Global Analyses of Sumoylated Proteins in *Saccharomyces cerevisiae*

INDUCTION OF PROTEIN SUMOYLATION BY CELLULAR STRESSES

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We have undertaken a global analysis of sumoylated proteins in *Saccharomyces cerevisiae* by tandem mass spectrometry. Exposure of cells to oxidative and ethanol stresses caused large increases in protein sumoylation. A large number of new sumoylated proteins were identified in untreated, hydrogen peroxide-treated, and ethanol-treated cells. These proteins are known to be involved in diverse cellular processes, including gene transcription, protein translation, DNA replication, chromosome segregation, metabolic processes, and stress responses. Additionally, the known enzymes, including E1, E2, and E3 of the sumoylation cascade were found to be auto-sumoylated. Taken together, these results show that protein sumoylation is broadly involved in many cellular functions and this mass spectrometry-based proteomic approach is useful in studying the regulation of protein sumoylation in the cells.

Protein ubiquitination is well known to be involved in protein degradation through the proteasome and intracellular protein sorting (1–3). A family of ubiquitin-like proteins has also been identified to be attached to substrate proteins through enzymatic processes similar to that of ubiquitination (4–6). However, the function of these ubiquitin-like proteins is unclear. Among them, SUMO,1 a small ubiquitin-like modifier, is the most widely studied to date. In mammals, SUMO was first identified to be conjugated to substrate protein such as Ran-GAP1 (7, 8). The Smt3 gene, in the budding yeast *Saccharomyces cerevisiae*, is essential for cell viability and is the only budding yeast homolog of mammalian SUMO. Smt3 was originally identified as a suppressor of Mif2 mutation, and its function in the nucleus has been suggested (9). With an approach similar to that used in the identification of ubiquitination enzymes, the enzymes of protein sumoylation in budding yeast were identified (10, 11).

In the yeast *S. cerevisiae*, Smt3 is first proteolytically processed to expose its Gly-Gly repeat at the C terminus by a SUMO-specific protease, Ulp1 (12). In an ATP-dependent reaction, the C terminus of mature Smt3 forms a thioester bond with the cysteine residue at the active site of the E1 enzyme, which is a heterodimeric Aos1/Uba2 complex (10). Through a trans-esterification reaction, Smt3 is transferred to the SUMO-conjugating E2 enzyme Ubc9, and the C terminus of Smt3 forms a thioester bond with the active site cysteine residue of Ubc9 (11). In the presence or absence of E3 SUMO ligases, the C terminus of Smt3 is then covalently attached to lysine residues of substrate proteins via formation of a stable isopeptide bond. In yeast, two SUMO E3 ligases were identified and are known as Siz1 and Siz2 (13, 14). No other E3 enzyme for Smt3 in the budding yeast has been identified.

A number of SUMO substrate proteins have also been identified in yeast, and these include the septin components, PCNA/Pol30 and Top2 (15–17). However, the function of protein sumoylation remains unclear, and questions remain about whether sumoylation plays a broad role in regulating cellular functions such as gene transcription (18–20). Yeast is a very useful model organism, because it allows both facile genetic and biochemical studies. Yet only a limited number of sumoylated proteins in yeast have been found so far (15–17). Identification of new sumoylated proteins in yeast would immediately reveal the cellular processes in which protein sumoylation plays a role. In addition, it could lead to new target proteins in which the function of sumoylation could be studied in detail. With these goals, we undertook a global analysis of sumoylated proteins in yeast cells under various cell growth conditions. This has revealed the auto-sumoylation of the enzymes involved in protein sumoylation. Furthermore, the newly identified sumoylation substrates have revealed many diverse cellular processes in which sumoylation plays a role.

**EXPERIMENTAL PROCEDURES**

**Materials**—Expression plasmid pYES2/NT was from Invitrogen. Plasmid pFA6a-3HA-kanMX6 was obtained from Mark Longtime (21). Yeast was grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) or in synthetic minimal defined medium with appropriate sugar at 30 °C. Ni2+-NTA resin was from Qiagen; anti-FLAG antibody and conjugated-agarose were from Sigma. Anti-HA antibody and anti-HA affinity matrix were from Roche Applied Science. Protease inhibitor mixture for purification of histidine-tagged proteins was from Sigma.

**Plasmid Construction**—The *S. cerevisiae* SMT3 gene was obtained by PCR using yeast genomic DNA as template, then cloned into the BamHI and XhoI sites of pYES2/NT plasmid. The Xpress epitope in pYES2/NT was mutated to the FLAG epitope, generating pYES-6His-FLAG-SMT3 plasmid, which contains Smt3 with an N-terminal extension MGHS-HHHHHGMASMTGGQQMGRDYKDDDDKVPGPS. The pYES-2HA-SMT3 plasmid was created by replacing the 6His-FLAG tag in the pYES-6His-FLAG-SMT3 plasmid with two copies of HA epitope (amino acid sequence: MGYPYDVPDYAGYPYDVPDYAGVPGPS). To make pFA6a-6His-FLAG-KanMX6 plasmid for C-terminal tagging of genes of interest, the 3HA tag in pFA6a-3HA-kanMX6 plasmid was replaced by...
the 6HisFLAG tag (amino acid sequence: GSSHHHHHGGSMSTG-GQQMGRDYKDDDDYVPGSSVVE), yielding the pFA6a-6HisFLAG-KanMX5 plasmid. DNA sequences in these plasmids were verified by sequencing.

**Genetic Methods Used in the Yeast S. cerevisiae**—To tag the SMT3 gene with 6HisFLAG at the N terminus chromosomal locus, the DNA sequence of 6HisFLAG-SMT3 from the above pYES-6HisFLAG-SMT3 plasmid was subcloned into the pFA6a-3HA-kanMX6 plasmid, generating the pFA6a-6HisFLAG-SMT3-kanMX6 plasmid. The chromosomal SMT3 was then replaced by 6HisFLAG-SMT3 through PCR-based homologous recombination. The forward primer sequence was 5’-GGAGAAGGGCACTTATGTCATTTAATAATACCGAGCGatgggggtactcactat, and the reverse primer sequence was 5’-TGCTCCGGGGGGATGGTGCTGCTATTGCAGTTAAAGTCCGTGatgggggtctcatcact.

**Purification of Sumoylated Proteins in Yeast**—Two liters of HZY1017 cells were grown in YPD and harvested at log phase \((A_600 \sim 1)\) by centrifugation. Collected cells were broken by glass beads using a beads-beater in denatured lysis buffer (100 mM NaHPO\(_4\), 10 mM Tris, pH 8.0, 0.1% SDS, 8 mM urea, and protease inhibitor mix). Cell debris was removed by centrifugation at 30,000 \(\times g\) for 30 min. The resulting supernatant was incubated with 2 ml of Ni\(^{2+}\)-NTA resin for 1 h, then washed by cold native wash buffer (50 mM NaHPO\(_4\), 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 8.0). Proteins were eluted by using 10 ml of cold elution buffer (50 mM NaHPO\(_4\), 300 mM NaCl, 250 mM imidazole, 0.05% Tween 20, pH 8.0) and mixed with 100 \(\mu\)l of anti-HA affinity matrix (Roche Applied Science). After incubating for 2 h at 4 °C, the beads were washed by PBST (phosphate-buffered saline containing 0.05% Tween 20), then washed by water to remove salt. Lastly, the sumoylated proteins were eluted with 1% acetic acid, then dried.

**Purification of Sumoylated Sod1**—Six liters of HZY1206 cells, transformed with pYES-2HA-SMT3 plasmid, were grown in HSM-ura dropout media (Qiogene) with 1% raffinose. At \(A_600 \sim 0.6\), galactose was added to a final concentration of 1%; cells were grown for an additional 4 h. The cells were harvested then broken by glass beads in ice-cold native lysis buffer (50 mM NaHPO\(_4\), 300 mM NaCl, 10 mM imidazole, 0.1% Tween 20, pH 8.0). Cell debris was removed by centrifugation at 30,000 \(\times g\) for 30 min. The resulting supernatant was incubated with 4 ml of Ni\(^{2+}\)-NTA resin for 1 h at 4 °C then washed with native wash buffer (50 mM NaHPO\(_4\), 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 8.0). His\(^{6}\)-FLAG-tagged Sod1 was eluted by 20 ml of elution buffer (50 mM NaHPO\(_4\), 300 mM NaCl, 250 mM imidazole, 0.05% Tween 20, pH 8.0). The volume of the eluted fraction was reduced to 5 ml by an Amicon Ultra centrifugal filter device (Millipore); then mixed with 50 \(\mu\)l of anti-HA affinity matrix (Roche Applied Science). After incubating for 2 h at 4 °C, the anti-HA affinity matrix was washed three times with PBST. Sumoylated Sod1 was eluted by boiling the resin two times with 0.6 M urea, then mixed with 50 \(\mu\)l of anti-FLAG agarose and further purification as described above. The sumoylated Sod1 was eluted by 1% acetic acid and dried.

**Protein Techniques**—Proteins were resuspended in SDS-PAGE sample buffer containing 100 mM DTT, boiled for 5 min, then loaded onto a 4–12% SDS-PAGE gel. After electrophoresis, the proteins were transferred onto a Hybond-C membrane (Amersham Biosciences). The membrane was blocked by TBS (20 mM Tris, pH 7.8, 150 mM NaCl) containing 5% dry milk, and incubated with anti-FLAG or anti-HA antibody for 1 h at 4 °C. The membrane was then washed three times with TBST (TBS containing 0.1% Tween 20) and incubated with a 1:10,000 dilution of the anti-mouse horseradish peroxidase conjugate (Amersham Biosciences) for 1 h. The membrane was washed and then developed with the enhanced chemiluminescence reagent ECL (Amersham Biosciences). Unless otherwise specified, ECL was always used in the Western blot analyses. In some instances, anti-mouse secondary antibody-alkaline phosphatase conjugate (Sigma) was used, and the membrane was developed in 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Sigma).

**Mass Spectrometry Analysis**—Purified protein was either subjected to in-solution digestion or in-gel digestion. For in-solution digestion, dried sample was resuspended in 20 \(\mu\)l of 6 M urea, and 100 \(\mu\)l of 50 mM Tris-HCl, pH 8.0, was added to dilute the urea to 1 m. Trypsin was then added to the solution for overnight digestion. In-gel digestion was done according to a standard procedure (22). Tryptic peptides were analyzed by microcapillary reverse-phase high-performance liquid chromatography microelectrospray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer. A 100-μm × 10-cm fused silica capillary column in-house packed with the C\(_18\) resin (Michrom Bio-Resources) was used, and the flow rate was typically 250 nL/min. COMET, a software package originally developed by the Institute for Systems Biology, was used in data analysis (23).

**RESULTS**

Identification of Sumoylated Proteins in Yeast Cells Grown in YPD—To facilitate purification of sumoylated proteins in yeast, we first replaced the chromosomal SMT3 gene by N-terminal 6HisFLAG-tagged SMT3, yielding the strain HZY1017. Several observations suggest that replacing the SMT3 gene by 6HisFLAG-SMT3 does not affect protein sumoylation events in yeast cells. First, Smt3 is essential for cell viability. We observed no difference in cell growth comparing WT cells and HZY1017 cells. In addition, it is well known that the C terminus of Smt3 is processed and covalently attached to the lysine residues of substrate proteins. N-terminal epitope-tagged Smt3 in a plasmid has been widely used for analysis of protein sumoylation, and its attachment to substrate proteins does not appear to be affected (11, 15). Second, as shown in Fig. 1, the sumoylation pattern in HZY1017 cells closely resembles those found by others using anti-Smt3 antibody (12, 14). Therefore, it appears that the sumoylation process in the 6HisFLAG-Smt3 cell is unaffected by protein tagging.

The sumoylated proteins from the HZY1017 cells were purified by tandem affinity purification under denaturing condition as described in the experimental procedure above. The results are shown in Fig. 2. The high molecular weight sumoylated proteins (around 100 kDa) in Fig. 1 are likely septsins (15). Septins tend to form aggregates during purification and lead to variable amount of their recovery. As shown in Fig. 2, thirteen silver-staining bands corresponding to those positive bands with the anti-FLAG Western blot were excised from the gel and subjected to in-gel trypsin digestion. The peptides were extracted from the gel and analyzed by mass spectrometry. Tryptic peptides from Smt3 were found in each band. In addition, a number of sumoylated protein candidates were identified (Table II). Because cova...
Identification of Sumoylated Proteins in Yeast by Mass Spectrometry

Verification of Sumoylation of Elp5 and Sod1—We asked whether some of these candidate proteins in Table II could be verified by methods other than mass spectrometry. Elp5 is one subunit of a six-subunit Elongator Complex that associates with the elongating form of RNA polymerase II (24). To verify Elp5 as a sumoylated substrate, the C-terminal 6HisFLAG-tagged Elp5 cells were transformed with pYES-2HA-Smt3 plasmid. Tagged Elp5 was purified by Ni²⁺-NTA resin under denaturing conditions and anti-FLAG immunoprecipitation. The purified Elp5 was readily detected by anti-FLAG (Fig. 3A), whereas the sumoylated Elp5 was detected by anti-HA (Fig. 3B). The sumoylated Elp5 appears in the exact same region as in Fig. 2, which was originally identified by mass spectrometry. The relatively faint upper band in Fig. 3A detected by anti-FLAG appears to coincide with the sumoylated Elp5 revealed by anti-HA in Fig. 3B. It thus appears that stoichiometrically, the sumoylation of Elp5 is fairly low. We also purified Elp5 under native conditions, in which Elp4 and Elp6 of the elongator complex were copurified; however, only Elp5 was found to be sumoylated.²

To verify the sumoylation of Sod1 in yeast, C-terminal 6His-FLAG-tagged Sod1 was purified from cells transformed with 2HA-Smt3. As shown in Fig. 3C, when the purified Sod1 was detected by anti-FLAG, an additional faint band corresponding to the molecular weight of sumoylated Sod1 could be seen. When the same purified protein sample was detected by anti-HA in Fig. 3D, the sumoylated Sod1 was clearly detected at a position that coincided to the upper band in Fig. 3C, as well as Band 1 in Fig. 2. Clearly, Sod1 is sumoylated in yeast under these growth conditions.

To further examine the sumoylation of Sod1, we purified the sumoylated Sod1 by three steps of affinity purification as described previously. Silver staining of the purified protein in Fig. 3C, when the purified Sod1 was detected by anti-HA, an additional faint band corresponding to the molecular weight of sumoylated Sod1 could be seen. When the same purified protein sample was detected by anti-HA in Fig. 3D, the sumoylated Sod1 was clearly detected at a position that coincided to the upper band in Fig. 3C, as well as Band 1 in Fig. 2. Clearly, Sod1 is sumoylated in yeast under these growth conditions.

| Band analyzed | Observed molecular mass on SDS-PAGE (kDa) | Proteins identified (expected molecular mass) |
|--------------|------------------------------------------|-----------------------------------------------|
| 1            | 40                                       | Smt3 (15), Ubc9 (18), and Sod1 (16)            |
| 2            | 48                                       | Smt3 (15) and Mrp8 (25)                        |
| 3            | 52                                       | Smt3 (15) and Ipd1 (32)                        |
| 4            | 55                                       | Smt3 (15), Elp5 (35), and Tdh3 (36)            |
| 5            | 60                                       | Smt3 (15), Aos1 (39), and Fba1 (40)            |
| 6            | 65                                       | Smt3 (15), Tif2 (45), and Tef2 (50)            |
| 7            | 68                                       | Smt3 (15), Tif2 (45), and Rpa49 (47)           |
| 8            | 70                                       | Smt3 (15) and Pgd1 (45)                        |
| 9            | 75                                       | Smt3 (15) and Pgi1 (61)                        |
| 10           | 85                                       | Smt3 (15) and Uba2 (71)                        |
| 11           | 90                                       | Smt3 (15) and Uba2 (71)                        |
| 12           | 100                                      | Smt3 (15) and Cdc3 (60)                        |
| 13           | 110                                      | Smt3 (15), Cdc3 (60), and Eif2 (93)            |

² W. Zhou, J. J. Ryan, and H. Zhou, unpublished results.

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To further examine the sumoylation of Sod1, we purified the sumoylated Sod1 by three steps of affinity purification as described previously. Silver staining of the purified protein in Fig. 3A indicates that the sumoylated Sod1 was highly pure, along with some additional unmodified Sod1. The identity of the sumoylated Sod1 was again confirmed by anti-HA Western blot (see Fig. 4B). Furthermore, tryptic peptides from only Sod1 and Smt3 were found in the band corresponding to the sumoylated Sod1 by mass spectrometry.³ Additionally, two sumoylated peptides from Sod1 were identified by tandem mass spectrometry analysis (Table III). Trypsin digestion of a sumoylated protein will produce sumoylated peptide(s) containing an internal lysine residue, modified by a fragment derived from the C terminus of Smt3 with the sequence EQIGG via an isopeptide bond. The expected mass modification on the lysine residue due to sumoylation is 484.5 Da, which was used in tandem mass spectra analysis. Single mutation of these sumoylation sites

³ W. Zhou, J. J. Ryan, and H. Zhou, unpublished results.
revealed that Sod1 was modified at more than one site by Smt3; however, it is presently unknown how many lysine residues of Sod1p could be sumoylated. Further studies are needed to reveal the biological function of the sumoylation of Sod1 in yeast.

Interestingly, enzymes that are known to catalyze sumoylation events in yeast, including the heterodimeric E1 enzyme consisting of Aos1 and Uba2 and the E2 enzyme Ubc9, were found to be sumoylated (see Table II). Ubc9 was found to be auto-sumoylated previously (25). The sumoylation of E1 and E2 enzymes are not due to thioester formation between E1 and E2 enzymes with Smt3p, because they are unaffected by 100 mM DTT under boiling conditions. One previously known sumoylation substrate Cdc3 was also identified in this experiment. Additionally, translation initiation factor TIF2, translation elongation factors Te2 and Eft2, and RNA polymerase I subunit Rpa49 were identified, along with several metabolic proteins. It thus appears that sumoylation is linked to protein translation in the ribosome and RNA polymerase function.

Identification of Sumoylated Proteins in Yeast Cells under Stress Conditions—we next asked whether protein sumoylation is affected by various cellular stresses, including oxidative stress and changes in growth media. We found that the sumoylation pattern was affected significantly under these conditions, as shown in Fig. 5. Although there is a relatively low level of sumoylation in yeast cells without stress, in the presence of 1 mM H₂O₂ or 10% ethanol, many more proteins become sumoylated. Particularly when cells were treated with ethanol, the abundance of monomer Smt3p dropped significantly, whereas a much higher level of protein sumoylation was found. We chose to purify the sumoylated proteins from cells treated with hydrogen peroxide and ethanol, and the purified proteins were visualized by silver staining in Fig. 6. Because no discrete bands were present from the Western blot analysis in Fig. 5, the gel in Fig. 6 was sliced into 20 equal segments. The proteins from each gel slice were identified, and the results are shown in Table IV. Smt3p was found in all gel slices analyzed, indicating that there is a high level of sumoylation of the other proteins identified in the same gel slice. Based on their electrophoretic mobility, most of these proteins have observed molecular weights significantly higher than their theoretical molecular weights, suggesting that they are likely sumoylated. These sumoylated protein candidates appear to be involved in many diverse cellular pathways, as summarized in Table V.

First, the known enzymes involved in the SUMO conjugation pathway were found to be auto-sumoylated (see Table V). Not only were E1 and E2 enzymes identified, but Siz1 and Siz2, the known E3 enzymes for SUMO conjugation, were also identified in this experiment. It thus appears to be a general property that the enzymes involved in the SUMO conjugation pathway are auto-sumoylated. Although a given protein can be modified by both ubiquitin and SUMO (16, 26), no ubiquitin was identified in these purified sumoylated proteins here, suggesting that they are alternate, rather than simultaneous modifications. Interestingly, Siz1 was identified in multiple gel slices spanning from the observed molecular masses of 150–250 kDa (see Table IV, ethanol-treated cells), indicating that Siz1 is multiply sumoylated.

A number of previously known sumoylated proteins were identified here. They include Cdc3, Cdc11, Shs1, and Pol30/PCNA. Pol30 was identified in both gel slices 7 and band 11 (see Table IV), indicating that Pol30 was modified by Smt3 more than once, an observation known previously (16). Here, two sumoylated peptides of Pol30 were identified directly by tandem mass spectrometric analysis as shown in Table III, and they correspond to the two known sumoylation sites in Pol30. Yeast Pol30 was previously reported to be sumoylated when cells were treated with the DNA-damaging agent methyl methane sulfonate (16). We asked whether the sumoylation of Pol30 is affected by the cellular stresses used here. C-terminal 6His-FLAG-tagged Pol30 cells were transformed with pYES-2HA-SMT3 plasmid, and the tagged Pol30 was purified. As shown in Fig. 7, the sumoylated Pol30 could be detected when cells are subjected to oxidative and ethanol stresses, while it is absent in cells without stress treatment. These results show that sumoylation of Pol30 has a general role in stress response, in addition to its role in the DNA damage response.

A large number of novel sumoylated proteins were identified (see Tables IV and V). Among them, H2B, a histone component, was identified along with Smt3 from cells treated with hydrogen peroxide. The observed molecular mass of H2B is about 35 kDa, consistent with H2B being modified by Smt3. Interestingly, sumoylation of H2B was not found in cells grown inYPD or ethanol-treated cells by mass spectrometry (see Table IV). The function of Smt3 in chromosome segregation was long implicated (9). Here Bir1, a 109-kDa protein involved in the kinetochore function (27), was identified in multiple gel slices 17–19, corresponding to apparent molecular masses from 130 to 190 kDa. It appears likely that Bir1 is multisumoylated.
Multisumoylation of Bir1 apparently depends on oxidative stress, because it was only found in cells treated with hydrogen peroxide, but not in cells grown in YPD or ethanol-treated cells. Many sumoylation substrates in mammalian cells were found to be involved in gene transcription (6). Consistent with this notion, one of the largest families of nuclear proteins found to be sumoylated here are involved in gene transcription (see Table V). Their identification should facilitate future investigation of the role of sumoylation in these processes.

The sumoylated candidates in Table IV were filtered based primarily on their observed and expected molecular weights. Because Smt3 (15 kDa with the tag) modification is expected to increase the molecular mass of substrate protein by at least 15 kDa, it is therefore likely that many of the proteins in Table IV are sumoylated substrates, particularly, those proteins identified in multiple regions of the gel. Furthermore, we sought to identify sumoylated peptides by tandem mass spectrometric analysis. Identification of sumoylated peptides from any given protein would directly validate it as an authentic sumoylated substrate. Through both in-gel digestion and in-solution digestion of the purified sumoylated proteins, we identified several sumoylated peptides, including both known (15) and novel sumoylation sites (see Table III).

### DISCUSSION

In this work, we undertook a global analysis of sumoylated proteins by mass spectrometry using the budding yeast *S. cerevisiae* as a model organism. In addition to the identification of all previously known sumoylated proteins in yeast, a large number of new sumoylated substrates was identified. Cellular stresses such as oxidative stress and ethanol stress led to a dramatic increase in the level of protein sumoylation in yeast cells. This increase in protein sumoylation does not appear to be a result from a single protein; rather it appears to be derived from a large number of proteins, including heat shock and ribosomal proteins. It is interesting to note that recent works on the sumoylation in mammalian cells and plants also showed marked increase of sumoylation due to cellular stresses (28, 29). The molecular mechanism of elevated sumoylation under cellular stresses could be due to a number of reasons, including changes in the enzymatic activities of sumoylation and desumoylation enzymes.
role of sumoylation in stress response and the mechanism should be of interest for further investigation.

We identified all known sumoylation enzymes in yeast by this mass spectrometric approach and found auto-sumoylation of E1, E2, and E3 enzymes to be a general property of the sumoylation enzyme cascade. This may not be too surprising considering that ubiquitination enzymes are also known to be auto-ubiquitinated (30). Furthermore, auto-sumoylation of the E3 enzymes, Siz1 and Siz2, was readily identified following ethanol stress, but not in the untreated cells. Thus, enhanced auto-sumoylation of E3 enzymes correlates with the overall increase in protein sumoylation in cells. Presently, whether and how the activities of Siz1 and Siz2 are regulated under cellular stresses remain poorly understood.

Here we identified a large number of sumoylated proteins involved in many cellular processes. Among them, the sumoylation of Pol30 was previously examined in detail in relation to the DNA damage response (16, 31). We further showed that the sumoylation of Pol30 is induced by both oxidative and ethanol stresses. Thus the sumoylation of Pol30 may be more generally involved in the stress response. Cu,Zn-superoxide dismutase (Sod1) is an abundant enzyme that scavenges superoxide anions that can cause damage to DNA, lipids, and protein. Mutations of Sod1 in humans are known to be involved in pathogenesis of amyotrophic lateral sclerosis (32). It is interesting to note here that Sod1 in yeast is modified by sumoylation, along with Tsa1, an antioxidant enzyme. Both Sod1 and Tsa1 are

| Gel slice | Observed molecular mass (kDa) | Proteins identified in H2O2-treated cells (expected molecular mass) | Proteins identified in ethanol-treated cells (expected molecular mass) |
|-----------|-------------------------------|---------------------------------------------------------------|-------------------------------------------------------------------|
| 1         | 35 Smt3 (15) and H2B (14)     | Smt3 (15) and Rps17B (16)                                     |                                                                   |
| 2         | 38 Smt3 (15)                   | Smt3 (15) and Rps8A (23)                                      |                                                                   |
| 3         | 40 Smt3 (15) and Ubc9 (18)     | Smt3 (15), Rps8A (23), Ubc9 (18), and Sod1 (16)               |                                                                   |
| 4         | 42 Smt3 (15) and Rpl40A (14)   | Smt3 (15), Rps8A (23), and Tsa1 (22)                          |                                                                   |
| 5         | 45 Smt3 (15) and Mrp8 (25)     | Smt3 (15), Rps8A (23), Rps0A (28), Mrp8 (25), Rhr2 (30), Gpm1 (28), and Hmo1 (28) |                                                                   |
| 7         | 52 Smt3 (15), Pol30 (29), Sce2 (27), and Rpb4 (25) | Smt3 (15), Pol30 (29), Sce2 (27), and Rps1 (29) |                                                                   |
| 8         | 55 Smt3 (15) and Elp5 (35)     | Smt3 (15), Elp5 (35), Hsp26 (24), Aoe1 (39), and Smt1 (30)    |                                                                   |
| 9         | 58 Smt3 (15) and Rps43 (36)    | Smt3 (15), Fba1 (40), Aoe1 (39), Toa1 (32), Adh1 (37), Adh2 (37), and Rps43 (36) |                                                                   |
| 10        | 62 Smt3 (15), Tif2 (45), and Toa1 (32) | Smt3 (15), Tif2 (45), Tif11 (48), Cdc11 (48), and Eno1 (47), and Toa1 (32) |                                                                   |
| 11        | 65 Smt3 (15), Pol30 (29), Cdc11 (48), and Rpe53 (47) | Smt3 (15), Pol30 (29), Cdc11 (48), Eno1 (47), and Pgk1 (45) |                                                                   |
| 12        | 70 Smt3 (15) and YMR111C (52)  | Smt3 (15)                                                     |                                                                   |
| 13        | 75 Smt3 (15) and Bec58 (58)    | Smt3 (15), Sho1 (63), Sho2 (63), and Rps8 (63)                |                                                                   |
| 14        | 80 Smt3 (15) and Cdc3 (60)     | Smt3 (15), Cdc3 (60), Sho1 (63), Ssa1 (70), Sho2 (67), and YDR336W (36) |                                                                   |
| 15        | 90 Smt3 (15) and Cdc3 (60)     | Smt3 (15), Cdc3 (60), Hsc82 (81), Hsc82 (81), Ssa1 (77), Gps1 (75), Ubc2 (71), and Siz2 (81) |                                                                   |
| 16        | 110 Smt3 (15), Cdc3 (60), Tup1 (78), and Gcd7 (43) | Smt3 (15), Cdc3 (60), Hsc82 (81), Siz2 (81), Cdc48 (92), Ef2 (93), Tfg1 (82), Abf1 (82), and Rps8A (23) |                                                                   |
| 17        | 130 Smt3 (15), Bir1 (109), and Sp5 (116) | Smt3 (15), Cdc3 (60), Hsc82 (81), Siz1 (101), and Siz2 (81) |                                                                   |
| 19        | 190 Smt3 (15), Rpo21 (192), and Bir1 (109) | Smt3 (15), Rpo21 (192), Siz1 (101), and Hsc82 (81) |                                                                   |
| 20        | 250 Smt3 (15), Rpo21 (192), and Rpa190 (186) | Smt3 (15), Rpo21 (192), and Siz1 (101) |                                                                   |

**Table V**

**Summary of sumoylated proteins identified in yeast cells under stresses**

| Biological process involved | Proteins identified |
|-----------------------------|---------------------|
| SUMO conjugation enzymes     | Uba2, Aos1, Ubc9, Siz1, and Siz2 |
| Components of the septins    | Cdc3, Cdc11, and Sha1 |
| Stress-related proteins      | Hsp26, Hsp82, Hsc82, Ssa1, Ssb1, Sse1, Sse2, Tsa1, and Sod1 |
| DNA replication-related proteins | Pol30 |
| Chromatin-related proteins   | H2B, Rse56, Pol3, and Abf1 |
| Chromosome segregation       | Bir1 |
| Transcription-related proteins | Rpo21, Rpa190, Rpa43, Rpe53, Elp5, Tfg1, Gps1, Toa1, Hmo1, Tup1, Sp5, and Spp41 |
| Translation-related proteins | Eft2, Tef2, Tif2, Gcd7, Yef3, Hcr1, Rpl28, Rpl40A, Rps3, Rps1A, Rps0A, Rps8A, Rps17B, and Mrp8 |
| Metabolism-related proteins  | Cdc19, Eno1, Rhr2, Adh1, Adh2, Gpm1, Fba1, and Pgk1 |
| Other                       | Stn1, Cdc48, Sce2, YMR111C, and YDR336W |

**Fig. 7.** Western blot analysis of the purified Pol30. 6His-FLAG-tagged Pol30 was purified from various conditions: without stress treatment (lane 1), with 1 mM H2O2 (lane 2), and 10% ethanol (lane 3), then analyzed by SDS-PAGE and detected with anti-HA antibody.
involved in protecting cells against oxidative stresses. Whether and how sumoylation of these enzymes contribute to their anti-oxidative functions in yeast cells would be interesting in further study. Recently, sumoylation of a component of histone, the H4 protein, in human cells was found (33). Here we identified H2B in yeast as a likely sumoylated substrate (see Table IV). Histone is known to be modified by a number of post-translational modifications on the amino side chain of lysine residues, including ubiquitination, methylation, and acetylation (34). The finding of histone sumoylation further adds to the complex nature of post-translational modifications of histone. Bir1 is involved in kinetochore function (27). Here, we found it to be multisumoylated in cells under oxidative stress (see Table IV). Further investigation of the regulation of sumoylation of Bir1 could reveal the role of sumoylation in chromosome segregation, a function of sumoylation long implicated (9). Consistent with the function of Smt3 in the nucleus, the largest families of proteins found to be sumoylated substrates are those involved in regulation of DNA replication, gene transcription, and protein translation (see Table V). It is interesting to note that several proteins were also found in multiple bands (see Table IV), suggesting that they are multisumoylated. They include Rps8A, Rps0A, Pol30, Stm1, Cdc11, Cdc3, Siz1, Rpo21, and Bir1, among others.

Identification of sumoylation sites of a sumoylated substrate is frequently the first step toward analysis of the function of sumoylation. Although sumoylated peptides of a given protein may not always be found by mass spectrometry, the mass spectrometric approach is unbiased and could reveal any new sumoylation sites not predicted from the consensus sumoylation sequence alone. Here we identified a number of sumoylated peptides using tandem mass spectrometric analysis (see Table III). The preferred sumoylation sites according to the consensus $\Psi$(K/X/D/E) motif were found to be quite common (see Table III). However, sumoylation of peptides with different amino acid sequences were also found. Proteins such as Pol30 and Sod1 have both consensus and non-consensus sumoylation sites. The existence of non-consensus sumoylation sites nevertheless raises questions about whether the commonly used lysine to arginine mutation alone, based on the consensus sumoylation sites, would be sufficient (6, 15, 16). Because many sumoylated proteins appear to be modified on more than one lysine residue, and each lysine residue could be modified by a number of other modifications, including ubiquitin, methylation, and acetylation. It is perhaps not surprising that mutation analysis has not been particularly informative. Understanding the role of protein sumoylation likely would require development of a suitable biochemical and cell biological assays for specific substrates. The identification of a large number of sumoylated substrates here should facilitate such effort in future study.

Collectively the data here showed that protein sumoylation is highly dynamic and is involved in a wide range of cellular processes. Sumoylation is clearly involved in gene transcription, protein translation, chromatin function, chromosome segregation, DNA replication and repair, stress response, and so on. The role of ubiquitin, for example, has been expanded significantly from protein degradation and the proteasome pathway to intracellular trafficking and nuclear functions (35). It is perhaps not so surprising that protein sumoylation also has many diverse functions. Detailed analysis of the sumoylated proteins identified here in future studies would be interesting and necessary to uncover the function of protein sumoylation in these cellular processes. Additionally, auto-sumoylation of the enzymatic cascade of sumoylation is evident; particularly, auto-sumoylation of E3 enzymes appears to correlate with the overall increase of sumoylation in cells.

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