Proofreading of pre-40S ribosome maturation by a translation initiation factor and 60S subunits

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In the final steps of yeast ribosome synthesis, immature translation-incompetent pre-40S particles that contain 20S pre-rRNA are converted to the mature translation-competent subunits containing the 18S rRNA. An assay for 20S pre-rRNA cleavage in purified pre-40S particles showed that cleavage by the PIN domain endonuclease Nob1 was strongly stimulated by the GTPase activity of Fun12, the yeast homolog of cytoplasmic translation initiation factor eIF5b. Cleavage of the 20S pre-rRNA was also inhibited in vivo and in vitro by blocking binding of Fun12 to the 23S rRNA through specific methylation of its binding site. Cleavage competent pre-40S particles stably associated with Fun12 and formed 80S complexes with 60S ribosomal subunits. We propose that recruitment of 60S subunits promotes GTP hydrolysis by Fun12, leading to structural rearrangements within the pre-40S particle that bring Nob1 and the pre-rRNA cleavage site together.

The maturation of pre-ribosomal particles to produce the functional small and large ribosomal subunits is a complex, multistep process, in which the processing of the pre-RNAs must be coordinated with other assembly steps. During this maturation process, the 20S pre-rRNA is cleaved to 18S rRNA only in late, cytoplasmic pre-40S particles, raising the question of how the timing of cleavage is related to 40S subunit maturation.

Nucleotide triphosphatases are an important class of ribosomal synthesis factors. Many ATPases and GTPases use the energy of NTP hydrolysis to drive or regulate structural changes in the pre-ribosomal particle. The RNA-stimulated ATPases, or RNA helicases, are a prominent group of ribosome synthesis factors. In yeast, 18 putative helicases have essential roles throughout the synthesis pathway, but, of these, only Prp43 is known to participate in the late, cytoplasmic steps of 40S subunit synthesis. GTPases are also required at multiple steps in the synthesis pathway. Although none had yet been clearly shown to participate directly in late steps of 40S synthesis in yeast, the GTPases Era and RsgA (also known as YjcQ) are required for 30S subunit synthesis in Escherichia coli. Yeast Tsr1 has homology to GTPases, is required for 40S synthesis and occupies a very similar binding site to Era2. In addition, a high-throughput analysis indicated that 20S pre-rRNA accumulated in strains lacking the translation-initiation factor and GTPase Fun12 (ref. 3). This suggested a potential role for this translation-initiation factor in ‘proofreading’ during the maturation of 40S subunits toward translation competence. Notably, the bacterial translation-initiation factor IF2, which is homologous to eukaryotic eIF5b homologs including Fun12, is a high-copy suppressor of mutations in RsgA4, suggesting that it might have a conserved function in small-subunit synthesis.

Steps in ribosome synthesis that occur in the nucleus are subject to very active quality-control systems. In contrast, surveillance of the late steps that occur in the cytoplasm appears to be less active, and 3′ unprocessed forms of the 18S and 5.8S rRNAs accumulate to high levels in strains with processing defects. Locations of ribosome synthesis factors in the pre-40S and pre-60S particles have been mapped, revealing that several proteins occupy sites that are required for key functional interactions during translation initiation and elongation2,5–9. The clear conclusion was that immature pre-ribosomes are actively prevented from interacting nonproductively with the translation machinery. In apparent contrast, other data indicated that late pre-40S particles, which accumulated in strains lacking processing factors, could associate with 60S subunits. This suggested that the last steps in pre-40S maturation might involve functional interactions with the translation machinery10.

The final RNA-processing step during 40S subunit maturation is the cleavage of the 3′ end of the mature 18S rRNA by Nob1, a PIN domain endonuclease11–15. Nob1 binds to early, nuclear pre-ribosomes that are exported to the cytoplasm before pre-rRNA cleavage, indicating that the timing of Nob1 cleavage is regulated and linked to 40S subunit maturation. Genetic analyses implicated Prp43 in this process, which acts together with its cofactor Pfa1 (refs. 11,16).

To better understand the late steps in 40S subunit maturation, we reproduced 18S rRNA 3′ cleavage in pre-40S particles and investigated

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the factors involved in the presumed structural reorganization that is required for the acquisition of cleavage competence.

RESULTS
Nob1 activity is not stimulated by prior cleavage at A2
During 40S subunit maturation, Nob1 associates with early, 90S preribosomes in the nucleus and is exported with the pre-40S particles to the cytoplasm. In the cytoplasm, Nob1 cleaves the pre-rRNA at site D, which forms the 3′ end of the mature 18S rRNA. This strongly indicates that the nuclease activity of Nob1 is regulated and linked to other steps in pre-40S maturation.

In our previous analyses of the in vitro activity of Nob1 we had used protein purified from yeast extracts. However, the lethal phenotypes of Nob1 mutants complicated functional analyses in vitro. We therefore purified recombinant wild-type and mutant forms of Nob1 expressed in E. coli (Fig. 1a). Wild-type Nob1 exhibited ~20% cleavage efficiency on an in vitro–transcribed RNA substrate that contained site D (Fig. 1b,c). We detected lower levels of cleavage at other sites with sequences related to those of site D, as previously observed for Nob1 purified from yeast. These may arise only in vitro because the positioning of Nob1 in vivo is constrained by the pre-40S structure. The mutation in the PIN domain (D15N) abolished cleavage, confirming that this activity is solely due to Nob1 activity (Fig. 1b).

During rRNA maturation, the pre-rRNA normally undergoes cleavage at a site designated A2 within the internal transcribed spacer I (ITS1) region (Fig. 1c) before cleavage at site D. A model has been proposed, in which prior cleavage at A2 and release of the 3′ fragment of the pre-rRNA triggers an RNA conformational switch that may activate D-site cleavage. To test this model, we compared four RNA substrates using in vitro endonuclease cleavage assays (Fig. 1c). Transcripts 1 and 2 have 5′ ends within the 18S rRNA 200 nucleotides (nt) 5′ of site D, whereas substrates 3 and 4 extend to site A1 at the 5′ end of 18S. Substrates 1 and 3 have 3′ ends at site A2, whereas substrates 2 and 4 extend to site A3, mimicking a pre-rRNA before A2 cleavage that is not predicted to act as a cleavage substrate.

For all four substrates, we analyzed the cleavage site by primer extension and quantified cleavage efficiency by northern hybridization (Figs. 1d,e and data not shown). On all substrates tested, we observed similar cleavage efficiencies (~20%), with no clear differences in the presence or absence of the A2–A3 region.

We also tested the pre-rRNA refolding model by extensive phylogenetic comparison of yeast ITS1 sequences and their secondary structures. This confirmed previously published and empirically tested models and showed that the secondary structure of the A2–A3 region was conserved. The primary sequence of the ITS1 region situated between A2 and A3 was variable, whereas the pre-rRNA refolding model postulates that this region interacts with a very highly conserved sequence in the 3′ end of 18S. In contrast, the actual A2 and A3 processing sites were highly conserved at sequence level, as was a stem-loop structure located just upstream of A3. Furthermore, we found no clear similarity between Saccharomyces sp. ITS1 sequences or predicted secondary structures and those of more distant fungi (Schizosaccharomyces or Pezizomyotina sp.) (Supplementary Fig. 1 and Supplementary Note).

Although these data do not demonstrate that the pre-rRNA refolding model is incorrect, they indicate that it is not sufficient to explain Nob1 activation in the cytoplasm and is unlikely to apply in organisms that are not closely related to S. cerevisiae.

Nob1 activity is stimulated by ATP and GTP addition
As the in vitro cleavage data and phylogenetic comparisons did not provide clear support for the role of RNA structure reorganization in the regulation of 20S pre-rRNA processing, we attempted to assess the roles of protein factors. To facilitate these analyses, we developed an in vitro cleavage assay using purified pre-40S particles. To ensure that late, cleavage-competent pre-40S particles were present in the precipitate, we used tagged Nob1 as the bait for purification (Fig. 2). The Nob1 tagged at the N terminus with Protein A–TEV-His6 (PTH-Nob1 fusion) we used has been previously shown to support Nob1 function in vivo.2
Like other PIN-domain nucleases, the in vitro cleavage activity of Nob1 is strongly stimulated by Mn$^{2+}$ (ref. 11). Therefore, we affinity-purified pre-ribosomes using PTH-Nob1 on an IgG column and extensively washed them in buffer containing 5 mM Mg$^{2+}$ but lacking Mn$^{2+}$ (Fig. 2a). Then we activated in vitro cleavage by buffer exchange to 5 mM Mn$^{2+}$, 2 mM Mg$^{2+}$, with or without NTPs. After incubating the particles for 30 min at 20 °C, we assessed cleavage by primer extension (Fig. 2b–d).

Addition of Mn$^{2+}$ without nucleotides induced only a very low level of site-D cleavage in the pre-40S particles (Fig. 2b). Inclusion of either ATP or GTP at 1 mM final concentration enhanced cleavage (about sevenfold and eightfold, respectively; Fig. 2b,c). In contrast, neither ADP nor GDP conferred clear cleavage stimulation (Fig. 2b). The effects of addition of both ATP and GTP were not synergistic (Fig. 2b) but were modestly additive when analyzed over multiple experiments (Fig. 2d). As Nob1 is bound to both early and late pre-ribosomes, it would be anticipated that only a fraction of the pre-ribosomes that purify together with Nob1 are cleavage-competent. Moreover, it is likely that some of the cleavage-competent particles will have undergone cleavage during the extended incubations needed for purification.

To assess the requirement for ATP and GTP hydrolysis, we tested nonhydrolyzable analogs AMP-PNP and GMP-PNP. Cleavage was stimulated about ninefold by AMP-PNP but was not stimulated by GMP-PNP (Fig. 2b). Many NTPases bind and hydrolyze either ATP or GTP, but this result clearly demonstrates that these are distinct activities in 40S processing.

To confirm that the observed cleavage was due to the activity of Nob1, we purified pre-40S particles using the catalytically inactive PTH–Nob1 D15N mutant protein (Fig. 2c). No cleavage activity was associated with pre-40S particles containing Nob1 D15N, in the absence or presence of ATP or GTP.

We conclude that Nob1 cleavage in pre-40S particles is activated by at least two different factors. One of these has ATP-binding activity and the other is a GTPase.

**Fun12 is a GTPase involved in 40S processing**

We sought to confirm the identity of the GTPase(s) involved. The inventory of late pre-40S components suggested an obvious candidate factor in Tsr1 (ref. 21), which blocks premature association of pre-40S particles with 60S subunits. Tsr1 has apparent sequence homology to GTPases and occupies a binding site on the 18S rRNA that overlaps that of the 746

Another candidate was the translation-initiation factor Fun12, a GTPase proposed to play a role in 20S processing in vivo. During initiation of translation, Fun12 promotes binding of tRNA$^{Met}$ and subunit joining. To assess whether Fun12 is directly involved in 20S processing, we tested its association with pre-40S particles by immunoprecipitation using Fun12 fused to a C-terminal His6–TEV-Protein A (HTTP) tag. We also compared the immunoprecipitation efficiency of wild-type Fun12 to that of a mutant (Fun12 T439A) that retains GTP-binding but lacks hydrolysis activity and is therefore predicted to show increased retention on its substrate. We analyzed by northern hybridization the RNA species that precipitated with Fun12 after purification on IgG sepharose (Fig. 3). Consistent with the function of Fun12 in translation, wild-type and mutant Fun12 bound mature 18S and 25S rRNA but did not bind to the snRNA U2, which is involved in pre-mRNA splicing. In addition, Fun12 interacted with 20S pre-rRNA and, to a lesser extent, with 27S pre-rRNA. We observed approximately twofold more Fun12 T439A precipitation with 20S pre-rRNA compared to wild-type Fun12. We observed lesser increases for the mature RNAs and 27S pre-rRNA.

Yeast Fun12 is non-essential for viability, at least in some strain backgrounds, but its loss confers a substantial growth defect. To test the involvement of Fun12 in pre-rRNA processing, we fused the FUN12 open reading frame to an N-terminal triple hemagglutinin (HA) tag and
placed it under the control of a \( P_{GAL} \) promoter (\( P_{GAL}::3HA-FUN12 \)), which is repressed in the presence of glucose. Western blotting (Fig. 4a) showed that Fun12 was strongly depleted 6 h after transfer to glucose medium and was undetectable by 12 h after transfer to glucose. Depletion of Fun12 did not strongly affect the abundance of Nob1 (Fig. 4a). Under these growth conditions, Nob1 migrated as two bands, which we speculate reflect post-translational modification. However, the ability of recombinant Nob1 to cleave 20S pre-rRNA in vitro shows that this putative modification is not required for activity.

We determined the effects of Fun12 depletion on pre-rRNA processing by northern hybridization (Fig. 4b). After 12 h of Fun12 depletion, we detected accumulation of the 35S primary transcript and a reduction in the amount of 27S pre-rRNA, indicating that cleavage at site A2 was impaired. This may reflect the inhibition of ribosomal protein synthesis because depletion of several different ribosomal proteins leads to inhibition of these cleavages\(^4\). Despite the reduction in 20S pre-rRNA synthesis implied by the reduction in A2 cleavage, we observed clear 20S pre-rRNA accumulation after 18 h of Fun12 depletion. These results strongly indicate that depletion of Fun12 impairs 20S pre-rRNA processing. We did not observe clear depletion of mature 18S rRNA relative to 25S rRNA probably because, unlike the 20S...
pre-rRNA, the mature 18S rRNA is stable and is depleted only by cell growth. Growth is inhibited by depletion of Fun12, but this is primarily due to the inhibition of translation. We predict that the residual 18S synthesis is sufficient to support the reduced requirement for ribosome synthesis.

To further assess the effects of Fun12 depletion, we performed pulse-chase labeling (Fig. 4c). The appearance of mature 18S rRNA was strongly retarded in the Fun12-depleted strain, showing that maturation of the entire 20S pre-rRNA population was delayed. Maturation of 27S pre-rRNA to 25S particles was also delayed, although this was less marked than for 20S pre-rRNA processing. It is unclear whether this reflects an additional involvement of Fun12 in 27S pre-rRNA processing or is a consequence of reduced protein synthesis in the Fun12-depleted strain.

The 20S pre-rRNA is processed to 18S rRNA after export of the pre-40S particles to the cytoplasm. The inhibition of processing is therefore expected to lead to the accumulation of cytoplasmic 20S pre-rRNA. To determine whether this is the case, we monitored the level of dimethylation of nucleotides A1781 and A1782. Dimethylation of nucleotides A1781 and A1782.

These results indicate that Fun12 is directly associated with pre-40S particles and suggested that GTP hydrolysis by Fun12 might be important for cytoplasmic 20S pre-rRNA cleavage.

**Fun12 activates Nob1 function in pre-40S particles in vitro**

To test whether the activity of Fun12 is responsible for the GTP-dependent stimulation of in vitro pre-rRNA cleavage, we performed this analysis using pre-40S particles purified from PGAL::FUN12 strains genetically depleted of endogenous Fun12 for 12 h and carrying either an empty plasmid or a plasmid expressing wild-type Fun12 under the control of an ADH1 promoter. Depletion of Fun12 reduced GTP-stimulated cleavage ~2.5-fold (Figs. 5a,b). Some GTP stimulation was still observed, but depletion of proteins under GAL control is never complete and a reduced level of Fun12 will remain in the depleted extract. We also observed a modest but reproducible reduction in ATP-stimulated cleavage, possibly reflecting a reduced amount of cleavage-competent pre-40S particles in the cell extract.

Specific mutations in the active sites of some GTPases allow the use of xanthine triphosphate (XTP) in addition to or instead of GTP. XTP does not occur naturally and is not normally a substrate for GTPases, so this allows specific determination of the roles of an individual GTPase in vitro, even when other GTPases are present. Such mutations have been previously described for human eIF5b and yeast Fun12, in which the active-site mutation (D533N) allows XTP hydrolysis. Expression of Fun12 D533N from a plasmid under the control of an ADH1 promoter had a strong dominant negative effect on cell growth even when PGAL::FUN12 was induced, presumably because of competition. We therefore used a much weaker MET25 promoter for this analysis. In cells expressing only wild-type Fun12, we saw no substantial stimulation of cleavage after XTP addition (Fig. 5c,d). In contrast, XTP substantially stimulated cleavage in lysates from strains expressing Fun12 D533N, demonstrating that the GTPase activity of Fun12 stimulates site D cleavage in vitro.
of Fun12 D533N with pre-40S particles was reduced in comparison with wild-type Fun12 (data not shown).

**Binding of Fun12 to 60S subunits stimulates 20S processing**

Fun12 is involved in 40S–60S subunit joining during translation initiation, suggesting the possibility that its role in stimulating 20S pre-rRNA processing in pre-40S particles might also involve interactions with 60S particles. Testing this hypothesis by depletion of 60S ribosomes is not feasible, so we sought to disrupt the interaction between Fun12 and the 25S rRNA. The 25S rRNA contact sites for Fun12 on the 25S rRNA are shown in Figure 2. The snoRNAs are expressed from the intron of the non-functional ADH1 (Tgy5) in a strain deleted for the SNR78–72 cluster. (c) Effects of expression of snR75 on pre-rRNA processing in vivo. FISH analysis (c) as in Figure 4d. Scale bar, 5 μm. Northern blot analysis (d) of pre-rRNAs indicated on the left. Mature rRNAs were visualized by ethidium bromide staining. (e) Cleavage assay as in Figure 2 in lysates from cells expressing wild-type snR75 (snR75WT) or snR75Mut2. Mock, cleavage without added GTP; GTP, cleavage with 1 mM GTP; and GTP + 60S, cleavage with 1 mM GTP plus 100 pmol of purified ribosomal subunits (ribs). (f) Quantification of cleavage efficiency.
snR75Mut2. Western blotting revealed no difference in recovery of HA-Fun12 (Supplementary Fig. 4c). Northern hybridization showed greatly reduced co-precipitation of mature 25S and 18S rRNA, confirming disruption of Fun12 interactions during translation initiation (Supplementary Fig. 4d). In addition, the association of Fun12 with 20S pre-rRNA was reduced in the strain expressing snR75Mut2 (Supplementary Fig. 4d), indicating that this interaction also requires association of 60S with pre-40S. We also observed some reduction in Fun12 association with 27S pre-rRNA on snR75Mut2 expression, consistent with specific interactions between Fun12 and pre-60S particles. It remains unclear, however, whether Fun12 also has a direct role in pre-60S maturation.

We tested the effects of snR75Mut2 expression and consequent 25S methylation on 20S processing by FISH and northern hybridization. In cells, we hybridized a Cy3-labeled oligonucleotide (probe F) complementary to the 5′ region of ITS1, which is present in the 20S pre-rRNA and all earlier precursors (Fig. 6c). The nucleoplasm was labeled with DAPI. We observed clear cytoplasmic accumulation of 20S pre-rRNA when methylation is present on Gm2307. The wild-type cytoplasmic 20S particle signal was lower in the experiments reported in Figure 6c than those in Figure 4d, owing to reduced ribosome synthesis on galactose compared to glucose medium. Northern hybridization (Fig. 6d) confirmed the accumulation of 20S pre-rRNA, whereas 35S and 27S pre-rRNA levels appeared unaffected. We conclude that the association of Fun12 with the 60S ribosomal subunit is required for efficient processing of cytoplasmic 20S in vivo.

To assess the involvement of 60S subunits in 20S pre-rRNA cleavage in vitro, we prepared 20S particles from lysates of strains expressing wild-type snR75 and snR75Mut2 (Figs. 6e,f). The wild-type strain showed approximately twofold more cleavage in the presence of GTP than the snR75Mut2 strain did (Fig. 6e), consistent with a requirement for Fun12–25S rRNA association for efficient cleavage. To test this, we supplemented the purified pre-40S particles with 60S subunits from wild-type lysates (Fig. 6e). Cleavage stimulation by 60S addition was substantially greater in the snR75Mut2 strain than in the wild type (Fig. 6f).

We interpret this as showing that the accumulated pre-40S particles in the snR75Mut2 strain identified by northern hybridization (Fig. 6d) can be activated for cleavage by association with exogenously added, unmodified 60S subunits, but that interaction with the endogenous subunits is blocked by 25S rRNA methylation.

We performed the initial analyses in strains deleted for the SNR78–72 cluster and complemented by expression of only snR75 (Fig. 6 and Supplementary Fig. 4). To confirm that the loss of other snRNAs in the cluster did not contribute to the observed phenotypes, we constructed plasmids that included the entire cluster except SNR75, with snR75 expressed independently (Supplementary Fig. 5a). In these strains, northern blot analyses confirmed the specific accumulation of 20S pre-rRNA, and RNase H resistance confirmed the specific methylation at Gm2307 (Supplementary Fig. 5b,c).

We conclude that both the GTPase activity of Fun12 and its functional association with the 60S subunit are required for efficient 3′ processing of the 18S rRNA.

**Pre-40S particles associate with 60S subunits**

To determine whether Fun12 shows stable or transient pre-ribosome association, we assessed its presence in pre-40S particles purified with HTP-Nob1 by western blotting (Fig. 7a). Around 2% of Fun12 precipitated with pre-40S particles, which is a notably high figure considering that most of
the Fun12 population is presumably engaged in translation initiation. This finding suggested that mature 60S subunits might also be stably associated with pre-40S particles. Ethidium bromide staining of RNA associated with the pre-40S particles showed a high level of mature 25S rRNA (Fig. 7b). This does not represent nonspecific recovery as we saw no corresponding signal in the mock precipitation using a nontagged strain. As noted above, it is likely that cleavage-competent pre-40S particles undergo some cleavage during the extended incubation steps needed for purification, and this may contribute to the 18S signal observed in the Nob1 precipitate. If cleavage during the extended incubation steps needed for purification, and this would expect this form to be increased in the pre-40S particles containing Nob1 D15N because these are specifically blocked at the cleavage step. As shown in the results in Figure 7b,c, this is indeed the case.

During translation initiation, Fun12 promotes 40S–60S subunit joining. To assess whether Fun12 also facilitates pre-40S–60S interactions, we expressed snR75Mut2 in a strain expressing PTH-tagged Nob1 (Supplementary Fig. 5e). The introduction of the Gm2307 modification by snR75Mut2 reduced recovery of 25S rRNA with Nob1 associated pre-40S particles, strongly indicating that Fun12 promotes pre-40S–60S joining.

To assess whether the pre-40S particles are assembled into discrete complexes of higher molecular weight, we analyzed pre-40S particles associated with Nob1 and Nob1 D15N by gel filtration. In both cases the RNA distribution comprised two major peaks (Fig. 7d). These were revealed by comparison to a calibration curve to be approximately 80S and 40S in size. Western blotting revealed two matching peaks of Nob1 distribution (Fig. 7d). Comparison of the pre-40S peaks associated with Nob1 and Nob1 D15N (Fig. 7d), exhibited a marked increase in the 80S particle fraction for the D15N mutant, which is in good agreement with the RNA precipitation data shown in Figure 7b,c.

These data strongly suggested that the pre-40S and mature 60S subunits form complexes resembling 80S ribosomes. We verified this by imaging fractions from the gel-filtration complex by electron microscopy (Fig. 7e). The distinctive mature 60S subunit structure was readily visible in the pre-40S associated complexes present in the 80S fractions.

**DISCUSSION**

Four yeast pre-rRNA endonucleases, Rnt1, RNase MRP, Rcl1 and Nob1, have been shown to cleave their cognate target sites in vitro in naked RNA substrates. In vivo, however, the timing of cleavage by MRP and Nob1, at least, is regulated and correlated with other events in ribosome synthesis. Detailed understanding of the mechanisms involved has been elusive, because these processes have been refractory to in vitro analyses in minimal systems. Here we report the development of an assay for in vitro maturation of purified pre-40S particles by the Nob1 endonuclease that allows the dissection of factors and interactions responsible for the acquisition of cleavage competence. Our results do not support the importance of a proposed RNA conformational switch in regulating 20S pre-rRNA cleavage but, instead, revealed independent ATP- and GTP-stimulated activities that each promote site-D cleavage by Nob1.

Stimulation of 20S cleavage in our in vitro assay required GTP hydrolysis, so we tested candidate potential GTPases. Our analyses in depletion strains showed that Fun12 is required for efficient processing of 20S pre-rRNA in vivo and in vitro. In vivo expression of a Fun12 mutant capable of hydrolyzing XTP conferred XTP-dependent stimulation of in vitro cleavage on purified pre-40S particles, demonstrating the direct involvement of Fun12.

During translation initiation, Fun12 binds both 40S and 60S subunits, suggesting that this might also be the case during pre-40S

![Figure 8](image-url) Model for the role of Fun12 in pre-40S processing. (a,b) Side view of mature 40S particles. Image in b is a magnification of the boxed region in a. Six nucleotides at the 3’ end of 18S particle are represented in green as a surface (nucleotides –6 to –4) or as beads (nucleotides –3 to 3). The Nob1 binding site is in blue. Positions of ribosomal proteins Rps5 and Rps14, which are involved in site D cleavage, are in black. The path of the mRNA across the 40S subunit is indicated. (c) Model of steps potentially driving cleavage at site D. ITS1 is proposed to be located in the mRNA-binding cleft (i). Fun12 binds pre-40S particles together with the 60S subunit (ii). GTP hydrolysis by Fun12 drives movement of the head domain and displaces ITS1 within the mRNA binding cleft, bringing site D toward Nob1; 40S head to body rotation is proposed to participate in bringing the Nob1 active site together with site D (iii). Cleavage of ITS1 and release of Nob1 generates mature, translation-competent 40S subunits (iv).
maturation. To test this hypothesis, we disrupted the interaction between Fun12 and 60S subunits. We introduced an epitope site of 2′-O-methylation at a nucleotide (G2307) previously shown to be a contact site for Fun12 in the 25S rRNA29,30. The single additional methyl group in 25S rRNA disrupted Fun12 association with 40S and 60S subunits and impaired 20S pre-rRNA processing in vivo and in vitro. This suggested that the functionally important interactions between Fun12 and pre-40S particles might take place in the context of a trimeric complex with mature 60S ribosomes.

Pre-40S particles containing 20S pre-rRNA that cannot be 3′ processed owing to depletion of synthesis factors previously have been reported to interact with 60S subunits10. We found that a substantial amount of 25S rRNA was associated with Nob1, and even more was bound to catalytically inactive Nob1 D15N protein11–15. Fractionation of Nob1-associated complexes by gel filtration, revealed two peaks corresponding to 40S and 80S complexes. Visualization by electron microscopy revealed that the 80S complexes included structures resembling mature 60S subunits. Consistent with the RNA precipitation data, a substantially higher fraction of the complexes associated with Nob1 D15N were in the 80S form. Nob1 is associated with 20S pre-rRNA in the nucleus and in early cytoplasmic pre-40S particles, as well as in late, cleavage-competent particles. The Nob1 D15N mutation causes specific accumulation of very late pre-40S particles. These data would therefore be consistent with 25S rRNA association and 80S complex formation only on late, cleavage competent pre-40S particles.

Hcr1, the yeast homolog of another translation-initiation factor eIF3j, physically interacts with Fun12 and has been reported to have a role in 20S particle processing36,37. However, depletion of several other translation-initiation factors did not affect 20S maturation10,38,39, making it unlikely that initiation of translation is required for processing.

The role of Fun12 in late 40S particle maturation
Fun12 promotes tRNA\text{Met} binding and subunit joining22,23, and is an attractive candidate to combine functional proofreading with late assembly steps in 40S particle synthesis (Fig. 8). The 20S pre-rRNA cleavage is inhibited by depletion of Fun12 (ref. 3) and also by mutation or depletion of ribosomal proteins located along the mRNA-binding cleft40. We speculate that the ITS1 5′ region, which forms an extended stem-loop structure, may associate with the mRNA binding cleft in the pre-40S particles40. Placement of ITS1 in the cleft would pull site D away from Nob1, which is bound to helix 40 in the 18S rRNA2,41. The 3′ terminal three nucleotides of 18S rRNA are missing from the ribosome crystal structures, and are therefore not included in the model shown (Fig. 8a) but are indicated with three green beads.

Detailed structural data on the role of Fun12 during eukaryotic translation initiation are currently lacking. However, the bacterial homolog IF2 has been shown to promote subunit joining and to promote small subunit rotation, leading to a translation–competent conformation42. Whereas subunit joining is promoted by GMP-PNP binding, subunit rotation requires GTP hydrolysis. The C-terminal domain of IF2 interacts with the 16S rRNA and rpsS12, and is responsible for positioning tRNA\text{Met} (ref. 43). Before GTP hydrolysis by IF2, the interaction between the Shine–Dalgarno sequence at the 3′ end of the 16S rRNA and the anti–Shine–Dalgarno sequence in the mRNA holds the mRNA in a ‘standby’ position, from which it must be moved for translation to initiate43,44.

Attainment of a structure close to the mature 40S and/or nuclear export may allow Fun12 to bind the pre-40S particle in association with mature 60S subunits. The reported Fun12 binding site on 18S H5 is distant from Nob1, but close to Prp43 and to the mRNA binding cleft40. During bacterial translation initiation, GTP hydrolysis by IF2 moves the Shine-Dalgarno–anti-Shine-Dalgarno duplex backwards (that is, to the right in the orientation shown in Fig. 8)33,44. We propose that a similar movement, possibly in combination with small-subunit rotation42, displaces ITS1 duplex from the mRNA-binding cleft and brings site D into proximity with the active site in the PIN domain of the endonuclease Nob1. On GTP binding, the effector domain of bacterial EF2 (a Fun12 homolog) moves by some 37 Å (ref. 45), consistent with estimates of the distance separating Nob1 from the 3′ end of 18S rRNA2,8.

Linking translation competence and subunit maturation
Previous analyses have reported that multiple ribosome synthesis factors occupy sites in both the pre-40S and pre-60S particles that are required for key functional interactions of the mature subunits during translation (reviewed in ref. 9). These are presumably important to ensure that immature pre-ribosomes do not engage nonproductively with the translation machinery. Given the high rate of pre-ribosome production (around 2,000 per minute in yeast) this would be a problem. However, the data presented here support the model that at later steps in maturation at least one translation-initiation factor and the 60S subunit interact with the pre-40S particle during final maturation. The obvious conclusion is that this represents a proofreading step to ensure that the subunits are close to translational competence.

In the slime mold Dictyostelium discoideum, pre-ribosomes that cannot engage with the translation machinery do not undergo late rRNA maturation steps46. In bacteria, the translation-initiation factor IF2, which is homologous to Fun12, is implicated in 3′ maturation of 16S and 23S rRNA. Moreover, there are clear links between maturation of the bacterial 30S and 50S subunits and genetic interactions between IF2 and ribosome synthesis factors4,47,48. Together these results indicate the existence of a conserved pathway, in which translation initiation factors and subunit interactions are involved in final steps of ribosome maturation. We postulate that functional proofreading of pre-ribosomes before their release for translation is an ancient process that was likely already present in the last universal common ancestor of all extant organisms.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
S.L., C.S., R.W.x.N., A.S., S.G., N.J.W. and D.T. designed experiments; S.L., C.S., R.W.x.N., A.S., D.W., B.B. and S.G. performed experiments; S.L., C.S., R.W.x.N., A.S., B.B., S.G., N.J.W. and D.T. analyzed data; and S.L., C.S., R.W.x.N., A.S., S.G., N.J.W. and D.T. wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Immature small ribosomal subunits can engage in translation initiation by 40S assembly intermediates. Science 333, 1449–1453 (2011).

Panse, V.G. & Johnson, A.W. Maturation of eukaryotic ribosomes: acquisition of functionality. Trends Biochem. Sci. 35, 260–266 (2010).

Soudet, J., Gelugne, J.P., Neuhäusler, A., Caizergues-Ferrer, M. & Mougin, A. Immature small ribosomal subunits can engage in translation initiation in Saccharomyces cerevisiae. EMBO J. 29, 80–92 (2010).

Pertsch, B. et al. RNA helicase Prp43 and its co-factor Pfa1 promote 20 to 18S rRNA processing catalyzed by the endonuclease Nob1. J. Biol. Chem. 284, 35079–35091 (2009).

Fatica, A., Offinger, M., Sllicak, M. & Tollervey, D. Nob1p is required for cleavage of the 3′ end of 18S rRNA. J. Mol. Biol. 235, 2537–2545 (2008).

Fatica, A., Tollervey, D. & Sllicak, M. The PIN domain of Nob1p is required for 20S ribosome assembly. J. Biol. Chem. 285, 14259–14264 (2009).

Wollenhaupt, H.A., Lamm, A.C. & Karst, K. Roles of Dim2 in ribosome assembly. J. Biol. Chem. 286, 2578–2586 (2011).

Lebaron, S. et al. The ATPase and helicase activities of Prp43p are stimulated by the G-patch protein Pfa1p during yeast ribosome biogenesis. EMBO J. 28, 3808–3819 (2009).

Lamanna, A.C. & Karst, K. An RNA conformational switch regulates pre-18S rRNA cleavage. J. Mol. Biol. 405, 3–17 (2011).

Allmang, C. et al. Processing of the yeast pre-rRNA at sites H2 and A3 is linked. RNA 2, 63–73 (1996).

Yeh, L.-C.C., Tsu, W.C., Tsai, M. & Lee, J.C. Internal transcription spacer 1 of the yeast pre-rRNA precursor ribosomal RNA. Higher order structure and common structural motifs. Biochemistry 29, 5911–5918 (1990).

Van Nues, R.W. et al. Separate structural elements within internal transcribed spacer 1 of Saccharomyces cerevisiae pre-rRNA direct the formation of 175 and 265 rRNA. Nucleic Acids Res. 22, 912–919 (1994).

Gelperin, D., Horton, L., Beckman, J., Hensold, J. & Lemmon, S.K. Bms1p, a novel nuclear export adaptor protein Nmd3 in Saccharomyces cerevisiae. J. Cell Biol. 199, 1079–1086 (2010).

Kemmler, S., Occhipinti, L., Veisu, M. & Panse, V.G. Yvh1 is required for a late maturation step in the 60S biogenesis pathway. J. Cell Biol. 186, 863–880 (2009).

Strunk, B.S. et al. Ribosome assembly factors prevent premature translation initiation by 40S assembly intermediates. Science 333, 1449–1453 (2011).

O׳Connor, D., Horton, L., Beckman, J., Hensold, J. & Lemmon, S.K. Bms1p, a novel nuclear export adaptor protein Nmd3 in Saccharomyces cerevisiae. J. Cell Biol. 199, 1079–1086 (2010).

Abou Elela, S., Igel, H. & Areas, M.J. RNase III cleaves eukaryotic pre-rRNA at a U3 snRNP-dependent site. Cell 85, 115–124 (1996).

Horn, D.M., Mason, S.L. & Karst, K. Rpl1 protein, a novel nuclelease for 18S rRNA production. J. Biol. Chem. 286, 34082–34087 (2011).

Allmang, C. & Tollervey, D. The role of the 3′ external transcribed spacer in yeast pre-rRNA processing. J. Mol. Biol. 278, 67–78 (1998).

Senger, B. et al. The nucleoliar Tip6 and Efi1p are required for a late cytoplasmic step of ribosome synthesis. Mol. Cell 8, 1363–1373 (2001).

Yarunin, A. et al. Functional link between ribosome formation and biogenesis of iron-sulfur proteins. EMBO J. 24, 580–588 (2005).

Neuder, A. et al. A local role for the small ribosomal subunit primary binder rps6 in final 18S rRNA processing in yeast. PLoS ONE 5, e10194 (2010).

Veith, T. et al. Structural and functional analysis of the archaeal endonuclease Nob1. Nucleic Acids Res. 40, 3259–3274 (2012).

Marshall, R.A., Alten, C.E. & Puglisi, J.D. GTP hydrolysis by IF3/HC1p in processing 20S pre-rRNA and translation initiation. J. Biol. Chem. 276, 43351–43360 (2001).

Collins, S.R. et al. Toward a comprehensive atlas of the physical interactome of Saccharomyces cerevisiae. Mol. Cell Proteomics 6, 439–450 (2007).

Lee, J.H. et al. Initiation factor eIF5B catalyzes second GTP-dependent step in eukaryotic translation initiation of the ribosome into elongation. Mol. Cell 35, 37–47 (2009).

Julian, P. et al. The cryo-EM structure of a complete 30S translation initiation complex from Escherichia coli. PLoS Biol. 9, e1001099 (2011).

La Terna, A., Guerali, C.O. & Brancic, R. Cysteine-mediated disulfide bond formation in the ribosome. J. Mol. Biol. 272, 27818–27822 (1997).

Kaczanowska, M. & Ryden-Aulin, M. Ribosome biogenesis and the translation process in Escherichia coli. Microbiol. Mol. Biol. Rev. 71, 477–494 (2007).

Belotserkovsky, J.M., Dabb, E.R. & Isaksson, L.A. Mutations in 16S rRNA that suppress cold-sensitive initiation factor 1 affect ribosomal subunit assembly. FEBS J. 278, 3508–3517 (2011).

Ben-Shem, A. et al. The structure of the eukaryotic ribosome at 3.0 A resolution. Science 334, 1524–1529 (2011).
ONLINE METHODS

Strains, media, plasmids and cloning. *Saccharomyces cerevisiae* strain BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) was used as the parental strain29. Strains with P_CAG promoters and tags were generated by PCR as described31. Strain genotypes are described in Supplementary Table 1. Strains were grown in YPD (1% yeast extract, 2% peptone and 2% dextrose), YPG (1% yeast extract, 2% peptone and 2% galactose), YPG/S (1% yeast extract, 2% peptone, 1% galactose and 1% sucrose) or yeast minimal medium (Formedium).

Oligonucleotides are listed in Supplementary Table 2. The plasmid pRS-PTH-NOB1 has been previously described24. The plasmid pRS415-Fun12-HTP was constructed after PCR amplification of the FUN12 ORF and cloning into pRS415-HTP52 using Xma1 restriction sites. The sequences encoding mutants Fun12D533N and Fun12T439A were generated by site-directed PCR mutagenesis as described11. The expression of Nob1 D15N was generated by site-directed PCR mutagenesis as described11. The variant pLS65 allowing expression of Nob1 D15N has been previously described16. Treatment with Tsr1 ORF was PCR-amplified and cloned into pCR4-TOPo. From this vector Tsr1 was subcloned into p415PL generating p415-PL-HIS used for in vivo analyses or into pGEX4-T, generating GST-Tsr1-HIS used to express and purify this protein from *E. coli*. Tsr1 mutations were generated by site-directed PCR mutagenesis. For intronic expression of snoRNAs from the P_FUTL promoter, oligonucleotides forming the genes for snR75 or its mutants were inserted in place of the artificial snoRNA in the actin/snoRNA cassette described previously27. Plasmids were transformed into derivatives of YPH499 (MATa, ura3-52, lys2-101, leu2-200, trp1-Δ63) or BY4741 (Supplementary Table 1) deleted for the SNR78–72 gene cluster26. For growth analysis, YPH499smR78–72 transforms were spotted as tenfold serial dilutions on YPD or YPD plates and incubated at 30 °C for 2 d.

In vitro RNA-cleavage assay. The recombinant proteins Nob1-HIS and Nob1 D15N–HIS were purified as described16. Ribosomal RNA substrates were transcribed in vitro using Xma1 restriction sites. The sequences encoding mutants Fun12D533N and Fun12T439A were generated by site-directed PCR mutagenesis as described previously28. Plasmids were transformed into derivatives of YPH499 (MATa, ura3–52, lys2–101, leu2Δ1, his3–Δ200, trp1–Δ63) or BY4741 (Supplementary Table 1) deleted for the SNR78–72 gene cluster. For growth analysis, YPH499smR78–72 transforms were spotted as tenfold serial dilutions on YPD or YPD plates and incubated at 30 °C for 2 d.

In vitro RNA-cleavage assay. The recombinant proteins Nob1-HIS and Nob1 D15N–HIS were purified as described16. Ribosomal RNA substrates were transcribed in vitro and preformed for 20 min at 55 °C in the presence of 10 mM MgCl2 and 50 mM HEPES (pH 7.4)35. Nuclease activity assays were performed at described11. The expression of Nob1 D15N was generated by site-directed PCR mutagenesis as described11. The variant pLS65 allowing expression of Nob1 D15N has been previously described16. Treatment with Tsr1 ORF was PCR-amplified and cloned into pCR4-TOPo. From this vector Tsr1 was subcloned into p415PL generating p415-PL-HIS used for in vivo analyses or into pGEX4-T, generating GST-Tsr1-HIS used to express and purify this protein from *E. coli*. Tsr1 mutations were generated by site-directed PCR mutagenesis. For intronic expression of snoRNAs from the P_FUTL promoter, oligonucleotides forming the genes for snR75 or its mutants were inserted in place of the artificial snoRNA in the actin/snoRNA cassette described previously27. Plasmids were transformed into derivatives of YPH499 (MATa, ura3-52, lys2-101, leu2-200, trp1-Δ63) or BY4741 (Supplementary Table 1) deleted for the SNR78–72 gene cluster26. For growth analysis, YPH499smR78–72 transforms were spotted as tenfold serial dilutions on YPD or YPD plates and incubated at 30 °C for 2 d.

Electron microscopy. For negative staining, 5-μl samples were placed on a freshly glow-discharged, carbon-coated grid and then washed three times with water, two times with uranyl acetate (2% w/v) and stained with uranyl acetate for 5 min before drying. Micrographs shown were recorded with a Tecnai F20 electron microscope equipped with a TVIPS F816 digital camera and operating at 200 kV.

Model building and phylogenetic analysis. For the models presented in Figure 8, PyMol software was used to manipulate crystal structures 3USC, 3UE5 and 1G7T49,58. For phylogenetic comparisons, ITS1 sequences were identified by BLAST using the 3′ and 5′ regions of 18S and 5.8S rRNAs, respectively, or retrieved from rDNA sequences accumulated previously20,28. Alignments were assembled manually using the 4SALE sequence editor (http://4sale.bioapps.biozentrum.uni-wuerzburg.de/) and formatted with Jalview (http://www.jalview.org/). In the pile-up sequences are abbreviated as in ref. 28; plus Torulaspora delbrueckii (Tdl), Hansenula wingei (Hw), Pichia canadensis)30, and Endomyces fibuliger (Ef; Genbank accession number U10409).

**Tsr1 purification and analysis.** Tsr1-HTP expressed in *S. cerevisiae* was purified on IgG sepharose beads or on GTP agarose beads; 250 μl of cells were used for each immunoprecipitation (IP). Cell extract was purified in lysis buffer (50 mM Tris-HCl (pH 7.5), 2.5 mM MgCl2, 150 mM NaCl and 5 mM dithiothreitol and protease inhibitors (Roche)) using Zirconia beads as previously described55. HA-tagged proteins were precipitated using 50 μl of Gamma-bind matrix (GE healthcare) coated with 2 μg anti-HA probe (sc-7392 Santa Cruz Biotechnology).
50. Brachmann, C.B. et al. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**, 115–132 (1998).

51. Petracek, M.E. & Longtine, M.S. PCR-based engineering of yeast genome. *Methods Enzymol.* **350**, 445–469 (2002).

52. Bohnsack, M.T. et al. Prp43 bound at different sites on the pre-rRNA performs distinct functions in ribosome synthesis. *Mol. Cell* **36**, 583–592 (2009).

53. Karbstein, K., Jonas, S. & Doudna, J.A. An essential GTPase promotes assembly of preribosomal RNA processing complexes. *Mol. Cell* **20**, 633–643 (2005).

54. Granneman, S., Kudla, G., Petfalski, E. & Tollervey, D. Identification of protein binding sites on U3 snoRNA and pre-rRNA by UV cross-linking and high throughput analysis of cDNAs. *Proc. Natl. Acad. Sci. USA* **106**, 9613–9618 (2009).

55. Lebaron, S. et al. The splicing ATPase prp43p is a component of multiple preribosomal particles. *Mol. Cell. Biol.* **25**, 9269–9282 (2005).

56. Tollervey, D. High level of complexity of small nuclear RNAs from fungi and plants. *J. Mol. Biol.* **196**, 355–361 (1987).

57. Leshin, J.A., Rakauskaite, R., Dimman, J.D. & Meskauskas, A. Enhanced purity, activity and structural integrity of yeast ribosomes purified using a general chromatographic method. *RNA Biol.* **7**, 354–360 (2010).

58. Roll-Mecak, A., Cao, C., Dever, T.E. & Burley, S.K. X-ray structures of the universal translation initiation factor IF2/eIF5B: conformational changes on GDF and GTP binding. *Cell* **103**, 781–792 (2000).