

Identification of Sumoylated Proteins by Systematic Immunoprecipitation of the Budding Yeast Proteome*

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The identification of post-translational modifications to proteins is critical for understanding many important aspects of biology. Utilizing a collection of epitope-tagged yeast strains, we developed a novel approach to determine which proteins are modified by the small ubiquitin-related modifier (SUMO). We crossed traits useful for the detection of SUMO conjugation into 4246 tandem affinity purification-tagged strains and successfully immunoprecipitated and screened 2893 of these proteins for association with SUMO (~70% of the expressed proteome detectable by immunoblot analysis). We found 82 proteins associated with SUMO, including many of low abundance. Because our screen was performed under non-denaturing conditions, we were able to identify multiple members of four complexes that were associated with SUMO: the RSC chromatin remodeling complex, the mediator complex, the TFIID complex, and the septin complex. In addition, we describe five new direct conjugates of SUMO, and we mutated SUMO conjugation sites in four proteins. This is the first attempt to immunoprecipitate a large fraction of the proteome of a eukaryote, and it demonstrates the utility of this method to identify post-translational modifications in the yeast proteome. *Molecular & Cellular Proteomics* 4:73–83, 2005.

Protein activity is altered by covalent post-translational modifications such as phosphorylation, ubiquitylation, acetylation, and methylation, and these modifications are essential for the growth and development of all organisms. Recently numerous ubiquitin-like polypeptides have been described that when conjugated to proteins regulate aspects of cell cycle progression, protein degradation, cell polarity, and morphogenesis (1–3). The identification of proteins that are post-translationally modified is the first step in determining how those modifications regulate important cell processes. In this study, we developed a new high throughput technique to identify post-translationally modified proteins and demonstrated the utility of this approach by identifying new targets of sumoylation.

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SUMO¹ was discovered as a small ubiquitin-related modifier that, like ubiquitin, is a polypeptide chain conjugated by an isopeptide bond to lysines in target proteins (4). SUMO conjugation, or sumoylation, is analogous to but biochemically distinct from ubiquitin conjugation (5, 6). SUMO, which is encoded by *SMT3* in *Saccharomyces cerevisiae*, is processed by the isopeptidase Ulp1 from its precursor form (7). SUMO conjugation requires an E1 heterodimer (Uba2/Aos1), E2 protein (Ubc9), and E3 protein (either Siz1 or Siz2) to form a covalent bond with target proteins (5, 8, 9). Removal of SUMO from proteins is mediated by the cysteine proteases Ulp1 or Ulp2 with Ulp1 appearing to mediate the majority of desumoylation (3, 10). The genes encoding *Smt3*, *Uba2*, *Aos1*, *Ubc9*, and *Ulp1* are required for cell growth and division (4). While ubiquitylation (conjugation of ubiquitin) often destabilizes targeted proteins, sumoylation appears to modify protein-protein interactions in some cases or acts as a ubiquitin antagonist in others (4, 11, 12). However, because a limited number of proteins modified by SUMO have been described, it is unclear whether these are the only roles for sumoylation in cells.

In budding yeast, seven proteins have been extensively characterized that are modified by SUMO. Three of these proteins are part of the essential septin complex (*Cdc11*, *Cdc3*, and *Shs1*), which plays a role in cytoskeletal organization and cell division. However, sumoylation of these proteins is not required for cell viability because when sumoylation is prevented by mutating the conjugated lysines, the septins retain function, and the cells continue to divide (6, 13). Proliferating cell nuclear antigen (*Pol30* in budding yeast), the DNA polymerase sliding clamp, is also sumoylated (14). When the lysine that is sumoylated is mutated to an arginine, the protein is also unable to be ubiquitylated (14). Sumoylation of proliferating cell nuclear antigen antagonizes ubiquitylation and allows the cells to replicate DNA, whereas the lack of sumoylation or either monoubiquitylation or polyubiquitylation stalls replication and allows for error-free DNA repair (14). Topoisomerase II in yeast is also sumoylated, and this modification

¹ The abbreviations used are: SUMO, small ubiquitin-related modifier; HA, hemagglutinin; IDA, iminodiacetic acid; ORF, open reading frame; SD, synthetic yeast medium with 2% dextrose; TAP, tandem affinity purification; E1, SUMO-activating enzyme; E2, SUMO carrier protein; E3, SUMO-protein isopeptide ligase; Ura, uracil.

alters its activity at the centromere but is not required for cell viability (15). Both proliferating cell nuclear antigen and topoisomerase II are sumoylated in mammalian cells, suggesting that regulation by sumoylation is evolutionarily conserved (14, 16). Recently Ycs4 and Pds5 have been identified as SUMO conjugates (17, 18). Sumoylation of Ycs4 mediates its localization to rDNA and Pds5 sumoylation mitigates chromatid cohesion (17, 18); however, the lysines modified by SUMO in Pds5 and Ycs4 have yet to be identified, and it is unclear how important SUMO conjugation is for the regulation of these two proteins.

Mass spectrometry-based screens have identified additional proteins in the budding yeast proteome that are modified by SUMO (19–21). In addition to reporting 22 *bona fide* sumoylation targets, which were confirmed by either secondary assays or identification of sumoylated peptides, these studies also identify other candidate sumoylated proteins that have yet to be confirmed by additional assays. Many are likely to be *bona fide* substrates, especially the SUMO substrates that were detected in multiple studies (19–21). Although these screens are particularly effective in detecting relatively abundant proteins, they may miss less abundant ones. The median abundance of the 10 proteins identified in one study is in the top 5% of expressed proteins (21), and the median abundance of the 67 proteins identified in a second study is in the top 10% (20, 22).

We began our studies with an alternative approach to screen systematically for sumoylated proteins. Utilizing a collection of yeast strains epitope-tagged with the tandem affinity purification (TAP) tag (22), we crossed traits useful for the detection of sumoylation into the TAP-tagged strains, immunoprecipitated each epitope-tagged protein, and assayed for sumoylation. Our technique is systematic in that we assay individually each protein in the yeast proteome, and our expectation was that specific immunoprecipitation of each tagged protein would allow for identification of less abundant SUMO substrates. Importantly our study demonstrated the feasibility of assaying for post-translational modifications in a method complementary to other approaches.

EXPERIMENTAL PROCEDURES

Generation of Yeast Strains for Discovering Sumoylated Proteins—To cross desirable traits into the TAP-tagged strains, we generated the following strain (EY1295): *MAT α can1 Δ ::MFA1p-LEU2 ulp1-333^{ts}::MET15 CUP1p-HA₃-SMT3(GG)::URA3*. Initially we generated a *can1 Δ ::MFA1p-LEU2* selectable marker in a *MAT α* strain isogenic to the TAP-tagged collection (EY1254). We used the following oligonucleotides to amplify two PCR products from the strain utilized in systematic genetic analysis studies (23) that when annealed to one another generated *can1 Δ ::MFA1p-LEU2*: 5'-TAGGGCGAACTTGAA-GAATAACC-3', 5'-CAGTCCATGGTTCTATTCGATGGCTTTGTACTT-ATTTTGG-3', 5'-CAGTCCATGGCTGCCCTAAGAAGATCGTCGTT-TTGCC-3', and 5'-ACGAAAAATGAGTAAAAATTATCTT-3'.

We transformed the resulting PCR product into a *MAT α* strain using standard lithium acetate transformation techniques (24) and selected for canavanine resistance and leucine prototrophy. We crossed this

strain (EY1254) to a *MAT α* strain that contained a temperature-sensitive allele of *ULP1* (*ulp1-333^{ts}*), which was generated by transformation with yIP353-ulp1-ts(3–33) (3). The *ulp1^{ts}* allele was marked with *MET15* (EY1274) by integrating a PCR product generated from the amplification of *MET15* in pBSKS (EB1435) with the following oligonucleotides: 5'-GATCATTGGATATGGAATAAAGTTCAATAAAC-CACTTGAACGCCATCCTCATGAAAAGTGT-3' and 5'-GTACAGACA-TCTAAATTGAGCTGGTGCTTATGCTATTCTTTACTTGTGAGAGAAA-GTAGG-3'.

CUP1p-HA₃-SMT3(GG) was generated by amplifying *SMT3(GG)* (processed Smt3 ends with two C-terminal glycines) with oligonucleotides that generated PacI and AscI sites, and the digested product was ligated into pRS426 (3, 25). To replace the native *SMT3* locus, we used the following oligonucleotides to amplify the new *SMT3* allele from pRS426-*CUP1p-HA₃-SMT3(GG)* plasmid (EB1445) and replace the genomic locus, marking the locus with *URA3*: 5'-CCGTACGGC-GGGGCACTTTTGAACGTTTTTGTGCATCCTCGCGGTTTCGGTG-ATGACG-3' and 5'-CCAGGGTTCCGAAGATAAAGTGGCATCTTTC-CATTACCGGCCGCTCTAGAAGTGTGG-3'.

We sequenced the integrated ORF to confirm that no additional mutations were introduced. The final *MAT α* strain (EY1295) was grown to logarithmic phase, mixed with the TAP-tagged strains in 96-well plates (Nalge Nunc International, Rochester, NY) and allowed to grow overnight at 25 °C. To select for diploids, the strains were pinned onto SD (synthetic medium with dextrose) lacking histidine (–His), uracil (–Ura), and methionine (–Met) utilizing a 96-pinning tool (V & P Scientific, San Diego, CA) and allowed to grow for 2 days at 30 °C. The strains were then pinned into liquid SD –His –Ura medium, grown overnight at room temperature, pinned onto solid sporulation medium, and allowed to sporulate for at least 7 days. Meiosis was monitored by light microscopy, and the resulting tetrads were pinned onto SD –His –Ura –Met medium also lacking leucine (–Leu) and arginine (–Arg) and supplemented with 50 μ g/ml canavanine and grown at room temperature for 4 days to select for appropriate haploid strains. The resulting colonies were pinned into 96-well plates with liquid SD –His –Ura –Met –Leu –Arg +canavanine medium and allowed to grow for 2 days at room temperature. Recipes for all media preparations are available upon request. Cells were diluted 1:1000 and allowed to grow to stationary phase in SD –His –Ura –Met –Leu. We screened the resulting strains in this study.

Generation of Cell Lysates and Assay for SUMO Conjugation—Strains in 96-well plates were grown to stationary phase in SD –His –Ura –Met –Leu and diluted 1:20 into YEPD (24) + 500 μ M CuSO₄ in 2-ml-deep-well plates (Greiner Bio-One, Frickenhausen, Germany) and grown at 25 °C for 8 h in a HiGro Shaker (Gene Machines, Ann Arbor, MI). Between 2 and 10 ml of cells were grown, harvested, and lysed as described previously (26) except that the lysis buffer consisted of 25 mM Tris-Cl, pH 8, 5 mM EDTA, 0.5% Triton X-100, 10 mM NaCl, 50 mM *N*-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride, 2.5 mM benzamide, and 1 μ g/ml pepstatin. After hypotonic lysis, we added NaCl to a final concentration of 250 mM and removed insoluble cell debris by centrifugation at 2000 \times *g* for 20 min. One microgram of biotin-IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and 5 μ l of buffer-equilibrated streptavidin-Sepharose (Amersham Biosciences) were added to the supernatant (26). After 20 min, we resuspended the Sepharose by pipetting the mixture. After 40 min total, we centrifuged the plates, removed the supernatant, washed the Sepharose once with fresh wash buffer (lysis buffer + 240 mM NaCl), and transferred the Sepharose to 96-well filter-tip plates (Orochem, Westmont, IL) (26). We washed the beads three times with 0.5 ml of ice-cold wash buffer and eluted the immunoprecipitation with 20 μ l of 2 \times SDS sample buffer at 65 °C. We loaded 13 μ l of the eluate into wells of 4–15% Tris Criterion gels (Bio-Rad) and blotted the electrophoresed proteins to nitrocellulose as described previously

(26). First we probed for hemagglutinin (HA) reactivity with 0.5 $\mu\text{g/ml}$ 12CA5 antibody and anti-mouse antibody conjugated to horseradish peroxidase (Bio-Rad), then boiled the blot in 1% SDS for 2 min, rinsed away the SDS, and probed for TAP reactivity with 0.1 $\mu\text{g/ml}$ Fc (Jackson ImmunoResearch Laboratories, Inc.) and anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad). We used SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) to detect horseradish peroxidase. We considered proteins to be immunoprecipitated if a band of approximately the predicted size was detected with a 1-min exposure to film. We repeated the screen twice for all proteins that were scored as SUMO-interacting and considered proteins as reproducibly associated with SUMO when they were scored as SUMO-interacting in at least one additional assay.

Parameters for Network Visualization System (Osprey)—We imported the 82 SUMO-interacting targets into Osprey 1.2.0 (27), selected them, and visualized all interactions among these proteins using data from three large scale data sets (28–30). We inverted the selection and removed nodes with the connection minimum filter set at 2. We grouped nodes into logical complexes; for example, all RSC subunits were grouped together.

Assay for Direct Targets of SUMO—TAP strains (*ULP1^{ts}*) that were scored as reproducibly SUMO-interacting were transformed with pRS426-*CUP1p*-HA-His₆-*SMT3*(GG) (EB1436). Strains with and without the plasmid were grown to midlogarithmic growth at 30 °C in 50 ml of SD –Ura or SD supplemented with amino acids, respectively. We induced the *CUP1* promoter with 0.5 mM CuSO₄ for 2.5 h. We lysed cells in 100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, pH 8, and 10 mM *N*-ethylmaleimide by bead-beating with glass beads at room temperature and cleared the extract by centrifugation at 13,000 $\times g$ for 10 min. The cleared extract was incubated with 50 μl of Co²⁺-iminodiacetic acid (IDA)-Sephacrose (Sigma) for 30 min. We washed bound proteins in 1.5-ml tubes four times with 1 ml of lysis buffer and then eluted proteins in lysis buffer + 50 mM EDTA. We added 2 \times sample buffer for 15 min, and 13 μl were loaded onto Criterion gels (26).

Generation of Myc-tagged ORFs—Each candidate ORF was amplified by PCR to generate 500 bp upstream of the start codon flanked by a NotI site and a PacI site at the 3'-end of the ORF without a stop codon so that the ORF was in-frame with 13 Myc epitopes. We ligated the Myc tag and ORF into pRS313 (25, 31) and transformed the plasmids into an S288c strain containing EB1436. Denatured extracts were prepared and processed as described above except that proteins bound to the Co²⁺-IDA resin were only washed twice with 1 ml of lysis buffer. We detected the Myc tag on immunoblots utilizing the mouse monoclonal antibody 9E10 at a concentration of 0.2 $\mu\text{g/ml}$. We generated mutations in codons encoding lysines by oligonucleotide-mediated overlapping PCR using Vent polymerase (New England Biolabs, Beverly, MA). We sequenced the ORFs to confirm there were no other mutations. To determine whether mutated proteins were able to substitute for wild-type function, we inactivated the *RPS3*, *TUP1*, and *RSC58* ORFs utilizing PCR gene replacement with the *Candida glabrata* *LEU2* gene in a diploid strain and sporulated and dissected tetrads with the appropriate covering plasmid (32).

RESULTS

Creation of 4044 TAP-tagged Strains That Contain *ulp1^{ts}* and *HA₃-SMT3*—To screen systematically each expressed protein in the yeast proteome for SUMO conjugation, we utilized a strategy in which we immunoprecipitated each yeast protein individually and assayed for SUMO conjugation by immunoblot analysis. This immunoprecipitation approach was made possible by the availability of a collection of TAP-tagged yeast strains (22). To enhance our ability to detect

SUMO conjugation, we introduced into the TAP-tagged yeast strains a *ulp1^{ts}* allele and a triple HA (HA₃) epitope-tagged version of *SMT3*. *ULP1* encodes the major desumoylating activity in budding yeast, and the *ulp1^{ts}* allele leads to the accumulation of more SUMO conjugates *in vivo* relative to wild type (3). Also, upon cell lysis, the Ulp1^{ts} protein becomes inactive, preventing loss of SUMO conjugates *in vitro* that are highly labile in wild-type cell extracts (10). We replaced the *SMT3* genomic locus with an *SMT3* coding sequence that is already processed, allowing the *ulp1^{ts}* strain to efficiently accumulate SUMO conjugates (10). Furthermore, to increase the steady-state level of sumoylation, we replaced the *SMT3* promoter with the *CUP1* promoter, which allows for high level expression of Smt3 in the presence of excess copper. Finally Smt3 is not an abundant protein in budding yeast, and while it is detectable with rabbit antibodies raised against Smt3, this antibody is highly cross-reactive with the TAP tag. Therefore, we fused a triple HA epitope tag to the N terminus of processed Smt3 that does not disrupt its function (3); furthermore we independently confirmed that the growth rate was unaffected by this modified form of Smt3 (data not shown). The HA epitope is recognized by mouse monoclonal antibodies, which are at least 10-fold less reactive with the TAP tag than rabbit antibodies (data not shown). It is worth noting that the above modifications to the TAP-tagged strains could lead to false positive or negative results, but we confirmed that these modifications allow for efficient and specific detection of sumoylation utilizing Cdc3-TAP and a control non-sumoylated protein, Msn5-TAP (Fig. 1A).

To introduce the *ulp1^{ts}* and HA₃-*SMT3* traits into the TAP-tagged strains we followed a crossing and selection scheme similar to that described previously (23) (Fig. 1B). Of the 4246 strains that were crossed, we recovered 4034 strains that were Ura⁺ Met⁺ Leu⁺ His⁺ and Can^R. As an independent assessment of whether we successfully recovered haploid strains containing the desired traits, we assayed the resultant strains for temperature sensitivity conferred by the *ulp1^{ts}* allele as this trait is linked to the *MET15* marker but is not selected for directly. Whereas all strains were scored as *MATa* based on a mating type test, ~80% of the strains were temperature-sensitive, indicating that the majority of strains were haploid and suggesting that some strains were *MATa* homozygous diploids. We subjected extracts from a subset of putative diploid strains to immunoblot analysis and established that the strains expressed both TAP-tagged proteins and HA₃-Smt3 (data not shown). We also confirmed that a diploid Cdc3-TAP strain heterozygous for the *ULP1* locus maintained SUMO conjugation during immunoprecipitation in the presence of *N*-ethylmaleimide, an inhibitor of desumoylating activity (data not shown).

Development of a High Throughput Immunoprecipitation Technique to Identify SUMO-associated Proteins—We designed a high throughput assay to immunoprecipitate TAP-tagged proteins and to determine whether the immunopre-

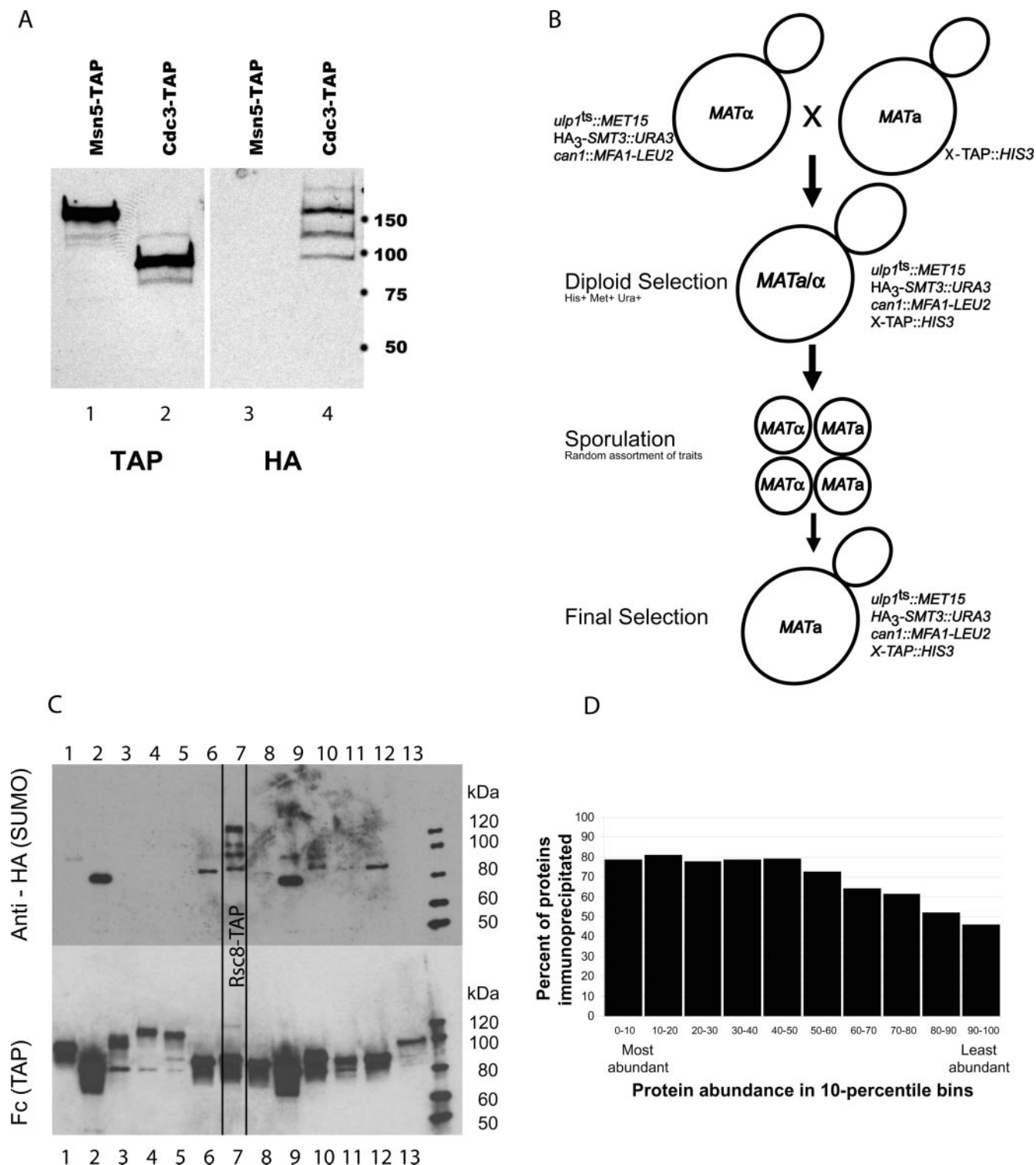


FIG. 1. A, demonstration that Cdc3-TAP sumoylation can be detected with our modifications to the TAP-tagged strains. Msn5-TAP and Cdc3-TAP were immunoprecipitated, and the immunoprecipitates were blotted for reactivity with the TAP (left panel, lanes 1 and 2) and HA (right panel, lanes 3 and 4) epitope tags. While both Msn5-TAP and Cdc3-TAP are immunoprecipitated, only Cdc3-TAP has HA reactivity. B, a schematic of the cross used to generate the TAP strains that were screened in this study. C, immunoblot analyses of immunoprecipitations of 13 proteins: Tbf1, Nde1, Not5, Pho2, Rtf1, Pcm1, Rsc8, Elp3, Pgi1, Pex29, Utp7, Cna1, and Ybr030w, respectively; the same analysis was performed for the rest of the proteome. The vertical lines surround the lane containing the Rsc8-TAP immunoprecipitation. D, abundance of each expressed protein was determined from a genome-wide data set (22), and proteins were binned into 10 fractions with 0–10 being the 10% most abundant proteins. The fraction of proteins immunoprecipitated and visualized in each bin is plotted relative to the abundance of the protein.

precipitated protein was conjugated to SUMO. We performed high throughput immunoprecipitations by adding biotinylated human IgG and streptavidin-Sepharose to extracts derived from the TAP-tagged strains containing *ulp1^{ts}* and HA₃-SMT3. IgG binds with high affinity and specificity to the TAP tag, and streptavidin binds with extremely high affinity to biotin. Importantly this protocol minimizes the cross-reactivity of IgG bands during immunoblot analysis because the biotin-streptavidin interaction is not disrupted by the elution buffer (Fig. 1C).

After immunoblot analysis to detect the TAP tag, we determined the efficiency of immunoprecipitation. Of the 4034 extracts analyzed, we were able to immunoprecipitate 2893 proteins (or 72%). We were able to immunoprecipitate 80% of the proteins that were above the median protein abundance in the proteome as determined by genome-wide immunoblot analysis (22). Importantly our success rate for immunoprecipitating and visualizing even the least abundant proteins was 46% (Fig. 1D). When we compared the ability to immunoprecipitate membrane-bound proteins relative to the proteins defined as soluble, there was no decrease in the immunoprecipitation efficiency of the membrane proteins. This may be a consequence of poor definition of membrane proteins (26) or more likely inclusion of the vesicle fraction in the immunoprecipitation because we performed a relatively low speed centrifugation to remove insoluble proteins during the preparation of cell lysate.

Next we probed the immunoblot to determine whether each TAP-tagged protein was sumoylated (Fig. 1C). Utilizing the 12CA5 mouse monoclonal antibody, we determined whether there was significant HA reactivity in the immunoprecipitate. We observed three different results. 1) There was no HA reactivity, suggesting that the protein is not sumoylated. 2) There was HA reactivity that corresponds to a protein migrating at the same size as the TAP-tagged protein. We observed this HA reactivity with the most abundant, efficiently isolated TAP-tagged proteins, and this reactivity is likely a consequence of 12CA5 cross-reacting with the TAP tag. We did not consider these candidate sumoylated proteins. 3) There was HA reactivity corresponding to a size larger than the major TAP-reactive band. We considered the proteins in category 3 candidate sumoylated proteins. For many sumoylated proteins, we observed multiple HA-reactive bands migrating slower than the major TAP-reactive band, suggesting these proteins may be multiply sumoylated. Because often only a small fraction of a given protein is sumoylated, when we probed for the TAP tag there was little or no TAP tag reactivity overlapping with the HA-reactive band.

Identification of Direct Targets of Sumoylation—Eighty-two proteins reproducibly associated with HA reactivity (category 3 from above) in our immunoprecipitation assay, suggesting an association with SUMO. Because our assay is performed under non-denaturing conditions, the HA reactivity can be a consequence of an associated protein that is sumoylated.

Therefore, we utilized a secondary denaturing screen with a six histidine (His₆) tag on SUMO to determine which candidates were directly conjugated to SUMO. To determine direct SUMO conjugates, we transformed the 82 TAP-tagged strains that scored positive in our primary screen with a plasmid expressing His₆-Smt3 under the control of the *CUP1* promoter. Utilizing the original TAP-tagged strain (with *ULP1^{wt}*) allowed us to eliminate the possibility that the protein was only sumoylated in the presence of the *ulp1^{ts}* allele. The TAP-tagged strains containing His₆-Smt3 were lysed in 8 M urea to disrupt non-covalent protein-protein interactions, and SUMO and its conjugates were purified on immobilized Co²⁺-IDA resin, eluted, and immunoblotted with antibodies that recognize the TAP tag. By comparing strains with and without His₆-Smt3, we identified proteins that were covalently modified by SUMO given that SUMO modification leads to slower migrating forms of the protein (Fig. 2). We demonstrated that 13 proteins were directly conjugated to SUMO. Eight proteins were previously demonstrated to be sumoylated: Aos1, Ubc9, Shs1, Cdc11, Sod1, Rsc8, Rsc58, and Tup1 (6, 19–21); and five were new direct sumoylation targets: Ysh1, Nut1, Rps3, Taf8, and Gsy2. These new targets are involved in transcription, ribosome function, mRNA processing, and cell survival during stress conditions (33–37).

In the primary screen of the 4034 strains, we identified 15 known SUMO-associated proteins. First we identified the septins; both Cdc11 and Shs1 scored positive in the screen. Additionally Cdc10, which is not directly sumoylated (6) but is part of the septin complex, was scored as positive. We also identified eight subunits of the RSC complex that interact with the recently demonstrated direct SUMO targets Rsc2, Rsc8, and Rsc58 (19, 20). Finally we identified other known direct SUMO targets, Aos1, Ubc9, Sod1, and Tup1. Other known sumoylation targets may not have been detected in this screen either because they were not present in the original TAP-tagged collection of strains, could not be immunoprecipitated, or were not sumoylated during logarithmic growth.

Complexes That Are Sumoylated—We performed our primary screen under non-denaturing conditions; therefore, we expected to identify SUMO-interacting proteins that, while not directly conjugated to SUMO, are associated in complexes with sumoylated proteins. To determine whether the proteins we identified were complexed with each other, we utilized a visualization program called Osprey (27) to display protein-protein interactions (Fig. 3). We chose stringent parameters, allowing there to be no more than one degree of freedom from the identified sumoylation targets, and only examined published protein-protein interaction data bases (28–30). Of the 82 proteins that interact with SUMO in our assay, 47 interact with proteins that we have demonstrated to be directly modified by SUMO. We identified four complexes associated with SUMO that are essential for growth: the TFIID, mediator, septin, and RSC complexes. By repeated immunoprecipitation of multisubunit complexes, the non-denaturing immuno-

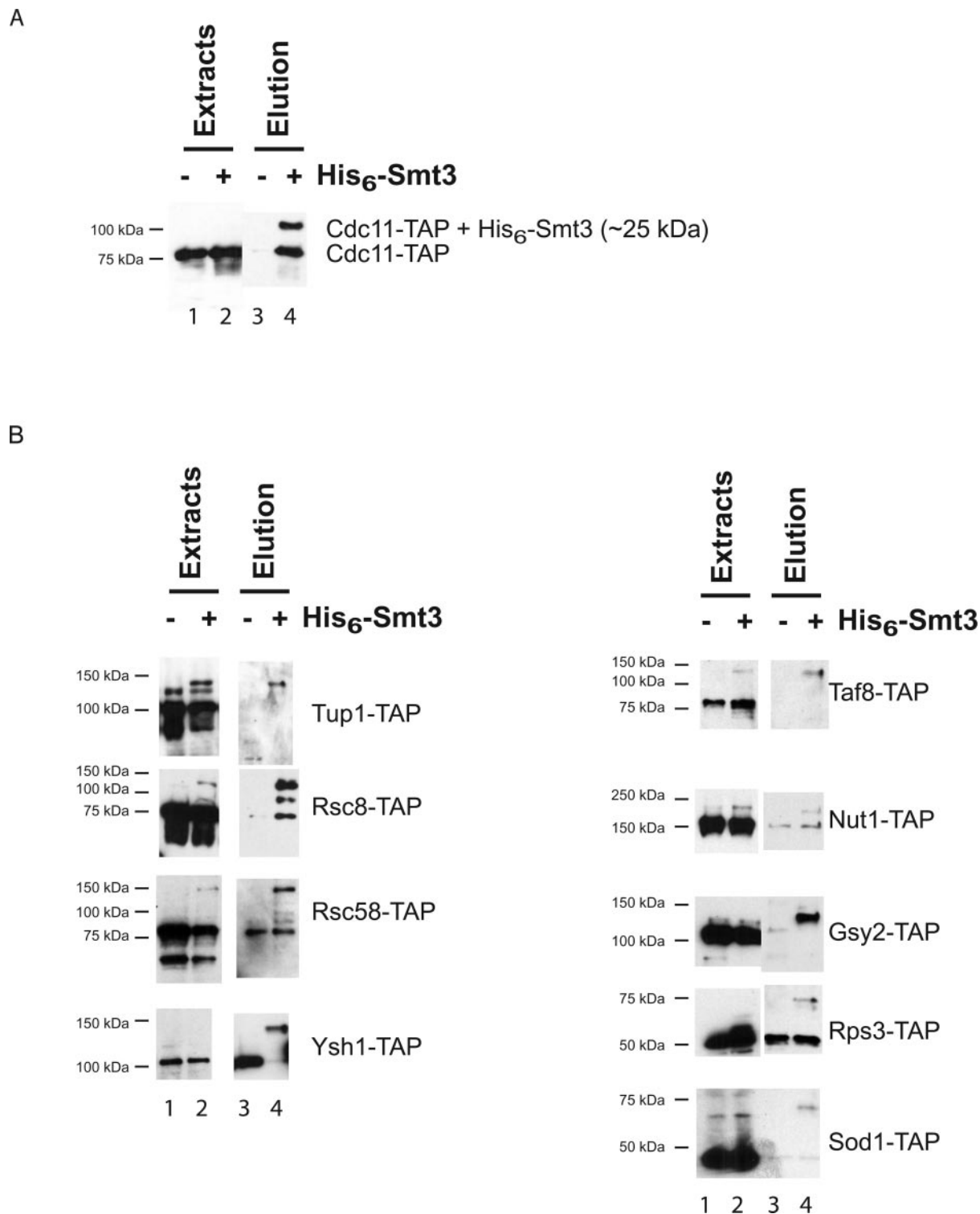


FIG. 2. **Demonstration of direct SUMO targets.** Denatured, cleared extracts from TAP-tagged strains that were either expressing or not expressing His₆-Smt3 were incubated with Co²⁺-IDA-Sepharose. After washing, the bound protein was eluted, and immunoblot analysis for the TAP tag was performed. Direct targets of sumoylation have slower electrophoretic mobility when His₆-Smt3 is expressed. *A*, assay with His₆-Smt3 (~25 kDa) and Cdc11-TAP. *Lane 1* is extract from the Cdc11-TAP strain, and *lane 2* is the same strain containing the plasmid expressing His₆-Smt3. If Cdc11-TAP is modified by SUMO, then after purification of His₆ proteins (*lane 4*), Cdc11-TAP conjugated to His₆-Smt3 will be enriched and detected as a mobility shift relative to the mock purification (*lane 3*) when probing for TAP reactivity. *B*, demonstration of other direct SUMO conjugation targets. *Lanes* are numbered in the same manner as *A* but with the TAP-tagged strains indicated. These assays were performed twice to confirm reproducibility of results.

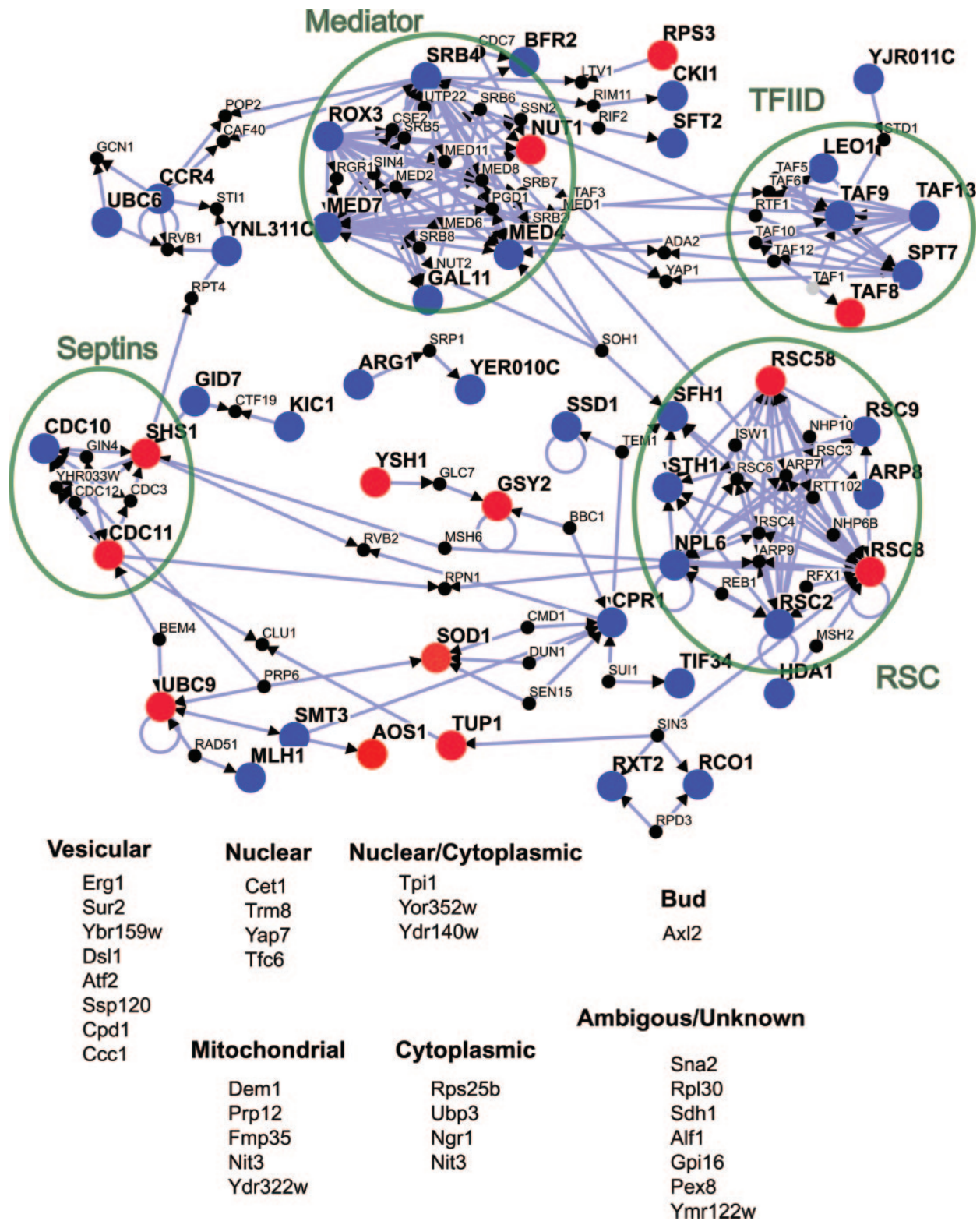


FIG. 3. Visualization of all 82 proteins identified in this screen for sumoylated proteins using Osprey (27). Red circles indicate direct SUMO targets, and blue circles represent proteins that were scored as HA-reactive. Blue lines indicate connection by two-hybrid or mass spectrometry data (28–30). Proteins that could not be connected to one another are listed with their localization (43).

precipitation minimizes two limitations of the TAP-tagged yeast collection: not all proteins are C-terminally tagged, and not all proteins are able to be immunoprecipitated.

The remaining 35 SUMO-interacting proteins provide useful avenues to identify more proteins modified by SUMO. For example, Sdh1 is involved in glucose metabolism, and other studies have suggested a role for SUMO in energy metabolism (21, 38). It may be useful to examine proteins with which Sdh1 is associated to determine whether they are direct targets of SUMO conjugation. It is possible that we did not identify this direct target because it was not tagged in our library, or we were unable to immunoprecipitate the direct target associated with Sdh1.

Confirmation of Proper Identification of Target Proteins—Although systematic strain collections are powerful tools, individual results must be confirmed in an independent manner. To confirm that the direct sumoylation targets that we identified are the annotated proteins we expect, we amplified nine target ORFs from a wild-type genome using PCR and cloned the ORF into a plasmid that C-terminally tagged the ORF with Myc epitopes. We chose these nine ORFs because they had not been identified as direct SUMO targets when we began these studies. We transformed the plasmids encoding the Myc-tagged proteins into a strain that contained a plasmid expressing His₆-Smt3. Exponentially grown cells were harvested and lysed under denaturing conditions. We enriched for sumoylated proteins on Co²⁺-IDA resin and immunoblotted for Myc reactivity. In the His₆-Smt3-enriched fraction, we looked for the presence of Myc-reactive bands that both migrated slower than the major Myc-reactive band present in crude extract and were enriched. By tagging the ORF with an epitope other than the TAP tag, we eliminated the possibility of an antibody binding to the TAP tag nonspecifically. Of the nine sumoylated proteins, we confirmed that eight are indeed modified by SUMO (Fig. 4A). However, Gsy2-Myc sumoylation was not detected in this assay. This could be caused by the changed epitope tag or more likely by the inconsistent determination of Gsy2-TAP as a direct SUMO target. Given that Gsy2 activity varies depending upon environmental conditions (35), it is possible that robust sumoylation of Gsy2 is dependent on growth conditions.

The creation of Myc-tagged plasmids of the eight ORFs allowed us to rapidly mutagenize lysines to identify SUMO conjugation sites. Because SUMO is thought to be conjugated only to lysines, we mutated lysines to arginines, a relatively conservative mutation (6, 14). A consensus site has been described for sumoylation, although it is not always used (14). We mutated 17 consensus sites within the eight proteins, assayed whether the sumoylation pattern of the mutant proteins changed, and identified five lysines that when mutated alter the sumoylation pattern of a protein (Fig. 4B). We confirmed that a sumoylation site previously identified in Sod1 by mass spectrometry is used *in vivo* (21) (Fig. 4B). Interestingly, whereas a majority of the sumoylation is abol-

ished by the Sod1^{K19R} mutation, there is residual sumoylation of altered mobility. Lysine 70 is also sumoylated (21), and it is possible that both lysines must be mutated to eliminate all sumoylation.

We mutated at least one conjugation site in three other proteins: Rps3, Rsc58, and Tup1 (Fig. 4B). Because *RPS3* is an essential gene, we examined whether removal of the majority of sumoylation causes lethality. We find that sumoylation of Rps3^{K212R} is dramatically decreased and that this mutant protein is able to replace the wild-type function of Rps3 during growth in rich medium (data not shown). It is possible that residual sumoylation is sufficient for Rps3 function or that sumoylation of Rps3 is not an essential modification during standard laboratory growth conditions. Interestingly lysine 212 has also been identified as a target of ubiquitylation (39), allowing for the possibility that sumoylation acts as a ubiquitin antagonist. Similarly Tup1^{K269R} is defective in sumoylation. To determine whether sumoylation regulates Tup1 activity, we assayed the *TUP1*^{K269R} allele for the ability to complement a *tup1Δ* strain (40). The mutant protein complemented the flocculation phenotype of the *tup1Δ*, and microarray analysis demonstrated that fewer than 10% of Tup1-dependent genes changed expression more than 2-fold between the wild-type and Tup1^{K269R} proteins (data not shown). The fact that the *TUP1*^{K269R} mutant does not have a dramatic phenotype could be explained by redundant sumoylation of Ssn6, a binding partner of Tup1 that has recently been demonstrated to be sumoylated (20), or by residual sumoylation of Tup1, which we observed in some experiments. Finally Rsc58^{K322R,K328R} is significantly less modified by SUMO than wild-type Rsc58, and this mutant protein allows the cells to grow in standard growth medium in the absence of the wild-type protein. Because we identified Rsc8 as a direct SUMO conjugate as well and others have demonstrated that Rsc2 is modified by SUMO (19), other RSC subunits may need to be mutated before the role of SUMO conjugation in RSC function becomes clear. Thus, it is likely that multiple sumoylation site mutants, either in single proteins or in complexes, will have to be generated to determine the role of SUMO in the regulation of these proteins.

DISCUSSION

Our screen for targets of sumoylation is different from screens performed previously. Whereas previous screens have purified the modification through affinity chromatography (6, 14, 20, 21), we use a systematic substrate-based screen utilizing a characterized TAP-tagged collection of yeast strains (22), minimizing the possibility that purification of highly abundant modified proteins will overwhelm the proteins of low abundance. Our screen is applicable to identify post-translational modifications, including acetylation and phosphorylation, and other ubiquitin-like modifiers such as Rub1, Hub1, or Urm1 (1, 2, 41, 42). This technique is also valuable for identifying interacting proteins of important cell regulators

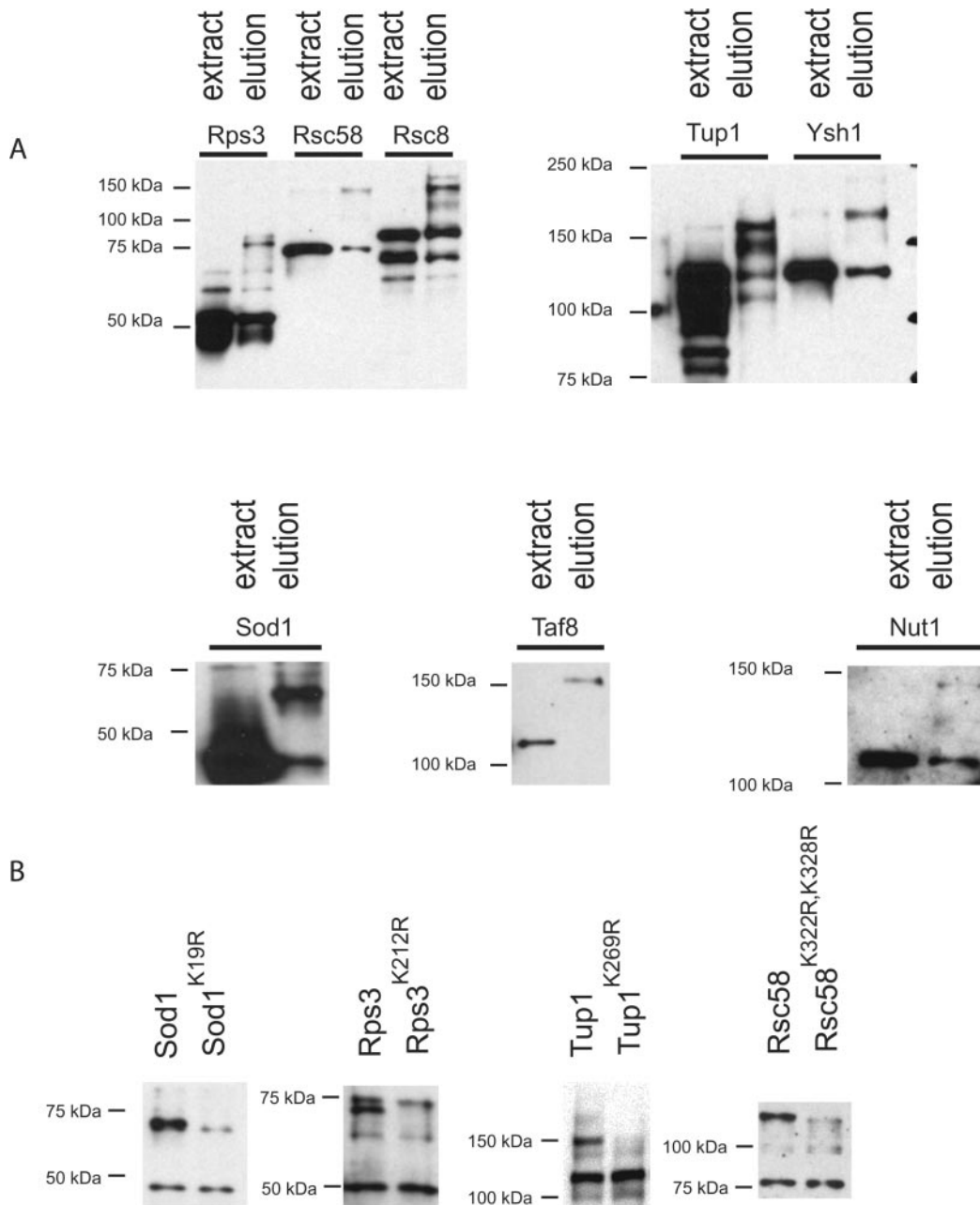


FIG. 4. *A*, demonstration that independent amplification of ORFs by PCR and using an alternative epitope tag (Myc) still allows for sumoylation of the candidate protein. All strains contained His₆-Smt3 and *ULP1*^{wt}. Sumoylation was detected after enrichment for His₆-Smt3 on Co²⁺-IDA-Sepharose. Immunoblot analysis was performed with 9E10 antibody, which detects the Myc epitope. Rps3, Sod1, and Ysh1 appear to be modified by SUMO once, whereas Rsc58, Rsc8, Tup1, and Taf8 appear to be modified by two SUMO moieties. *B*, extracts from the mutants containing His₆-Smt3 were purified on Co²⁺-IDA-Sepharose, washed twice with lysis buffer, eluted, and analyzed by immunoblot with 9E10 antibody.

as it is a proteome-wide co-immunoprecipitation experiment. Furthermore our technique can allow for quantification of the fraction of protein that is modified and for an estimate of the number of modification sites. It should be noted that the technique described here was not as facile and productive as the mass spectrometry screens for SUMO substrates (19–21); however, when screening for substrates of other post-trans-

lational modifications that are difficult to purify, our approach could be a more attractive alternative screen.

We identified new sumoylated proteins that are involved in important processes within the cell. Concurrent mass spectrometry studies have confirmed that Sod1, Rsc8, Rsc58, and Tup1 are sumoylated (19–21), supporting the utility and complementary nature of our screen. We identified Nut1, Ysh1,

and Taf8 as directly sumoylated proteins, and these proteins are present in the cell at less than 2500 copies/cell, which is below the median protein abundance (22). Furthermore the new direct SUMO conjugates that we identified were nuclearly localized (43), which is consistent with the primarily nuclear localization of Smt3, Aos1, Uba2, and Ubc9 (6, 43). The mass spectrometry-based approaches and our approach have identified some of the same substrates, e.g. Sod1 and Tup1, but each study has unique candidates as well (19–21). While it is likely that the identified substrates that overlap in all of the studies are *bona fide* SUMO conjugates, further research is required to validate the unique candidates in each study.

Many of the recently identified targets of sumoylation have demonstrated roles in transcription (34–36, 44, 45). Important mammalian transcription factors that control cell proliferation are sumoylated (11), and our results along with those of others suggest that the regulation of transcription by sumoylation is an evolutionarily conserved process (19). Furthermore the loss of activity of two of the substrates we identified, Rps3 and Sod1, contributes to the human diseases xeroderma pigmentosum class D (46) and familial amyotrophic lateral sclerosis (47), respectively, and understanding how sumoylation regulates the function of these proteins in budding yeast may provide insights into these diseases.

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