D., Cox, B., Rossant, J., and Emili, A. (2003) *Mol. Cell Proteomics* **2**, 96–106
25. Eng, J. K., McCormack, A. L., and Yates, J. R., III (1994) *J. Am. Soc. Mass Spectrom.* **5**, 976–989
26. Tabb, D. L., McDonald, W. H., and Yates, J. R., III (2002) *J. Proteome Res.* **1**, 21–26
27. Harris, M. A., Clark, J., Ireland, A., Lomax, J., Ashburner, M., Foulger, R., Eilbeck, K., Lewis, S., Marshall, B., Mungall, C., Richter, J., Rubin, G. M., Blake, J. A., Bult, C., Dolan, M., Drabkin, H., Eppig, J. T., Hill, D. P., Ni, L., Ringwald, M., Balakrishnan, R., Cherry, J. M., Christie, K. R., Costanzo, M. C., Dwight, S. S., Engel, S., Fisk, D. G., Hirschman, J. E., Hong, E. L., Nash, R. S., Sethuraman, A., Theesfeld, C. L., Botstein, D., Dolinski, K., Feierbach, B., Berardini, T., Mundodi, S., Rhee, S. Y., Apweiler, R., Barrell, D., Camon, E., Dimmer, E., Lee, V., Chisholm, R., Gaudet, P., Kibbe, W., Kishore, R., Schwarz, E. M., Sternberg, P., Gwinn, M., Hannick, L., Wortman, J., Berriman, M., Wood, V., de la Cruz, N., Tonellato, P., Jaiswal, P., Seigfried, T., and White, R. (2004) *Nucleic Acids Res.* **32**, 258–261
28. Robinson, M. D., Grigull, J., Mohammad, N., and Hughes, T. R. (2002) *BMC Bioinformatics* **3**, 35
29. Johnson, E. S., Schwienhorst, I., Dohmen, R. J., and Blobel, G. (1997) *EMBO J.* **16**, 5509–5519
30. Biggins, S., Bhalla, N., Chang, A., Smith, D. L., and Murray, A. W. (2001) *Genetics* **159**, 453–470
31. Hochstrasser, M., Ellison, M. J., Chau, V., and Varshavsky, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 4606–4610
32. Henikoff, J. G., Henikoff, S., and Pietrokovski, S. (1999) *Nucleic Acids Res.* **27**, 226–228
33. Gattiker, A., Gasteiger, E., and Bairoch, A. (2002) *Appl. Bioinformatics* **1**, 107–108
34. Song, J., Durrin, L. K., Wilkinson, T. A., Krontiris, T. G., and Chen, Y. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 14373–14378
35. Minty, A., Dumont, X., Kaghad, M., and Caput, D. (2000) *J. Biol. Chem.* **275**, 36316–36323
36. Ghaemmaghani, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., De-phoure, N., O'Shea, E. K., and Weissman, J. S. (2003) *Nature* **425**, 737–741
37. Zhou, W., Ryan, J. J., and Zhou, H. (2004) *J. Biol. Chem.* **279**, 32262–32268
38. Panse, V. G., Hardeland, U., Werner, T., Kuster, B., and Hurt, E. (2004) *J. Biol. Chem.* **279**, 41346–41351
39. Wohlschlegel, J. A., Johnson, E. S., Reed, S. I., and Yates, J. R., III (2004) *J. Biol. Chem.* **279**, 45662–45668
40. Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M., and Finley, D. (2000) *Cell* **102**, 67–76
41. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2003) *J. Cell Biol.* **162**, 71–84
42. Schubert, C., Richly, H., Rumpf, S., and Buchberger, A. (2004) *EMBO Rep.* **5**, 818–824