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Formation and Nuclear Export of Preribosomes Are Functionally Linked to the Small-Ubiquitin-Related Modifier Pathway

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Abstract

Ribosomal precursor particles are initially assembled in the nucleolus prior to their transfer to the nucleoplasm and export to the cytoplasm. In a screen to identify thermosensitive (ts) mutants defective in the export of pre-60S ribosomal subunit, we isolated the *rix16-1* mutant. In this strain, nucleolar accumulation of the Rpl25-eGFP reporter was complemented by *UBA2* (a subunit of the E1 sumoylation enzyme). Mutations in *UBC9* (E2 enzyme), *ULP1* [small-ubiquitin-related modifier (SUMO) isopeptidase] and *SMT3* (SUMO-1) caused 60S export defects. A directed analysis of the SUMO proteome revealed that many ribosome biogenesis factors are sumoylated. Importantly, preribosomal particles along both the 60S and the 40S synthesis pathways were decorated with SUMO, showing its direct involvement. Consistent with this, early 60S assembly factors were genetically linked to SUMO conjugation. Notably, the SUMO deconjugating enzyme Ulp1, which localizes to the nuclear pore complex (NPC), was functionally linked to the 60S export factor Mtr2. Together our data suggest that sumoylation of preribosomal particles in the nucleus and subsequent desumoylation at the NPC is necessary for efficient ribosome biogenesis and export in eukaryotes.

Keywords

Mtr2; nuclear export; ribosome biogenesis; ribosome export; SUMO; Ulp1; yeast Smt3

Eukaryotic ribosome biogenesis is a highly dynamic and coordinated multistep process that starts with transcription of rDNA (ribosomal DNA) repeats by RNA polymerase I [25S, 18S and 5.8S ribosomal RNA (rRNA)] and RNA polymerase III (5S rRNA). The RNA polymerase-I-derived transcript (35S pre-rRNA) undergoes extensive processing and modification (1,2). Concomitantly, ribosomal proteins are assembled onto the maturing

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rRNA to form preriboso-mal particles (3). In the past, genetic screens have identified many factors involved in the assembly of the ribosomal subunits (1). Recently, a major advance in the field was achieved by isolation of maturing preribosomal particles via tandem affinity purification (TAP) methods, aided by sensitive mass spectrometry. This greatly expanded the inventory of nonribosomal factors that participate in the assembly, maturation and transport of preribosomal particles (4–7). These factors include rRNA-modifying enzymes, endonucleases, exonucleases, RNA helicases, adenosine triphosphatases associated with various cellular activities, guanosine triphosphatases and proteins associated with small nucleolar RNAs (3). Subsequent proteomic approaches helped unravel changes in the protein and rRNA composition of preribosomal particles (60S and 40S) as they travel from their site of initial assembly in the nucleolus to their final site of maturation in the cytoplasm (6,8–10).

We have previously described a visual assay to isolate factors involved in 60S export that detects the nucleolar/ nuclear accumulation of an Rpl25-eGFP reporter in thermosensitive (ts) mutants (11). By screening a ts mutant collection using this assay, numerous trans-acting factors that are required for the intranuclear transport or export of pre-60S ribosomal subunits were identified (12–16). Here, we report that the ts phenotype and nuclear accumulation of Rpl25-eGFP of one of the isolated mutants *(rix16-1)* was complemented by *UBA2,* which encodes a subunit of the heterodimeric E1 enzyme of the small ubiquitin-related modifier (SUMO) pathway. The ubiquitin-like protein SUMO-1 (Smt3 in yeast) (17,18) is conjugated to substrate proteins by sequential thioester transfer reactions via E1 (Uba2/Aos1) and E2 (Ubc9) enzymes (19,20). Unlike ubiq-uitination, sumoylation of target proteins does not lead to degradation by the proteasome but can affect diverse protein functions including subcellular localization, protein/DNA interactions and enzymatic activity (21–23). Here, we show that an impaired SUMO pathway causes defects in 60S subunit biogenesis and export. Importantly, preribosomal particles on both 60S and 40S biogenesis pathways are decorated by SUMO. Moreover, we show several *bona fide* factors involved in ribosome biogenesis to be modified by SUMO. Together, our data implicate SUMO as a novel regulator of ribosome biogenesis/export in eukaryotes.

Results

The SUMO pathway mutants are impaired in ribosome export

To identify factors involved in nuclear export of ribosomes, we previously screened a bank of randomly generated ts mutants for nuclear accumulation of a large subunit reporter protein (Rpl25-eGFP) (12). One of the isolated mutants *rix16-1,* which showed a strong nucleolar/nuclear concentration of Rpl25-eGFP (Figure 1A), was complemented by *UBA2* (Figure 1B). Uba2 together with Aos1 constitutes the heterodimeric E1 enzyme of the SUMO conjugation pathway (19). To test whether impairment of other steps in the sumoylation pathway inhibits ribosome export, we expressed Rpl25-eGFP in ts mutants of *UBC9* (E2 enzyme), *ULP1* and *ULP2* (deconjugation enzymes), and *SMT3.* As shown in Figure 1C, all three mutants, except *ulp2D* (data not shown), significantly accumulated Rpl25-eGFP in the nucleus when shifted to the restrictive temperature. Similar accumulation was found for another large subunit reporter, Rpl11-eGFP (data not shown). However,

rix16-1, *ubc9-ts* and *ulp1-ts* cells were not detectably impaired in small subunit export (Figure 1A and C), as judged by the localization of the Rps2-eGFP reporter. Moreover, these mutant cells were not impaired in export of poly(A)$^+$ RNA or transfer RNAs (tRNAs).

However, we observed a moderate nuclear accumulation of poly(A)$^+$ RNA (data not shown) and the 40S reporter in the *smt3-ts* strain at restrictive temperature (Figure 1C). Together, these data suggest that defects in the sumoylation pathway lead to a relatively specific inhibition of 60S subunit export. We did not observe an apparent defect in nuclear protein import in *rix16-1, ubc9-ts* and *ulp1-ts* cells, as assayed by NLS^{L25}-GFP and NLS^{Npl3}-GFP reporter constructs (data not shown). In contrast, Stade et al. (24) reported that *ulp1-ts* cells exhibit an import defect of a cNLS reporter construct at restrictive temperature. This discrepancy could be due to the experimental method used to monitor nuclear import.

The SUMO pathway mutants show defects in pre-rRNA processing

Next, we tested for pre-rRNA processing defects (for pre- rRNA processing scheme, see Figure 2A and B) in strains impaired in SUMO conjugation and deconjugation, *rix16-1, ulp1-ts* and *smt3-ts* (Figure 2C and D). Strains were grown at permissive temperature (23°C) and shifted to 37°C for 4 h or 8 h. Northern analysis of the high-molecular-weight RNAs (Figure 2C) revealed that the *rix16-1, ulp1-ts and smt3-ts* cells each showed a reduction in the levels of the 27S pre- rRNA species. In addition, the *ulp1-ts* and *smt3-ts* strains showed reduced 32S and 20S pre-rRNA levels, while *rix16-1* also showed accumulation of the 35S precursor, indicating a delay in pre-rRNA processing at the early sites A_0, A_1 and A_2 (see Figure 2C). Analysis of the low-molecular-weight RNAs (Figure 2D) revealed a decrease in 6S pre-rRNA level in *rix16-1, ulp1-ts and smt3-ts* strains, indicating a defect in processing from 7S pre-rRNA to 5.8S rRNA at restrictive temperature. We conclude that impaired SUMO conjugating and deconjugating activities adversely affect both early and late pre-rRNA processing steps.

Small-ubiquitin-related modifier conjugation is genetically linked to 60S biogenesis

Recent proteomic approaches in yeast have implicated SUMO in several essential nuclear processes (25–30). Synthetic lethality or enhancement has been widely used to reveal functionally linked biological pathways. To test whether the SUMO pathway was genetically linked to ribosome assembly and export, we asked whether a compromised SUMO conjugation pathway exacerbates the growth phenotypes of ribosome assembly factor mutants. We therefore assessed genetic interactions between the alleles of *nop7, nsa1, rix7*and *nugl* and the *ubc9-ts* (P28L, Y64H) mutant. As shown in Figure 3, *nop7, nsa1* and *rix7* mutants were synthetically enhanced by the *ubc9-ts* allele at 35°C, whereas the *nug1/ ubc9-ts* combination showed almost no synthetic enhancement phenotype.

Preribosomes on both the 60S and the 40S pathway are subject to SUMO modification

The observation that the SUMO pathway is functionally linked to ribosome biogenesis and export could indicate that preribosome formation is regulated by this post-translational modification. To determine whether this reflects a direct involvement of sumoylation in ribosome biogenesis, we attempted to detect SUMO modification on preribosomal particles. Therefore, preribosomal particles representing different intermediates on the 60S and 40S

maturation pathways were isolated and analyzed for the presence of sumoylated proteins. To this end, we constructed yeast strains expressing different TAP-tagged nonribosomal factors together with a functional Myc-tagged version of yeast SUMO (3xMyc-SMT3). The isolation of the preribosomal particles was carried out in presence of iodoacetamide (IAA) to inhibit isopeptidases (e.g. Ulp1 and Ulp2), which deconjugate SUMO from substrate proteins (31,32). Preribosomal particles were TAP purified in two steps (immunoglobulin G-Sepharose and calmodulin-Sepharose chromatography), and the EGTA eluates were analyzed by SDS-PAGE and Coomassie staining and by Western blotting using anti-Myc antibodies. The following TAP-tagged preribosomal factors were purified: (1) Nsa3, Nop7, Rix1, Sda1, Arx1 and Kre35, which are associated with different (early to late) pre-60S particles (9) and (2) Utp22, Utp8, Nop14, Dim1, Tsr1 and Rio2, which are associated with various (early to late) pre- 40S particles (10). As shown in Figure 4A and B, specific Western signals representing putative sumoylated substrate proteins were detected in both pre-60S and pre-40S particles. As controls, we performed TAP-purifications in the absence of IAA (data not shown) or from strains that do not contain Myc-Smt3 (Nsa3-TAP and Utp22-TAP). Because the putative SUMO-modified proteins were of molecular masses > 70 kDa, they likely correspond to trans-acting factors involved in ribosome biogenesis (see below). Notably, the pattern of sumoylated bands differed from early to intermediate nuclear preribosomes (compare Nsa3, Nop7, Rix1, Sda1 and Arx1 baits). In addition to the few distinct prominent bands, we observed several weak bands in early preribosomal particles, suggesting that not all trans-acting factors are modified to a similar extent. However, late pre-60S (as specified by the Kre35 bait) and pre-40S particles (as specified by Tsr1 and Rio2 baits), which have a predominant cytoplasmic location (9,10), did not contain detectable amounts of sumoylated proteins. Thus, pre-60S and pre-40S particles are subject to SUMO modification.

We next sought to assign major bands present in the pre-60S and pre-40S preparations to specific proteins. We suspected the major band on the Nsa3-derived preribosome to be the sumoylated version of Nop7, as inferred by the slower mobility of this band in the Nop7-TAP. To address whether the suspected band was indeed Nop7, we purified Nsa3-TAP from a strain expressing GFP-tagged version of Nop7. As shown in Figure 5A, the band corresponding to sumoylated Nop7 (115 kDa) was shifted to ~140 kDa in the strain expressing Nop7-GFP. Similarly, a ~140 kDa band in the Utp8-TAP preparation, which we assumed to be Myc-Smt3-modified Nop14, was shifted to a ~160 kDa when Nop14-GFP was expressed (Figure 5B). These data show that Nop7 and Nop14 are modified by SUMO in early pre-60S and pre-40S ribosomal particles, respectively.

We next assessed whether abolition of Nop7 sumoylation would affect 60S biogenesis/ export. Mutation of the SUMO consensus acceptor lysine (K285R) in Nop7 abolished sumoylation (Figure 5C), as assessed by the ‘SUMO finger print’ method (25). However, this mutation conferred neither a growth defect nor a 60S export defect (Figure 5D and data not shown). These data suggest that Nop7 is not the only relevant SUMO substrate on the 60S biogenesis pathway. The modification of one or more additional factors is likely to be required for correct assembly of pre-60S particles. It may be that the simultaneous loss of sumoylation on multiple factors would be needed for a deleterious effect on pre-60S biogenesis/ export to be observed.

Pre-60S and pre-40S assembly factors are subject to SUMO modification

While this work was in progress, several groups used proteomic approaches to unravel sumoylated proteins in yeast (25–30). These studies suggested that some proteins involved in ribosome biogenesis might be modified by SUMO. In order to get an overview of the subset of proteins in the SUMO proteome involved in 60S biogenesis, we conducted a comprehensive analysis. To this end, we purified sumoylated proteins via His_8-SUMO under denaturing conditions from a battery of strains expressing TAP-tagged candidate proteins. The purified sumoylated proteins were separated on an SDS-PAGE gel and subjected to Western blotting. Figure 6A shows a Ponceau S- stained nitrocellulose membrane prior to Western blotting (left panel). Enrichment for SUMO conjugates was observed as judged by Western blotting using anti-PentaHis antibodies (middle panel, compare lane 2 and lane 3). Furthermore, Western blotting using anti-ProteinA antibody was performed for assessing sumoylation of Kre35- TAP. As shown in Figure 6A (right panel, anti-ProteinA blot) lane 1 shows the mobility of the unmodified protein Kre35- TAP in whole cell lysates. Lanes 2 and 3 correspond to Nickel-nitriolotriacetic acid (Ni-NTA) purifications carried out on Kre35-TAP yeast strains expressing untagged and tagged versions of SUMO, respectively. A slower migrating band in lane 3 corresponds to the sumoylated form of Kre35-TAP. In addition to allowing detection of the sumoylated version of Kre35-TAP, the highly sensitive anti-ProteinA antibody is able to detect minor pool of nonspecifically bound unmodified protein Kre35-TAP to the Ni-NTA beads (faster migrating band, compare lane 1 with lanes 2 and 3). Using this denaturant-based method for analysis of sumoylation, we could show that many early pre-60S trans-acting factors (Nop7, Nsa3, Nug1, Ytm1, Rlp7, Rix7 and Nsa1), a few late factors (Bud20, Arx1, Ecm1 and Kre35) and the ribosomal protein Rpl5 are modified by SUMO (Figure 6A–D). In addition, we found that the early-acting factors on the 40S biogenesis pathway, namely Nop14, Enp1, Pwp2 and Utp8, are subject to SUMO modification (Figure 6B). Importantly not all factors that we tested were found to be sumoylated, e.g. Noc4, Nmd3, Rrp5, Krr1, Utp22, Ipi3 and Rrp9 (data not shown). The sumoylated forms of the above ribosome biogenesis factors appear to be of varying degrees of low abundance and were estimated to be in the range of ~0.01-0.5% of nonmodified protein. Together, many assembly factors along 60S and 40S pathways appear to be modified by SUMO.

The SUMO deconjugating Ulp1 is functionally linked to a late 60S export step

The yeast SUMO deconjugating enzyme, Ulp1 is anchored to the nuclear pore complex (NPC) via its interaction with the nuclear import factors Kap60, Kap95 and Pse1(33). This complex also interacts at the NPC with Mlp1 and Mlp2, filamentous proteins involved in retaining defective messenger ribonucleic proteins inside the nucleus (34,35). It is possible that this strategic localization of Ulp1 at the NPC allows SUMO deconjugation of cargo either prior to or during nuclear export. We tested genetic interactions between Ulp1 and factors that associate with late, export-competent pre-60S particles (Ecm1, Arx1, Nmd3 and Mtr2). Mutant alleles of *nmd3, arx1* and *ecm1* did not exacerbate the *ulp1-ts* phenotype (data not shown). The heterodimeric Mex67/Mtr2 complex is the major messenger RNA (mRNA) export factor in eukaryotes (36). While the *mex67-5* allele (defective in mRNA export) did not exacerbate the *ulp1-ts* phenotype (Figure 7A), the *mtr2-33* allele, which specifically inhibits pre-60S subunit export (4), showed a significant synthetic enhancement when

combined with *ulp1-ts* at both 23°C and 30°C (Figure 7B). Importantly, strong accumulation of Rpl25-eGFP was observed in the *ulp1-ts/mtr2-33* double mutant at 30°C, while the single mutants showed no accumulation (Figure 7C). However, the observed synthetic enhancement did not result in the nuclear accumulation of poly(A)$^+$ RNA (Figure 7D) or the 40S reporter Rps2-eGFP (data not shown). Thus, deconjugation at the NPC by Ulp1 may be an important step for the export of the pre-60S particle.

Discussion

In recent years, the inventory of nonribosomal factors involved in 60S and 40S biogenesis has dramatically increased (3). However, understanding of the regulation of the ribosome synthesis pathway has progressed more slowly. In particular, little is known about the impact of post-translational modifications, such as phosphorylation, ubiquitination and sumoylation, which play key roles in many other pathways, on preribosomal particle assembly. Here, we provide evidence that both sumoylation and desumoylation are required for efficient export of pre-60S particles.

By purifying preribosomal particles that represent different stages of maturation along both the 40S and the 60S pathways, we could show that early, nucleolar particles, which are undergoing dynamic changes in their protein and RNA composition, are modified by SUMO (Figure 3). Notably, we have been able to identify Nop7 as one of the abundantly sumoylated proteins on an early pre-60S particle (Figures 4 and 5). However, mutation of the major SUMO acceptor site on Nop7 conferred neither a growth defect nor a 60S export defect. Abolition of sumoylation sites within components of large macromolecular assemblies, such as Rpc128 and Rpc82 (RNA polymerase III), Rsc58 (RSC chromatin remodeling complex), and Rps3 (40S ribosomal protein), did not lead to any observable phenotype (25,30). The SUMO proteome has revealed some ribosomal proteins and *trans*-acting factors as to be ‘potential’ SUMO substrates (25–30). Using a directed proteomic approach, we show that, indeed, many early and few late trans-acting factors along the 60S and 40S maturation pathways are subject to sumoylation (Figure 6). Thus, the inactivation of single sumoylation sites is not sufficient to affect ribosome assembly/export or the analyzed protein is not the critical substrate for SUMO. However, the observed ribosome export defect in strains impaired in sumoylation is likely to reflect a direct effect. In line with the above findings, an impaired sumoylation machinery exhibited defects in pre-rRNA processing along both the 40S and the 60S pathways (Figure 2).

How could sumoylation of nonribosomal factors affect ribosome biogenesis? It is conceivable that sumoylation of several proteins on the preribosomal particle may aid in maintaining the overall stability or intranuclear targeting of preribosomal particles. Small-ubiquitin-related modifier could be part of a nuclear surveillance mechanism that ensures correct building of large macromolecular complexes, for example by inhibiting incorrect interactions between proteins. Indeed, under stress conditions that promote aggregation, such as heat shock, a strong increase in sumoylation of proteins, which are part of large macromolecular complexes, has been observed in yeast and plant cells (26,37). It is also notable that fusion proteins with SUMO have been found to be less prone to aggregation (38). The high incidence of substrate clustering in large complexes may reflect the

regulation of macromolecular complex assembly and/or disassembly by sumoylation (27). Thus, the global impairment in SUMO conjugation of preribosomal particles is likely to compromise the process of building this assembly. Such a role for SUMO is supported by the genetic links between *trans*acting factors (Nop7, Rix7 and Nsa1) and Ubc9.

Does desumoylation by Ulp1 influence directly the nuclear assembly/export of the pre-60S particle? The NPC-associated, heterodimeric Mex67/Mtr2 is the primary export factor for mRNA in eukaryotes (36). In addition, Mtr2 associates with the export-competent late pre-60S particle that is loaded with the 60S export factor Nmd3 (9). The strong genetic interaction between Ulp1, the NPC-associated SUMO deconjugating enzyme and Mtr2 at permissive temperature, however, may reflect a direct role of desumoylation during pre-60S export. In support of this, only the *ulp1-ts/mtr2-33* double mutant showed a strong nuclear pre-60S accumulation in the *mtr2-33/ulp1-ts* strain (Figure 7). Indeed, we find that late pre-60S-associating factors (Bud20, Arx1, Ecm1 and Kre35) are modified by SUMO. However, TAP purified, late pre-60S (Arx1-TAP) did not contain detectable amounts of sumoylated proteins (Figure 4). It is thus likely that the SUMO isopeptidases have not been efficiently inactivated or that the levels of sumoylated forms of late associating factors are too low to be detected by this method. Only by affinity enrichment of sumoylated conjugates after a harsh denaturing cell lysis method, we were able to detect, using sensitive anti-ProteinA antibodies, the sumoylated forms of the late pre- 60S-associating factors.

The maintenance of the nucleolar organization relies on the equilibrium between transcription, processing and export of the ribosomal subunits, and inactivation of any of these processes ultimately leads to nucleolar breakdown. Notably, mouse cells deficient for the Ubc9 protein showed a striking nucleolar disassembly phenotype (39). This suggests an important role for sumoylation in ribosome synthesis that is conserved from yeast to mammals.

Materials and Methods

Yeast strains and microbiological methods

Microbiological techniques, plasmid transformation and recovery, mating, sporulation of diploids and tetrad analysis were done according to standard procedures. Genomic integration of GFP and TAP tag in an *SMT3* shuffle strain was performed according to Rigaut et al. and Longtine et al. (40,41). The yeast strains used in this study are given in Table S1. The list of plasmids used is given in the Table S2 and details of plasmid constructions will be provided on request.

Affinity purifications

TAP-purification of preribosomal particles was carried out as previously described (25,40). The final eluates from calmodulin-Sepharose were trichloroacetic acid precipitated, resuspended in SDS sample buffer and analyzed on an SDS 4-12% gradient polyacrylamide gel (Invitrogen, Karlsruhe, Germany). Antibodies used for Western blotting were horse-radish-peroxidase-conjugated polyclonal rabbit anti-ProteinA antibody (1:3000; DakoCytomation, Hamburg, Germany), polyclonal rabbit anti-Myc (1:2000; BioMol,

Hamburg, Germany) and horseradish-peroxidase-coupled goat anti-rabbit (1:3000; Bio-Rad, Munich, Germany). For Western blotting, enhanced chemiluminescence detection was performed (Amer-sham Biosciences, Frieburg, Germany). The SUMO finger print was performed as previously described (25). For the His8-SUMO purifications, strains were grown to an OD600nm of 0.5 at 308C. CuSO4 was added to a final concentration of 1 mM to induce the expression of His_s-SUMO for 3 h. Subsequent purification of the His8-SUMO conjugates was performed as previously described (22).

RNA analysis

Northern hybridization and primer extension were performed as described previously (42,43). Oligonucleotides used were as follows: 003, 5'-TGTTAC CTC TGG GCC C-3'; 004, 5'-C GGTTTTAATT GTC CTA-3'; 006, 5'-AGATTA GCC GCA GTT GG-3'; 007, 5'-CTC CGC TTA TTG ATA TGC-3'; 008, 5'-CAT GGC TTA ATC TTT GAG AC-3'; 017, 5'-GCG TTG TTC ATC GAT GC-3'; 020, 5'-TGA GAA GGA AAT GAC GCT-3'; 041, 5'-CTA CTC GGT CAG GCT C-3'; 250, 5ATCCCG GCC GCC TCC ATC AC-3'; and 306, 5'-GCATCTTAC GAT ACC TG-3^0.

Miscellaneous

Analysis of nuclear export of ribosomal subunits using Rpl25-eGFP and Rps2-eGFP reporters was performed as described (12,15). The intranuclear accumulation of poly$(A)^+$ RNA and tRNA by *in situ* hybridization was carried out as described (44). Cloning of the gene complementing the *rix16-1* ts mutant was carried out by transforming a yeast genomic library as described previously (12). Site-directed mutagenesis was performed using fusion polymerase chain reaction techniques. Mutations were verified by DNA sequencing.

Supplementary Material

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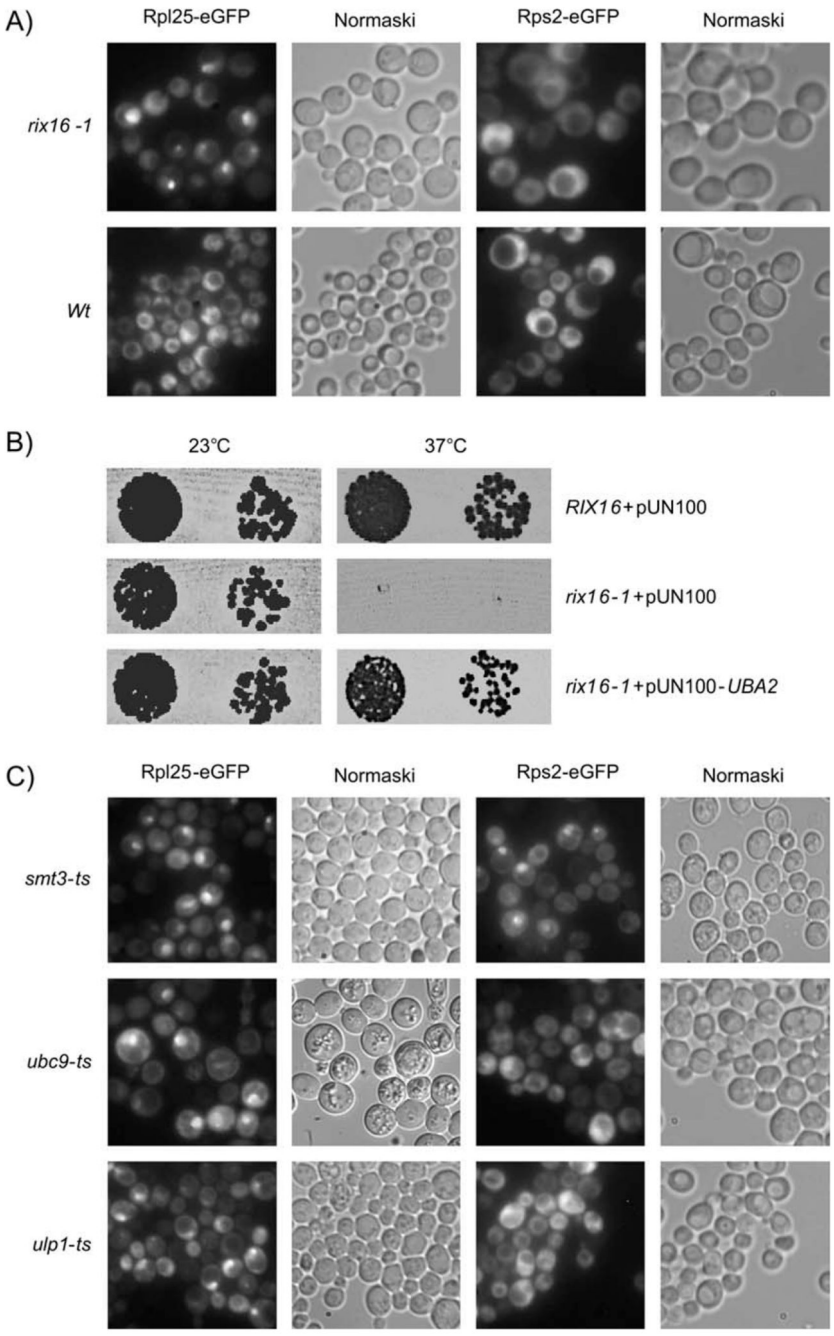

Figure 1. Yeast ts mutants of the SUMO pathway are impaired in the export of pre-60S ribosomal subunits.

A) Inhibition of nuclear export of pre-60S subunits in the *rix16-1* ts mutant. Cells were shifted to the temperature of 37°C for 2 h, and the location of Rpl25-eGFP and Rps2-eGEP was analyzed by fluorescence microscopy. B) *rix16-1* is complemented by *UBA2.* Growth of Wt *(RIX16)* and *rix16-1* strains transformed with an empty plasmid (pUN100) or pUN100-UBA2. Cells were spotted in 10^{-1} dilution steps on yeast-peptone-dextrose (YPD) plates and incubated for 4 days at 23°C and 37°C. C) *smt3-ts, ubc9-ts* and *ulp1-ts* mutants were shifted

to the temperature of 37°C for 2 h before location of Rpl25-eGFP and Rps2-eGFP was analyzed by fluorescence microscopy. Wt, wild-type strain.

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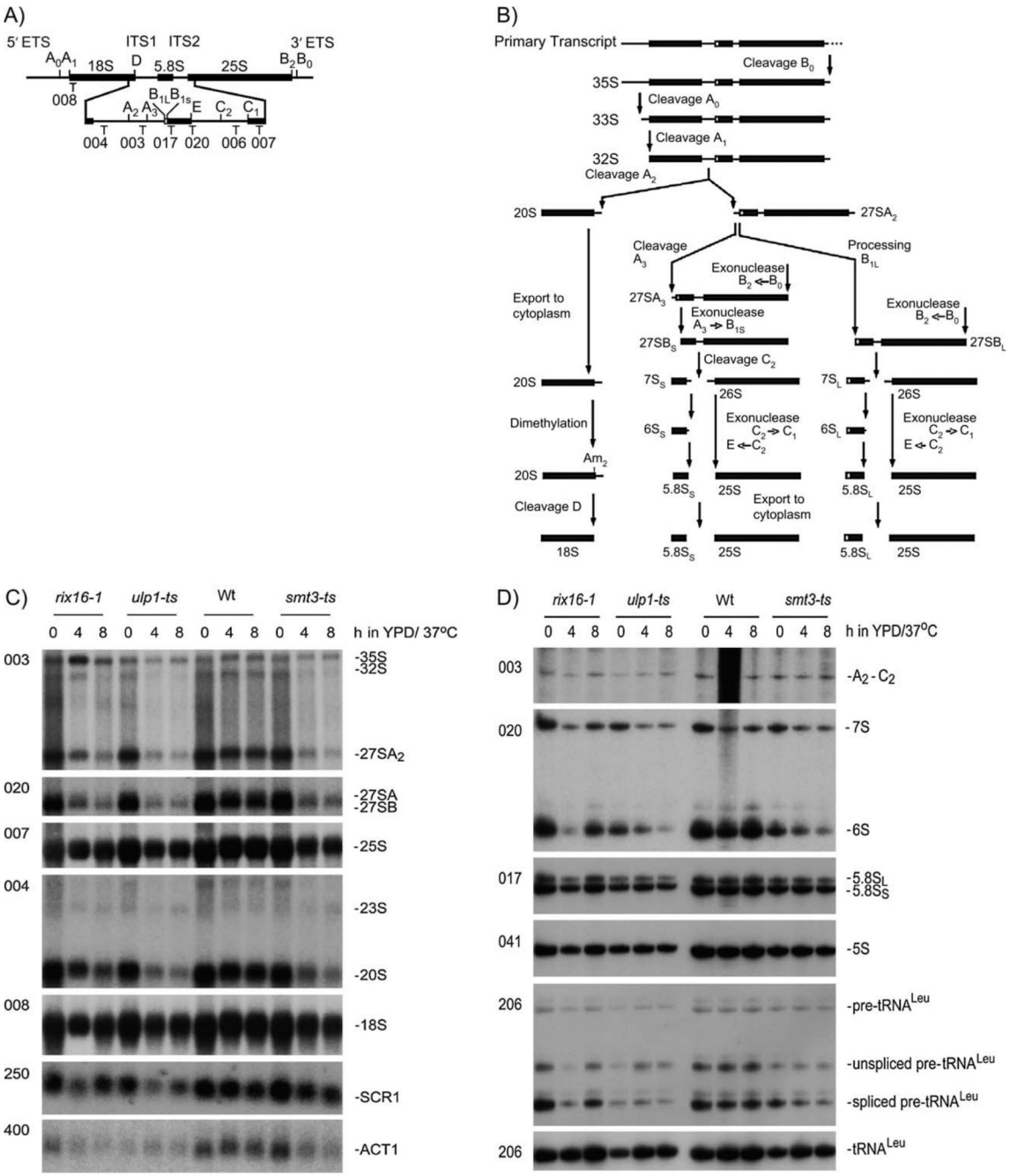

Figure 2. The SUMO pathway mutants are inhibited in pre-rRNA processing.

A) The primary pre-rRNA transcript and its processing sites. The transcribed spacer is indicated by narrow lines and the mature rRNA species are represented as filled rectangles. Locations of the oligonucleotides used for Northern analysis are indicated. B) Schematic of pre-rRNA processing. Yeast strains *rix16-1, ulp1-ts* and *smt3-ts* along with the isogenic Wt strain were grown in yeast-peptone-dextrose (YPD) medium at 23°C and then shifted to 37°C for the indicated times. C) Northern hybridization analyses of high-molecular-weight RNA separated on a 1.2% agarose gel containing formaldehyde. D) Northern hybridization

analyses of low-molecular-weight RNAs separated on a 8% polyacrylamide gel containing urea. ETS, external-transcribed-spacer; ITS, internal-transcribed-spacer; Wt, wild-type strain.

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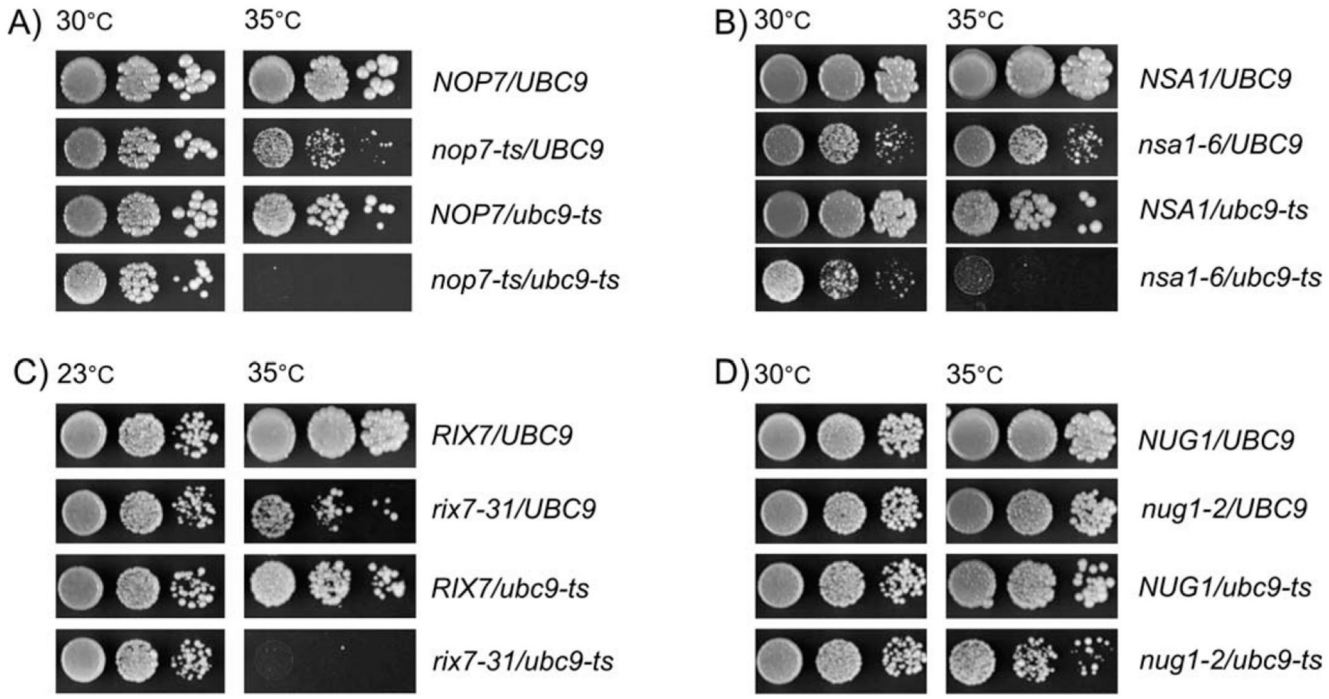

Figure 3. The SUMO conjugating enzyme Ubc9 is genetically linked to 60S biogenesis factors.

Synthetic enhancement of the *ubc9- ts* mutant when combined with the indicated A) *nop7* B) *nsa1,* C) *rix7* and (D) *nug1* alleles. For genetic analyses, the respective double shuffle strains (strain; Table S1) were transformed with plasmids that carry the indicated wild-type or mutant alleles. Transformants that grew on 5-fluororotic acid (5-FOA) plates were further streaked on YPD and grown at 238C. The growing colonies were spotted in 10^{-1} dilution steps on YPD plates and incubated for 3 or 4 days at the indicated temperatures.

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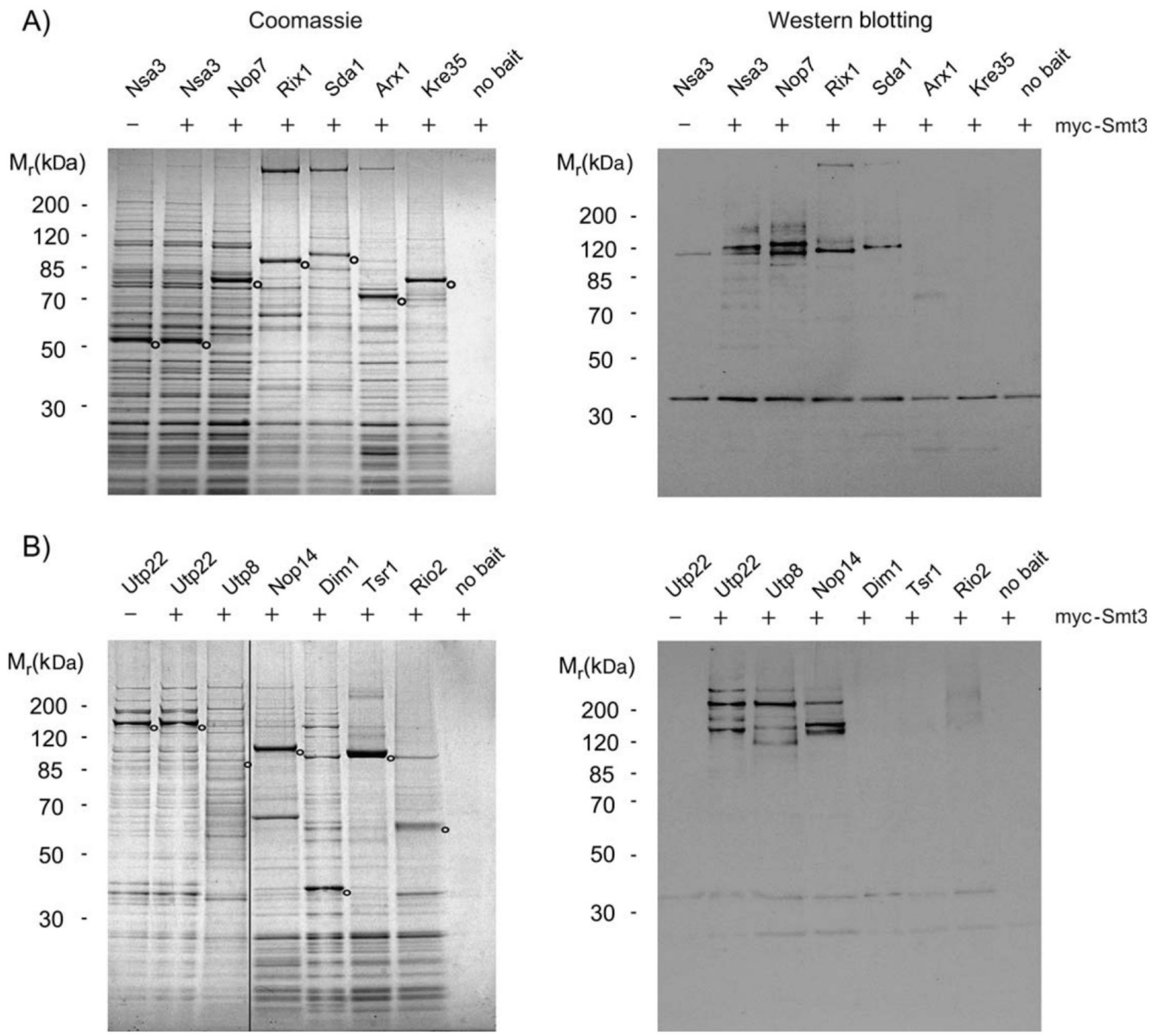

Figure 4. Preribosomal particles of the 40S and 60S biogenesis pathways contain sumoylated proteins.

The indicated TAP-tagged protein baits for A) 60S and B) 40S pathway were isolated from cell lysates expressing *Myc*$_3$-*SMT3* (+) or untagged *SMT3* (-) by two-step affinity purification (TAP method). TAP-purified bait proteins were separated on an SDS 4-12% gradient polyacrylamide gel and stained with Coomassie (left panel) or analyzed by Western blotting using **a**-Myc antibodies to detect sumoylated proteins (right panel). The positions of the bait proteins are indicated by open circles. A molecular weight standard is also shown.

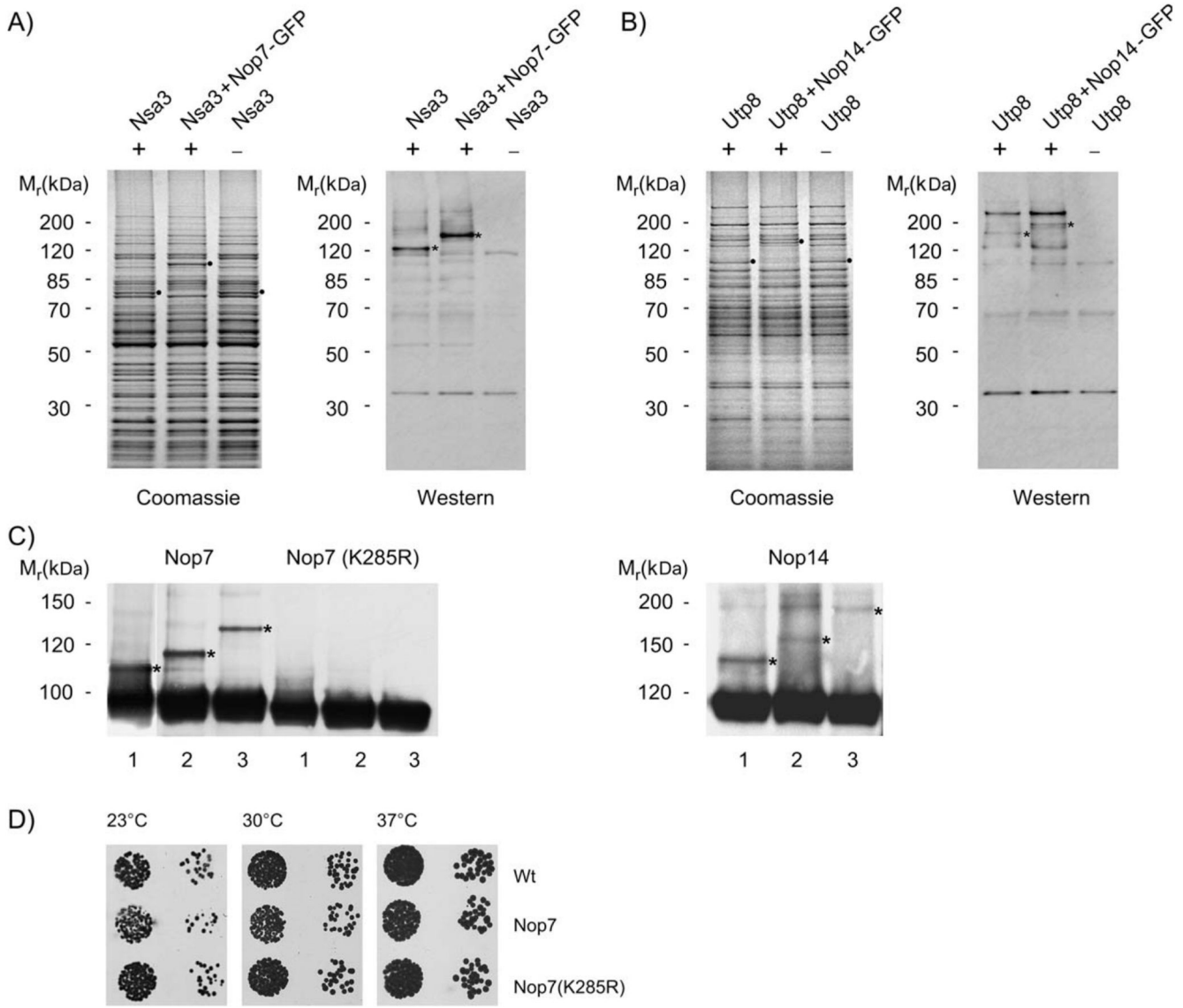

Figure 5. Identification of sumoylated Nop7 and Nop14 in pre-60S and pre-40S particles, respectively.

The Nsa3 (a pre-60S bait) and Utp8 (a pre-90S/40S bait) baits were purified from strains containing either untagged Nop7 or Nop7-GFP and either untagged Nop14 or Nop14-GFP, respectively, in strains expressing *Myc*$_3$*-SMT3* (+). The positions of Nop7, Nop7-GFP, Nop14 and Nop14-GFP proteins are indicated by closed circles, the positions of sumoylated Nop7 and Nop14 by asterisks. As negative control, A, TAP-tagged Nsa3, or B, TAP-tagged Utp8, was affinity purified from a strain expressing an untagged version of *SMT3* (-). The purified proteins were separated on an SDS 4-12% gradient polyacrylamide gel and analyzed by Western blotting using **a**-Myc antibodies to detect sumoylated proteins. A molecular weight standard for each purification is shown. C) Nop7 and Nop14 'SUMO finger print' analysis. Cell lysates of Nop7-TAP and Nop14-TAP strains expressing plasmid-borne *SMT3* (lane 1), *Myc*$_3$*-SMT3* (lane 2) and *GFP-SMT3* (lane 3) were analyzed by

Western blotting using **a**-ProteinA antibodies to detect TAP-tagged baits and slower migrating SUMO conjugated forms. The asterisks indicate the sumoylated forms. A molecular weight standard is shown on the left of the Western blot. D) The K285R mutation in Nop7 does not confer a growth defect. A Nop7 shuffle strain was transformed with Nop7 (Wt), Nop7-TAP (Nop7) and Nop7(K285R)-TAP [Nop7(K285R)] plasmids. The colonies that grew on 5-FOA were analyzed by spotting cells in 10^{-1} dilution steps on YPD plates and incubation for 3 days at the indicated temperatures. Wt, wild-type strain.

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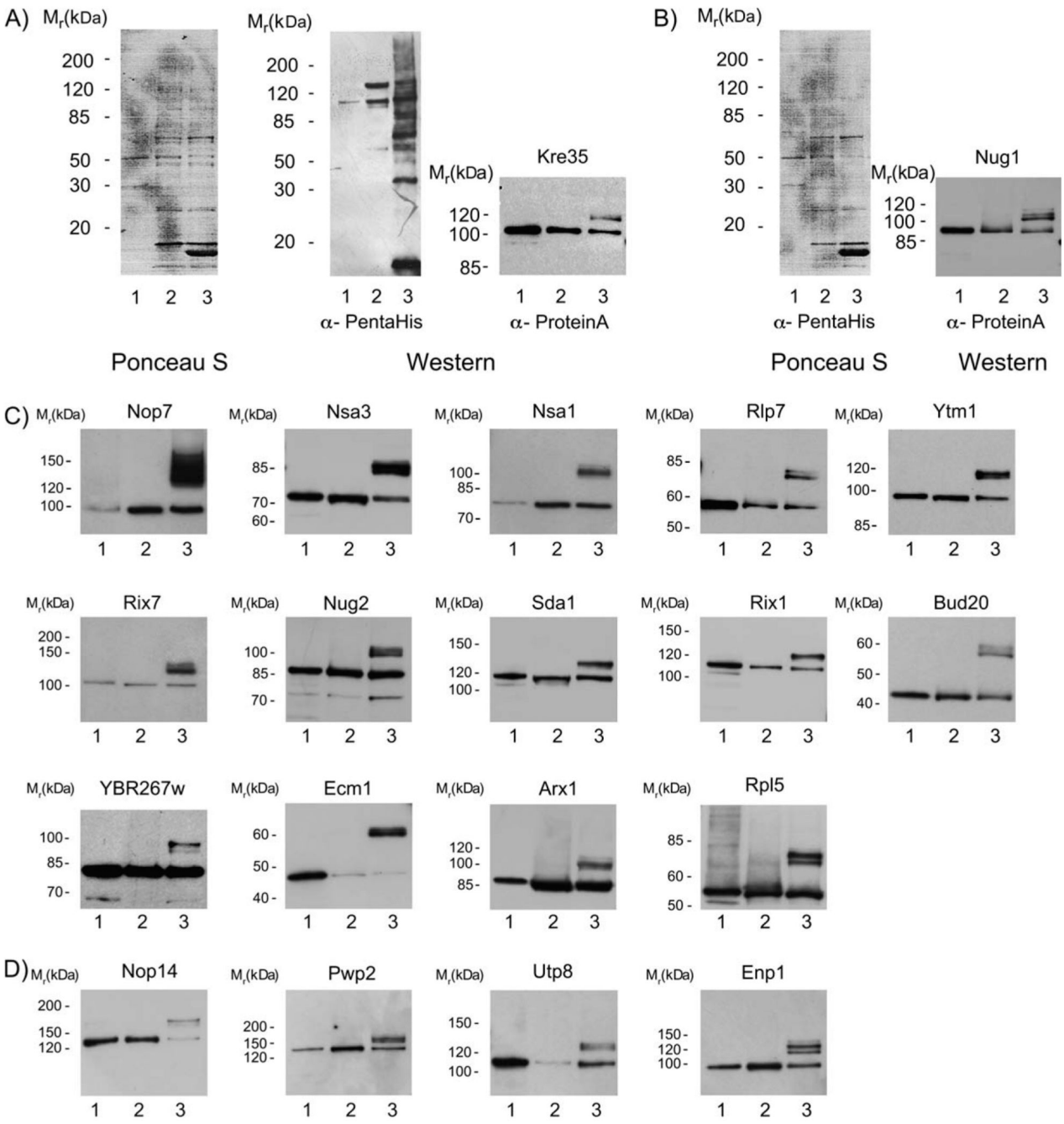

Figure 6. Trans-acting factors involved in 60S and 40S ribosome biogenesis are modified by SUMO.

Small-scale Ni-NTA purifications as described in experimental procedures were performed under denaturing conditions on cell lysates of strains expressing *SMT3* (as a negative control) or *His$_8$-SMT3* to isolate sumoylated proteins. The purified proteins were analyzed on an SDS 4-12% gradient polyacrylamide gel and then subjected to Western blotting. A) and B) shows Ponceau S-stained nitrocellulose membrane used for Western blotting using anti-PentaHis antibodies (to detect His$_8$-SUMO) and anti-ProteinA antibodies (Kre35 and Nug1). The Western signal, using anti-ProteinA antibodies, in lane 1 shows the mobility of

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the TAP-tagged protein in whole cell lysates. Lanes 2 and 3 represent purifications performed in the TAP-tagged strain by expressing untagged *SMT3* and His_8-SMT3, respectively. Ni-NTA purifications performed for C) 60S and D) 40S TAP-tagged trans-acting factors.

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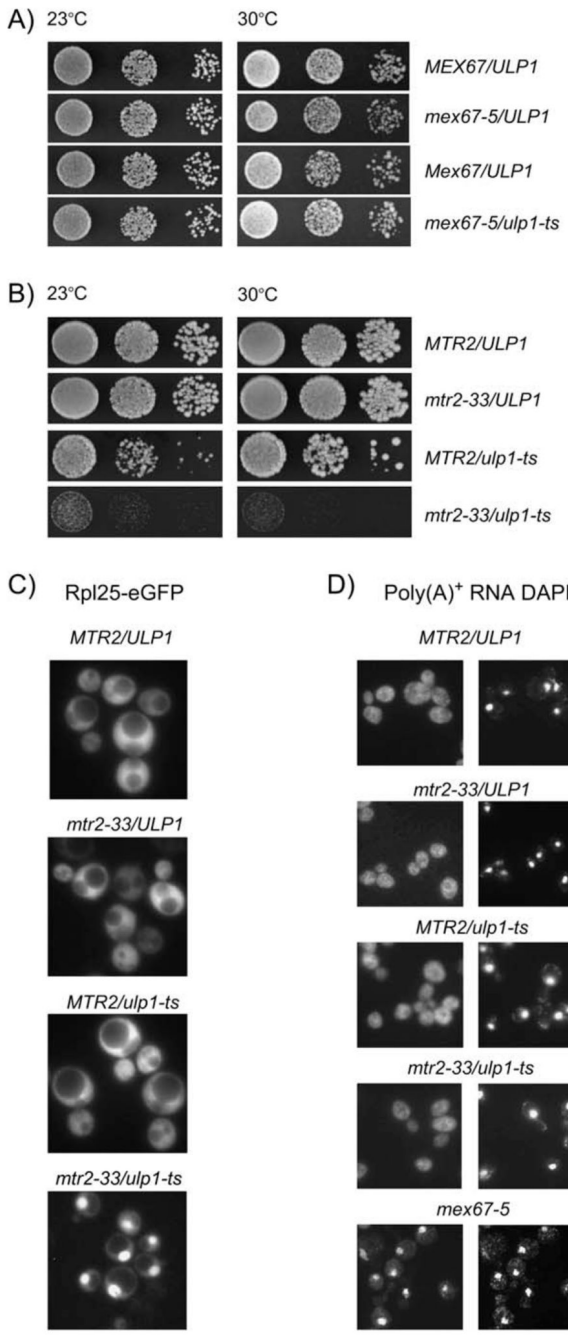

Figure 7. The SUMO deconjugating enzyme Ulp1 is genetically linked to the 60S export factor Mtr2.

A) The *mex67-5*allele is not synthetically enhanced when combined with *ulp-ts* mutant. The *ULP1/MEX67* double shuffle strain (strain Table S1) was transformed with plasmids that carry the indicated wild-type or mutant alleles. Transformants that grew on 5-FOA plates at 238C were streaked out on YPD. The growing colonies were spotted in 10^{-1} dilution steps on YPD plates and incubated at 23°C and 30°C for 3 days. B) Synthetic enhancement of the *ulp1-ts* mutant when combined with the *mtr2-33* allele. The *ULP1/MTR2* double shuffle strain (strain; Table S1) was transformed with plasmids that carry the indicated wild-type or

mutant alleles. Transformants that grew on 5-FOA plates at 23°C were streaked on YPD. The growing colonies were spotted in 10^{-1} dilution steps on YPD plates and incubated at 238C and 308C for 3 days. C) The *ulp1-ts/mtr2-33* double mutant is inhibited in 60S export. The indicated strains were transformed with pRS316-Rpl25-eGFP, and the location of the Rpl25-eGFP reporter was analyzed by fluorescence microscopy at 308C. D) The *ulp1-ts/mtr2-33* double mutant does not exhibit an mRNA export defect. Poly(A)$^+$ RNA accumulation in the depicted strains at 30°C was determined by fluorescence microscopy following *in situ* hybridizations of Cy3-labeled oligo-(dT) probes. The *mex67-5*strain was grown at 23°C and then shifted to 37°C for 1 h, it served as a positive control for the *in situ* hybridization.