Ytm1, Nop7, and Erb1 Form a Complex Necessary for Maturation of Yeast 66S Preribosomes

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The essential, conserved yeast nucleolar protein Ytm1 is one of 17 proteins in ribosome assembly intermediates that contain WD40 protein-protein interaction motifs. Such proteins may play key roles in organizing other molecules necessary for ribosome biogenesis. Ytm1 is present in four consecutive 66S preribosomes containing 27SA₂, 27SA₃, 27SB, and 25.5S plus 7S pre-rRNAs plus ribosome assembly factors and ribosomal proteins. Ytm1 binds directly to Erb1 and is present in a heterotrimeric subcomplex together with Erb1 and Nop7, both within preribosomes and independently of preribosomes. However, Nop7 and Erb1 assemble into preribosomes prior to Ytm1. Mutations in the WD40 motifs of Ytm1 disrupt binding to Erb1, destabilize the heterotrimer, and delay pre-rRNA processing and nuclear export of preribosomes. Nevertheless, 66S preribosomes lacking Ytm1 remain otherwise intact.

Biogenesis of eukaryotic ribosomes is a highly regulated and dynamic process that begins in the nucleolus with transcription of a precursor rRNA (pre-rRNA) that is rapidly packaged into the 90S ribonucleoprotein particle containing ribosomal proteins, nonribosomal proteins, and snoRNA-containing ribonucleoprotein particles (snoRNPs). The 90S pre-RNPs are converted into 43S and 66S ribosome assembly intermediates, which ultimately give rise to mature 40S and 60S ribosomal subunits (Fig. 1).

Molecular genetic approaches in yeast identified more than 70 trans-acting factors required for ribosome assembly (12, 14, 46). Subsequent advances in proteomics enabled purification of pre-rRNPs from yeast and identification of an additional 80 assembly factors present in preribosomes, as well as most of those proteins previously discovered using genetic screens (3, 7, 11, 17, 20, 21, 24, 26, 37, 38, 41, 49–51). Metazoan homologues of most of the yeast ribosome assembly factors were discovered by proteomic analysis of purified nucleoli (2, 52).

Among the assembly factors found in yeast preribosomes are 17 proteins containing WD40 motifs (14). These motifs function as protein-protein interaction domains (53). Therefore, such WD40-containing proteins may nucleate assembly of preribosomes by interacting sequentially or simultaneously with other assembly factors or ribosomal proteins. Previously, we identified the WD40 protein Ytm1 as a constituent of purified 66S preribosomes and showed that depletion of Ytm1 results in a deficiency of 60S ribosomal subunits (21).

In this study, we have further investigated the role of Ytm1 in ribosome biogenesis. Ytm1 is a constituent of multiple consecutive 66S preribosomes containing 27SA₂, 27SA₃, 27SB, 25.5S, and 7S pre-rRNAs plus a collection of ribosomal and nonribosomal proteins. Ytm1 is present in a heterotrimer with two other assembly factors, Nop7 and Erb1, both within 66S preribosomes and as a subcomplex independent of preribosomes. Mutations in Ytm1 disrupt interactions between Ytm1 and Erb1, destabilize the heterotrimer, and significantly reduce association of these three proteins with 66S preribosomes. These 66S pre-rRNPs otherwise remain intact in the ytm1-1 mutant, but processing of 27SA₃ pre-rRNAs is delayed and release of 66S preribosomes from the nucleolus is partially blocked. Thus, Ytm1 is necessary to nucleate the assembly of a heterotrimer that is important for intermediate-to-late steps in maturation of 66S preribosomes.

MATERIALS AND METHODS

Strains, plasmids, and media. Yeast strains used in this work (Table 1) were grown in YEPD medium (2% dextrose, 2% peptone, and 1% yeast extract) or YEPGal medium (2% galactose, 2% peptone, and 1% yeast extract) at 30°C and harvested at 5·10⁷ cells/ml unless otherwise indicated. The ytm1-1 mutant strain JWY7128 was generated by mutantizing plasmid pRS317 containing wild-type YTM1 and LYS2 with hydroxylamine and transforming it into yeast strain SM412 cells which have GAL-YTM1 at the YTM1 locus. The ytm1-1 mutant plasmid that conferred temperature sensitivity to strains grown on selective medium containing 2% glucose was rescued, shuttled through Escherichia coli, and used to replace the chromosomal YTM1 gene in strain JWY7132 to generate ytm1-1 strain JWY7128. The GAL-YTM1 strain JWY6992 was described previously (21).

Three-hemagglutinin epitope (HA3)- and tandem affinity purification (TAP)-tagged strains were generated as described in references 21 and 26. Integration of the HA3 tag or the TAP cassette in-frame with the last codon of each open reading frame was confirmed by genomic PCR and Western immunoblotting. Yeast strain JWY6790 expressing enhanced green fluorescent protein (eGFP)-tagged rlp25 showed the expression of rlp25eGFP (28). Transformants were screened by fluorescence microscopy for expression of rlp25eGFP. Yeast strains JWY7808 (GST-YTM1), JWY7810 (GST-ERB1) and JWY7807 (GST-NOP7) expressing glutathione S-transferase (GST) fusion proteins were obtained from Mike Snyder (Yale University).

Sucrose gradient analysis. Ribosomes, preribosomes, and polyribosomes in yeast strains JWY3400 (YTM1), JWY7124 (YTM1-TAP), and JWY7128 (ytm1-1) were analyzed as described previously (26). JWY7128 was grown at 25°C and harvested or else shifted from 25 to 37°C for 3 h and harvested. Ytm1-HA3 or
Ytm1-TAP were identified in gradient fractions by Western blotting using mouse monoclonal antibody 12CA5 or rabbit anti-mouse immunoglobulin G (Pierce), respectively.

Analysis of rRNA. Steady-state levels of rRNAs were analyzed by Northern blotting (26) or primer extension assays (58). To carry out primer extension, radiolabeled oligonucleotide primers complementary to 35S, 27S, or 25.5S pre-rRNA were first annealed for 90 min at 46°C to total RNA or affinity-purified RNA. Primer extension reaction mixtures containing the annealed oligonucleotide primer/RNA hybrid, 10 mM deoxynucleoside triphosphates (dNTPs) (Amersham Biosciences), 12.5 U avian myeloblastosis virus reverse transcriptase (Promega), and 20 U RNasin (Promega) were incubated for 40 min at 46°C. To hydrolyze the RNA, 6 μl of 1 M NaOH and 1 μl of 0.5 M EDTA were added to each primer extension reaction mixture for 30 min to 1 h at 55°C. Next, 6 μl of 1 M HCl was added to each reaction mixture and DNA was precipitated with 4 μg glycogen, 30 μl 7.5 M NH₄OAc, and 250 μl of 100% ethanol. DNA was suspended in DNA dye (95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue in 20 mM EDTA, pH 8.0) and subjected to electrophoresis on a 6% polyacrylamide-urea sequencing gel. Gels were dried and directly exposed to film for autoradiography. Oligonucleotide sequences are available upon request.

Pulse-chase assays of pre-rRNA processing were carried out as previously described (26).

Ribosome export assays. Release of preribosomes from nucleoli and export from the nucleoplasm to the cytoplasm were assayed as previously described (28), except that strains were grown overnight in C-Trp medium, washed and suspended in YEPD medium, and grown at 25°C or shifted to 37°C for 5 h.

Affinity purification and mass spectrometry. Cell extracts were prepared, tandem affinity purification was carried out, and identification of copurifying proteins and RNAs was performed as previously described (26, 47).

Ribosome assembly subcomplexes were separated from 66S preribosomes and 60S ribosomal subunits by centrifugation on 7% to 47% sucrose gradients (26) or by differential centrifugation as performed previously (32) with the following modifications: whole-cell extracts were centrifuged for 2 h at 180,000 × g at 4°C, followed by a second centrifugation of supernatants for 30 to 45 min at 180,000 × g at 4°C. Subcomplexes were affinity purified from gradient fractions or from the 180,000 × g supernatant using TAP-tagged Nop7.

Assembly subcomplexes were isolated directly from whole-cell extracts by adding to the lysis buffer and calmodulin binding buffer a phosphatase inhibitor cocktail (20 mM pyrophosphate, 10 mM sodium azide, 20 mM sodium fluoride, 1 mM sodium orthovanadate, and 100 mM β-glycerophosphate) that disrupts pre-rRNPs.

Generation of anti-Ytm1 antibodies and Western immunoblotting. Rabbit antibodies generated against the synthetic peptide ITREDKSVQKGVDK (Alphapha Diagnostics, Inc.) were used to detect Ytm1. Antibodies were concentrated by ammonium sulfate precipitation, dialyzed, and affinity purified using full-length filter-bound Ytm1 protein previously subjected to electrophoresis through
respectively.

GST pull-down assays. GST fusion proteins were harvested from yeast by glass bead lysis of frozen cell pellets suspended in 1.6 ml sorbitol buffer (300 mM sorbitol, 5 mM MgCl₂, 10 mM Tris·HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 μM leupeptin). One milliliter of protein extract was incubated with 50 μl glutathione-agarose beads by boiling in 35 g/ml aprotinin, 1 g/ml pepstatin, 1 g/ml leupeptin). Beads were washed three times with 1 ml high-salt wash buffer (10 mM HEPES, 100 mM KCl, 0.5% Triton X-100, 5% bovine serum albumin) and once with 1 ml sorbitol buffer (300 mM sorbitol, 5 mM MgCl₂, 10 mM Tris·HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin).

One milliliter of protein extract was incubated with 50 μl glutathione-agarose beads overnight at 4°C. Beads were washed three times with 1 ml high-salt wash buffer (10 mM HEPES, 100 mM KCl, 0.5% Triton X-100, 5% bovine serum albumin) and once with 1 ml sorbitol buffer (300 mM sorbitol, 5 mM MgCl₂, 10 mM Tris·HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin).

To investigate the importance of WD40 motifs in Ytm1, we used the ytm1-1 temperature-sensitive mutant that contains two mutations, G398D and S442N, in WD40 repeats 6 and 7 of Ytm1 (Fig. 2A). This mutant strain was generated by random mutagenesis of cloned YTM1 and replacement of chromosomal YTM1 with the mutant allele. The counterpart of Ytm1 residue G398 in the G8 protein contacts phosducin (54). Thus, residues at this position may be important for mediating interactions with associated proteins. Within the conserved WD40 repeat consensus sequence is the structural tetrad, which contributes to local and global stability through intradomain hydrogen bonding (40, 53, 54). The G398D substitution occurs in the position adjacent to the highly conserved aspartate residue in the structural tetrad. Similarly, the S442N substitution occurs in a residue that is next to the serine/threonine

**RESULTS**

The ytm1-1 mutant contains mutations in two of the WD40 repeat motifs of Ytm1. Ytm1 is an essential 51-kDa yeast nucleolar protein that is highly conserved from fungi to humans. Seven WD repeats are clustered at the C terminus of the protein (Fig. 2A). WD repeat-containing proteins are predicted to share a common circularized beta-propeller structure (Fig. 2B) based on the crystal structure of the G8 subunit of heterotrimeric G proteins (16, 33, 54, 59). A paramount feature of WD repeat proteins is their ability to participate in multiple interactions either simultaneously or sequentially. The WD repeat motif is not known to have any enzymatic activity. Therefore, WD repeat proteins acting in disparate pathways may share a common function of establishing and regulating interactions within multiprotein complexes.

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residue of the structural tetrad. Thus, the ability of Ytm1 to bind to ligands may be compromised in the ytm1-1 mutant, which may result in defects in ribosome assembly. The ytm1-1 mutant was unable to grow at 37°C and grew slower than the wild-type control strain at all temperatures below 37°C (data not shown). The G398D and S442N mutations might inactivate Ytm1 at 37°C, perhaps by distorting the structure of Ytm1 or by disrupting interactions with ligands of Ytm1. The ytm1-1 mutant protein is relatively stable: amounts of Ytm1-1 protein did not change drastically when compared to those of wild-type Ytm1 in strains grown at 25°C or shifted from 25°C to 37°C for 5 h (data not shown).

The ytm1-1 mutant is deficient in 60S ribosomal subunits. To determine the effect of inactivation of Ytm1 on ribosome biogenesis, we assayed levels of ribosomal subunits, monoribosomes, and polyribosomes in ytm1-1 cells grown at 25°C or shifted from 25°C to 37°C for 3 h. Upon shifting the ytm1-1 mutant cells to 37°C, amounts of 60S ribosomal subunits and 80S monosomes were greatly reduced and half-mer polyribosomes were apparent. At 25°C, ytm1-1 mutant cells contained fewer free 60S subunits and 80S monoribosomes and accumulated half-mer polyribosomes, compared to wild-type YTM1 cells (Fig. 3). This suggests that the function of Ytm1-1 is compromised even at the permissive temperature and is consistent with the slow-growth phenotype at 25°C. These findings are consistent with previous results obtained when Ytm1 is metabolically depleted (21).

Pre-rRNA processing is slowed when Ytm1 is inactivated or depleted. The kinetics of pre-rRNA processing were analyzed by pulse-labeling YTM1 wild-type and ytm1-1 mutant cells, grown at 25°C, and shifted to 37°C for 5 h, as well as the GAL-YTM1 cells shifted from galactose- to glucose-containing medium, in which Ytm1 is depleted. Processing of 35S pre-rRNA to mature 25S and 18S rRNAs occurred rapidly in the YTM1 strain. The 27S pre-rRNA processing intermediate was completely converted to 25S rRNA by the 60-min chase point. In the ytm1-1 mutant, however, 27S pre-rRNA was still present at the 60-min chase point; 27SA pre-rRNAs were converted to 27SB pre-rRNA less efficiently than in wild-type cells (Fig. 4B). Consequently, 3.6-fold less mature 25S rRNA was produced relative to 18S rRNA in the ytm1-1 mutant than in the wild-type cells (Fig. 4B, compare lanes 5 and 10). Both 35S pre-rRNA and 23S pre-rRNA accumulated in the ytm1-1 mutant. The effects on 35S and 23S are thought to be indirect and often are observed when 60S ribosomal subunit assembly is perturbed (4, 19). Effects on pre-rRNA processing observed in Ytm1-depleted cells are identical to those found in the ytm1-1 mutant (data not shown).

We analyzed the steady-state levels of rRNA intermediates and mature rRNAs by primer extension and Northern blotting.
(Fig. 4C and D). Ytm1 was depleted from GAL-YTM1 cells by shifting galactose-grown cells to glucose for 0 h, 10 h, 12 h, 15 h, or 18 h. The ytm1-1 cells were grown at 25°C and shifted to 37°C for 0 h, 3 h, or 6 h. Primer extension using an oligonucleotide that detects 27S pre-rRNAs (Fig. 4A) indicated that under nonpermissive conditions amounts of 27SA1 pre-rRNA decrease in ytm1-1 cells but increase in GAL-YTM1 cells (Fig. 4C). 27SA1 pre-rRNA strongly accumulated upon shifting GAL-YTM1 and ytm1-1 cells to the nonpermissive conditions (Fig. 4C). Amounts of 27SB pre-rRNAs were also affected: 27SB1 pre-rRNA was drastically diminished relative to 27SB2 pre-rRNA, making these species nearly equal in Ytm1-depleted or Ytm1-inactivated cells (Fig. 4C).

Phosphorimaging analysis of Northern blots also indicated that 7S pre-rRNA and 5S rRNA decrease slightly upon shifting GAL-YTM1 and ytm1-1 cells to nonpermissive conditions (Fig. 4D). Despite the observed effects on processing of early and intermediate pre-rRNAs present in 66S pre-rRNPs, amounts of 25S and 5.8S rRNA were largely unaffected. Because preexisting 25S and 5.8S rRNAs are present in vast quantities, changes in steady-state amounts of 25S and 5.8S rRNAs may be masked and therefore difficult to observe by standard assays.

Taken together, these results indicate that in ytm1 mutants pre-rRNA processing delays begin early, at the step when 27SA1 pre-rRNA is converted to 27SA2 pre-rRNA. Subsequent steps in pre-rRNA processing are similarly slowed down, but no step in pre-rRNA processing is completely blocked. Mutation of Ytm1 through depletion or inactivation results in nearly identical phenotypes, suggesting that Ytm1-1 protein is largely inactive.

**Ytm1 is necessary for release of 66S preribosomes from the nucleolus.** To further investigate the timing and role of Ytm1 in ribosome biogenesis, we assayed the ability of 60S preribosomes to exit the nucleolus and nucleus in the ytm1-1 mutant, using the ribosome export assay (28), in which eGFP-tagged rpl25 functions as a reporter. In ytm1-1 cells grown at the permissive temperature, 66S preribosomes were released to the cytoplasm (Fig. 5C). In wild-type cells grown at 25°C or shifted from 25°C to 37°C, rpl25-eGFP signal was cytoplasmic (data not shown). When the ytm1-1 mutant strain was grown at 25°C and shifted to 37°C for 5 h, rpl25-eGFP was strongly retained in the nucleolus in most cells (Fig. 5D, arrows), although in some cells signal was distributed throughout the nucleoplasm. Thus, Ytm1 is important for nucleolar release of 66S preribosomes and perhaps for subsequent nuclear export.

**Ytm1 is a component of 66S preribosomes.** Previously Ytm1 was identified in 66S pre-rRNPs purified using TAP-tagged ribosome assembly factors (3, 21, 26, 41). Consistent with this result, HA3-tagged Ytm1 peaks in sucrose gradient fractions 15 to 17 containing 66S preribosomes (21) (Fig. 6). A small amount of Ytm1 can be detected sedimenting at the top of the gradient in lighter fractions and larger amounts at the bottom of the gradient in heavier fractions. The significance of the sedimentation in heavier fractions is unclear.

To examine in more detail ribosome assembly intermediates containing Ytm1, we identified the pre-rRNAs and proteins associated with affinity-purified Ytm1-TAP. TAP-tagged Ytm1 is fully functional: the tagged strain grows at wild-type rates and has a wild-type polysome profile, and Ytm1-TAP sediments on sucrose gradients with a peak at 66S (data not shown).

To determine in which preribosomes Ytm1 is present, we assayed which pre-rRNAs copurify with TAP-tagged Ytm1. The amounts of 27SA2, 27SA3, 27SB, 25.5S, and 7S pre-rRNAs recovered relative to each other were similar to those found in whole cells. Smaller relative amounts of 5.8S RNA and no 35S or 20S pre-rRNA or 18S rRNA copurified with Ytm1-TAP (Fig. 7). Enrichment of Ytm1 with these RNA molecules is consistent with our finding that Ytm1 is important for assembly of 60S ribosomal subunits and for processing of 27S pre-rRNA (Fig. 3 and 4).

Fifty-three different proteins that copurify with TAP-tagged Ytm1 were identified by SDS-PAGE and mass spectrometry (Fig. 8). Among these are ribosomal proteins from both the large and small ribosomal subunits. Since these ribosomal proteins frequently contaminate TAPs (3, 11, 21), their significance cannot be assessed. Seventeen nonribosomal proteins specifically required for biogenesis of 60S ribosomal subunits are present in the Ytm1-containing particles (Fig. 8 and Table 2). Copurification of these pre-rRNAs and proteins with TAP-tagged Ytm1 indicates that Ytm1 first enters the ribosome assembly pathway by associating with 66S preribosomes and remains stably associated with 66S pre-rRNPs until late stages of maturation in the nucleoplasm.

**Ribosome assembly factors Ytm1, Nop7, and Erb1 are present together in a subcomplex.** Several different experiments demonstrate that Ytm1, Erb1, and Nop7 form a heterotrimeric subcomplex that is present within 66S preribosomes and also exists independently of pre-rRNPs. (i) Affinity purification from whole-cell extracts using TAP-tagged Ytm1, Nop7, or Erb1 yielded 50 to 60 proteins present in 66S pre-rRNPs (Fig. 8) (21; data not shown). In each case, greater amounts of Ytm1, Nop7, and Erb1 (Fig. 8, bands 33, 37, and 40, respectively) were recovered than those of any of the other proteins. This suggests that a mixture of 66S pre-rRNPs and a heterotrimer of Ytm1, Nop7, and Erb1 copurify with each of these TAP-tagged proteins (Fig. 8 and see Fig. 11). (ii) Affinity purification using TAP-tagged Nop7 or Ytm1 from rrl1-1 or norp4-3 mutants in which 66S pre-rRNPs are unstable (21, 26) yielded mostly Ytm1, Nop7, and Erb1, and greatly diminished amounts of molecules comprising 66S pre-rRNPs (21) (Fig. 9A). Thus, under these conditions, many fewer 66S pre-rRNPs were recovered, but the heterotrimeric subcomplexes remained intact. (iii) The Ytm1/Nop7/Erb1 heterotrimer could be separated from 66S pre-rRNPs by sucrose gradient fractionation or differential centrifugation of whole-cell extracts. Affinity purification from such enriched fractions using TAP-tagged Ytm1, Nop7, or Erb1 yielded primarily Ytm1, Nop7, and Erb1 (8, 32) (Fig. 9B, lane 1, and C, lane 2). (iv) Treatment of whole-cell extracts with a cocktail of phosphatase inhibitors caused pre-rRNPs to be disrupted (P. Harnpicharnchai, unpublished), while the Nop7/Ytm1/Erb1 subcomplex remained intact. Nop7-TAP or Ytm1-TAP under these conditions resulted in the recovery of only the Ytm1/Nop7/Erb1 heterotrimer (Fig. 9D, lane 2) (5; data not shown). (v) Nop7-TAP from sucrose gradient fractions containing 66S preribosomes yielded most protein components of the 66S pre-rRNPs (Fig. 9E, lane 1). However, when gradient fractions containing 66S pre-rRNPs were treated with the phosphatase inhibitor cocktail prior to affinity purification with Nop7-TAP, mostly the heterotrimer was recovered (Fig. 9E, lane 2). The last result indicates that Ytm1,
Erb1, and Nop7 form a stable complex within preribosomes sedimenting at 66S and can be released from the 66S pre-rRNPs by treatment with the phosphatase inhibitor cocktail. The molecular basis of the effect(s) of the phosphatase inhibitors is unknown.

Our current data suggest that the components of the Ytm1/Nop7/Erb1 heterotrimer join preribosomes separately since significant or small amounts of 35S pre-rRNA coprecipitate with Nop7 and Erb1, respectively, although no 35S pre-rRNA copurifies with Ytm1 (Fig. 7C). Thus, Nop7 likely joins nascent preribosomes first, followed by Erb1. Ytm1 later associates with 66S preribosomes containing 27SA2 pre-rRNA (Fig. 7B). Ytm1 and Nop7 directly interact with Erb1. To assay pairwise interactions between components of the heterotrimer and to determine whether the interactions are direct, we carried out GST pull-down assays. Ytm1 bound specifically to GST-Erb1, and Erb1 bound to GST-Ytm1, while Nop7 displayed strong binding to GST-Erb1 but not to GST-Ytm1 (Fig. 10A) (data not shown). Consistent with these observations, Pes1 and Bop1, the mammalian homologues of Nop7 and Erb1, bind to each other in vitro and interact in two-hybrid assays in vivo (34). The interactions between Erb1 and Ytm1 were corroborated by two-hybrid assays in vivo. Cells expressing AD-YTM1 and BD-ERB1 or AD-ERB1 and BD-YTM1 displayed strong expression of the GAL-HIS3 reporter gene (growth on 50 mM 3-aminotriazide) (data not shown). Thus, Ytm1 and Nop7 each bind directly to Erb1 but not to one another (Fig. 10C). Erb1, like Ytm1, contains WD40 repeats. Nop7 also contains a known protein-protein interaction motif, the BRCT domain (1). Further analysis is necessary to test whether these or other domains dictate the strong interactions among these three proteins.

Association of heterotrimer with 66S preribosomes is significantly weakened in the ytm1-1 mutant. Since the two ytm1-1 mutations are in residues that may be important for interactions with ligands, we determined the effects of these mutations on the integrity of the heterotrimer and 66S preribosomes. We used TAP-tagged Nop7 or Brx1 to purify 66S preribosomes from YTM1 and ytm1-1 strains, since both of these proteins are present in all seven different 66S pre-rRNPs (21, 60) (Fig. 1B). SDS-PAGE, silver staining, and Western blotting revealed that most of the proteins present in wild-type 66S pre-rRNPs are also present in equivalent amounts in the particles isolated

FIG. 4. Processing of pre-rRNAs is altered in the ytm1-1 mutant. (A) Oligonucleotide probes or primers used to detect rRNAs and pre-rRNAs. (B) Yeast strains JFY3400 (YTM1) and JFY7128 (ytm1-1) were grown in YEPD medium at 25°C and shifted to 37°C for 5 h. Cells were pulse-labeled with [5,6-3H]uracil for 5 min and chased with an excess of unlabeled uracil for 2, 5, 10, and 60 min. Equal cpm of RNA isolated from cells at each time point were subjected to electrophoresis on agarose-formaldehyde gels to separate each pre-rRNA and rRNA and detected by autoradiography. (C) Primer extension was performed to determine steady-state levels of 27SA2, 27SA, 27SB, plus 7Sb, 27Sb, plus 7Sb, pre-rRNAs. RNA was extracted from whole-cell extracts from strains JFY3400 (YTM1) and JFY7128 (ytm1-1) grown in YEPD medium at 25°C or shifted from 25°C to 37°C for 3 h or 6 h or from strain JFY6149 (YTM1) or JFY6992 (GAL-YTM1) grown in galactose-containing medium and shifted to glucose-containing medium for 0, 10, 12, 15, or 18 h. (D) Northern blotting was used to determine steady-state levels of 25S, 18S, 5.8S, and 5S rRNA and 7S pre-rRNA. High-molecular-weight RNAs were subjected to electrophoresis on agarose-formaldehyde gels, whereas acrylamide-urea gels were used to separate low-molecular-weight RNAs. U3 snoRNA was used as a loading control. RNA was quantified by phosphorimaging and normalized to U3 snoRNA.

FIG. 5. Inactivation of Ytm1 in the ytm1-1 mutant causes 66S preribosomes to accumulate in the nucleolus. The ytm1-1 mutant strain JFY6790 expressing eGFP-tagged rpL25 was grown in C-Trp medium at 25°C, washed and suspended in YEPD, and grown at 25°C (A and C) or shifted to 37°C for 5 h (B and D). Nuclei stained with 4′,6′-diamidino-2-phenylindole (DAPI) are shown in panels A and B (typically, nucleoli do not stain with DAPI). The signal from Rpl25eGFP is shown in panels C and D. Arrows indicate nucleolar accumulation of rpL25eGFP (D) and corresponding DAPI staining (B).
from the ytm1-1 mutant, indicating that the 66S preribosomes are largely intact in the ytm1-1 mutant (Fig. 11). However, Ytm1-1 mutant protein was greatly diminished or absent from the pool of purified 66S preribosomes (Fig. 11A, lanes 2 and 4, and B, lanes 2 and 4). Nop7 and Erb1 were still present, but in reduced amounts (Fig. 11A, lanes 2 and 4). Thus some of the mutant preribosomes contain Nop7 and/or Erb1 but not Ytm1, and others lack all three proteins. The relative amounts of each pre-rRNA copurifying with TAP-tagged Nop7 or Brx1 in 66S pre-rRNPs parallel those in whole-cell extracts (data not shown).

The Ytm1/Nop7/Erb1 heterotrimer is destabilized in the ytm1-1 mutant. The effects of the ytm1-1 mutations on 66S pre-rRNPs and pre-rRNA processing could result from alterations of the heterotrimer containing Ytm1. Therefore, we purified the heterotrimer from the ytm1-1 mutant and examined its integrity, using three assays: sucrose gradient centrifugation, differential centrifugation, and treatment of whole-cell extracts with phosphatase inhibitors. In each case, only small amounts, if any, of Erb1 copurified with Nop7 and no Ytm1 could be detected (Fig. 9B, lane 2; C, lane 4; and D, lane 4). GST-pulldown assays confirmed that Ytm1-1 does not bind to Erb1 in vitro at 37°C (Fig. 10B). These results suggest that at the nonpermissive temperature, Ytm1-1 fails to interact with Erb1 and the Nop7-Erb1 association is significantly weakened, leading to destabilization of the heterotrimer and perturbations of preribosome maturation.

**DISCUSSION**

Although there has been much progress to identify yeast ribosome assembly intermediates and their protein and RNA constituents (reviewed in references 12 and 14), nothing is known about the architecture of these pre-rRNPs. For example, it is unclear which proteins are nearest neighbors within assembling ribosomes and to what extent neighboring molecules function together. Since Ytm1 contains multiple WD40 protein-protein interaction motifs, it is an excellent candidate for a molecule present in a multiprotein subcomplex comprising a neighborhood in assembling ribosomes.

Here we have shown that Ytm1 is a constituent of four consecutive 66S preribosomes and is necessary for steps in their maturation to 60S ribosomal subunits. Ytm1 associates with ribosome assembly factors Erb1 and Nop7 to form a stable subcomplex that is present within 66S preribosomes and that also exists separately from pre-rRNPs. Erb1 and Nop7 assemble into preribosomes prior to Ytm1. Mutations in WD40 motifs 6 and 7 of Ytm1 destabilize the heterotrimer and weaken association of each of the three proteins with 66S pre-rRNPs. Consequently, processing of pre-rRNAs and release of preribosomes from the nucleolus are delayed, resulting in production of fewer 60S...
Ytm1 involves with 60S preribosomes. The copurification of Ytm1 with the same relative proportions of 27SA2, 27SA3, 27SB, 25.5S, and 7S pre-rRNAs as found in whole-cell extracts (Fig. 7) indicates that Ytm1 is present in each of the four consecutive 60S pre-rRNPs containing these pre-rRNAs, through out most or all of their lifetimes. No 20S pre-rRNA or 18S rRNA copurifies with Ytm1, consistent with Ytm1 participating in biogenesis of 60S ribosomal subunits but not 40S subunits. Although Ytm1-TAP particles contain moderate amounts of 5.8S rRNA, we failed to detect significant levels of 25S rRNA, consistent with a previous report indicating that conversion of 7S pre-rRNA to 5.8S rRNA occurs more rapidly than production of 25S rRNA from 25.5S pre-rRNA (18).

We also identified 53 proteins that copurify with TAP-tagged Ytm1 (Fig. 8, Table 2). Of the proteins isolated, 17 are ribosome assembly factors previously shown to be components of 60S preribosomes (Table 2). None of the proteins found specifically in late nucleoplasmic or cytoplasmic 60S pre-rRNPs (41) are present in preribosomes purified using Ytm1-TAP. This is consistent with our RNA and subcellular localization data indicating that Ytm1 dissociates from pre-rRNPs in the nucleoplasm prior to the latest nucleoplasmic and cytoplasmic stages of ribosome maturation. Thus 60S preribosomes purified using Ytm1-TAP may represent core complexes of molecules important for intermediate steps in 60S subunit ribosome biogenesis.

Ytm1 is present in a heterotrimeric complex with Nop7 and Erb1. Our results and those of others indicate that Ytm1, Erb1, and Nop7 form a stable heterotrimeric complex (8, 21, 32) (Fig. 8, 9, and 10). The sedimentation of Nop7, Ytm1, and Erb1 on sucrose gradients indicates that in wild-type cells most or all of their lifetimes. No 20S pre-rRNA or 18S rRNA copurifies with Ytm1, consistent with Ytm1 participating in biogenesis of 60S ribosomal subunits but not 40S subunits. Although Ytm1-TAP particles contain moderate amounts of 5.8S rRNA, we failed to detect significant levels of 25S rRNA, consistent with a previous report indicating that conversion of 7S pre-rRNA to 5.8S rRNA occurs more rapidly than production of 25S rRNA from 25.5S pre-rRNA (18).

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| Gel ID | Protein | ORF | Characteristic |
|-------|---------|-----|----------------|
|       |         |     | Metazoan homolog | Essential | Sediments at 66S | Decreased 60S Subunits | Pre-rRNA processing | Localization | Reference |
| 33    | Ytm1    | YOR272w | +        | +        | +        | +        | +        | 27SA2, ↑ 25.5S ↓ 7S ↓ | No/Nu | This work (21) |
| 36    | Nop7    | YPL211w | +        | +        | +        | +        | 27SA3, ↑ 27S B ↓ | No/Nu/C | 61 |
| 24    | Nop16   | YER002c | +        | +        | +        | 27SA2, ↑ 27SB ↓ | No/25 | 20 |
| 25    | Btx1    | YOL077c | +        | +        | ND       | +        | 27SA2, ↑ 27SB ↓ 7S ↓ | No/25 | 21 |
| 27    | Rlp7    | YNL002c | +        | +        | ND       | +        | 27SB ↑ 7S ↓ | No/25 | 21 |
| 28    | Rpt2    | YKR081c | +        | +        | +        | 27SB ↑ 7S ↓ | No/25 | 21 |
| 29    | Cic1/Nsa3 | YHR052c | +        | +        | ND       | +        | 27SB ↑ 7S ↓ | No/25 | 21 |
| 30    | Nsa1    | YGL111w | +        | +        | +        | +        | 27SB ↑ 7S ↓ | No/25 | 21 |
| 31    | Has1    | YMR290c | +        | +        | +        | +        | 27SB ↑ 7S ↓ | No/25 | 21 |
| 32, 33 | Ebp2    | YKL172w | +        | +        | ND       | +/−      | 35S ↑ 27SB ↑ | No/25 | 21 |
| 35, 36 | Nog1    | YPL093w | +        | +        | +        | +        | 27SB ↑ 7S ↓ | No/25 | 21 |
| 37    | Nop7    | YGR103w | +        | +        | +        | 27SB → 25S slow | No/25 | 21 |
| 38    | Noc3    | YLR002c | +        | +        | +        | 35S ↑ 27SB ↑ | No/25 | 21 |
| 39    | Noc2    | YOR206w | +        | +        | +        | 35S ↑ 27SB ↑ | No/25 | 21 |
| 40    | Nop2    | YGL111w | +        | +        | ND       | +        | 27SB ↑ 7S ↓ | No/25 | 21 |
| 41    | Dsr1    | YLL008w | +        | +        | ND       | +        | 27SB ↑ 7S ↓ | No/25 | 21 |
| 42    | Erb1    | YMR049c | +        | +        | +        | +        | 27SB ↑ 7S ↓ | No/25 | 21 |

* Protein band 30 was identified as Tsf2 and protein band 36 also contained Ssal. These proteins are common contaminants of affinity-purified complexes (17, 24).
* No: nucleolar; Nu: nuclear; C: cytoplasmic.
* ↑ and ↓, increased and decreased amounts of indicated RNAs, respectively.
* ND, not determined.
bind tightly to Erb1; however, we find no evidence for direct interactions between Ytm1 and Nop7 (Fig. 10). The ability to purify the heterotrimer directly from 66S preribosomes using the phosphatase inhibitor cocktail that disrupts 66S pre-rRNPs (Fig. 9E) suggests that interactions among these three proteins are stronger than those with other molecules in the 66S pre-rRNP. Nevertheless, it is reasonable to assume that this subcomplex influences the assembly or function of a larger protein or RNP neighborhood within assembling ribosomes necessary for their efficient maturation (see below). Thus, it will be important to identify other molecules that are adjacent to Ytm1, Erb1, and Nop7 within preribosomes.

The heterotrimeric complex also exists independently of 66S preribosomes. It can be purified from fractions near the top of gradients or from high-speed supernatants (Fig. 9B and C). Our present data suggest that Nop7 and Erb1 may assemble into preribosomes before Ytm1. Consistent with this finding is the presence of Nop7 and Erb1, but not Ytm1, in 66S pre-rRNPs isolated from the ytm1-1 mutant. Nop7 and Erb1 may not require Ytm1 to assemble into or remain in preribosomes. Upon completion of their functions, Nop7, Erb1, and Ytm1 might dissociate from preribosomes together as a heterotrimer, prior to recycling into other nascent preribosomes. The substantial amount of heterotrimer that we purify from cells might also result from disassembly in vivo of unstable abortive assembly intermediates or upon dissociation from preribosomes in vitro during fractionation or purification.
A conserved network of protein interactions. Interactions among Ytm1, Erb1, and Nop7 required for ribosome biogenesis are likely to be conserved. Interactions of Pes1, the mouse homologue of Nop7, with Bop1, the mouse homologue of Erb1, are required for assembly of Pes1 into preribosomes (34). Like their yeast counterparts, Pes1 and Bop1 are required for similar steps in processing of pre-rRNAs to mature 25S and 5.8S rRNA (1, 35, 44, 55, 56).

Mutations in the WD40 motifs of Ytm1 destabilize the heterotrimer and weaken its association with preribosomes. The G398D and S442N mutations in WD40 repeats 6 and 7 of YTM1 prevent binding of Ytm1-1 to Erb1 in vitro and significantly weaken association between Nop7 and Erb1 (Fig. 9 and 10). Destabilization of the heterotrimer also weakens association of Ytm1, Erb1, and Nop7 with 66S pre-rRNPs, resulting in recovery of greatly diminished amounts of Ytm1 and slightly decreased amounts of Erb1, relative to Nop7, in the pool of purified 66S preribosomes (Fig. 11). The mixture of 66S pre-rRNPs purified from the ytm1-1 mutant using Brx1-TAP contains no detectable Ytm1-1 and less Nop7 and Erb1 compared to preribosomes isolated from wild-type cells (Fig. 11). (Note that no free heterotrimer could copurify with Brx1-TAP.) The greater decrease of Ytm1 compared to Erb1 in Nop7-TAP-purified ytm1-1 mutant particles suggests that the Ytm1-Erb1 interaction may be necessary to recruit or maintain Ytm1 in 66S pre-rRNPs. This interaction may also influence assembly of Nop7 and Erb1 into preribosomes or may be required to stabilize their association with preribosomes, since amounts of Nop7 are reduced slightly in the 66S preribosomes purified from ytm1-1 mutant cells expressing Brx1-TAP.

Ytm1 is required for pre-rRNA processing and trafficking of 66S preribosomes. Our data show that pre-rRNA processing is slowed in the ytm1 mutants. The conversion of 27SA pre-rRNA to 27SB pre-rRNA is delayed, resulting in changes in amounts of 27SBa and 27SBb pre-rRNAs. Subsequent steps in pre-rRNA processing are also slowed, resulting in reduced amounts of 7S pre-rRNA and 5S rRNA (Fig. 4). Processing of 27SA to 27SB pre-rRNA involves rapid exonucleolytic trimming of the 5′ end of the 27SA pre-rRNA by Rat1 and Xrn1 (23). Ytm1 might function directly in all of these steps. Alternatively, the decreased rate of processing of 27SA pre-rRNA in the ytm1 mutants might indirectly affect subsequent processing of 27SB pre-rRNA, for example, by perturbing the architecture of pre-rRNPs. Depletion of Nop7 and Erb1 has effects on pre-rRNA processing similar but not completely identical to those of ytm1 mutants: increased levels of 27SA pre-rRNA and decreased amounts of 27SB and 7S pre-rRNAs (42, 44).

Thus, the pre-rRNA processing phenotypes of the ytm1 mutants may reflect a combination of effects on the presence and/or functions of all three proteins in the Ytm1/Erb1/Nop7 heterotrimer.

Most, but not all steps of processing of precursors to 25S and 5.8S rRNA are thought to occur prior to nucleolar release and most likely require or are accompanied by many changes in the topology of these pre-rRNPs. Although the mechanism whereby preribosomes exit the nucleolus remains a mystery, it may require...
some changes in the composition and structure of preribosomes, including those resulting from pre-rRNA processing. Nuclear export of preribosomes requires binding of export factors to preribosomes, perhaps timed by the availability of ligands on the surface of preribosomes (22). Thus pre-rRNA processing, nucleolar release, and nuclear export may be in-itated from samples affinity purified from extracts from these strains.

Functions of the heterotrimer in ribosome biogenesis. Taken together, our results suggest the following model for effects of ytm1-1 mutations on ribosome assembly. In wild-type cells, early in ribosome assembly during or after synthesis of 35S pre-rRNA, Nop7 and Erb1 join nascent preribosomes. Slightly later, many 60S ribosome assembly proteins, including Ytm1, associate with the 275S A pre-rRNA to form the earliest detectable 60S pre-rRNPs. In the ytm1-1 mutant, Ytm1-1 may not efficiently assemble into this pre-RNP or may not remain stably associated with preribosomes, also resulting in destabilization of association of Nop7 and Erb1 with pre-60S ribosomes. Although it is also possible that Ytm1, Nop7, or Erb1 dissociates from the mutant particles during purification rather than in vivo, these mutant particles are likely otherwise intact. However, their overall architecture might be perturbed by the absence or weakened association of Ytm1, Erb1, and Nop7. When preribosomes lack these putative scaffolding proteins, they might be unable to establish or maintain structures necessary for efficient pre-rRNA processing, ribosome assembly, release of nascent ribosomes from the nucleolus, or export of preribosomes from the nucleus to the cytoplasm. Moonlighting functions of the heterotrimeric proteins. Clearly Ytm1 is necessary for efficient ribosome production. Depletion or inactivation of Ytm1 decreases the rate of 60S subunit biogenesis below levels necessary to sustain viability. However, Ytm1 may have a second “moonlighting” function, such as mitosis or chromosome transmission (36, 43; Matsumoto et al., personal communication). Interestingly, Nop7 and its metazoan homologue Pescadillo, and Bop1, the mammalian homologue of Erb1, also have independent functions. Nop7 and Pescadillo are implicated in DNA replication or cell proliferation (8, 35). Mutations in BOP1 induce p53-dependent cell cycle arrest (45). Thus, the heterotrimeric complex of Ytm1, Erb1, and Nop7 might be a depot for directing multiple functions of these proteins.

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