The Roles of Puf6 and Loc1 in 60S Biogenesis Are Interdependent, and Both Are Required for Efficient Accommodation of Rpl43*

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Puf6 and Loc1 have two important functional roles in the cells, asymmetric mRNA distribution and ribosome biogenesis. Puf6 and Loc1 are localized predominantly in the nucleolus. They bind ASH1 mRNA, repress its translation, and facilitate the transport to the daughter cells. Asymmetric mRNA distribution is important for cell differentiation. Besides their roles in mRNA localization, Puf6 and Loc1 have been shown to be involved in 60S biogenesis. In puf6Δ or loc1Δ cells, pre-rRNA processing and 60S export are impaired and 60S subunits are underaccumulated. The functional studies of Puf6 and Loc1 have been focused on ASH1 mRNA pathway, but their roles in 60S biogenesis are still not clear. In this study, we found that Puf6 and Loc1 interact directly with each other and both proteins interact with the ribosomal protein Rpl43 (L43e). Notably, the roles of Puf6 and Loc1 in 60S biogenesis are interdependent, and both are required for efficient accommodation of Rpl43. Loc1 is further required to maintain the protein level of Rpl43. Additionally, the recruitment of Rpl43 is required for the release of Puf6 and Loc1. We propose that Puf6 and Loc1 facilitate Rpl43 loading and are sequentially released from 60S after incorporation of Rpl43 into ribosomes in yeast.

Almost every process in a cell is mediated by proteins, the products of translating mRNA by ribosomes. Ribosomes are composed of two subunits, a small subunit and a large subunit, 40S and 60S, respectively, in eukaryotic cells. In Saccharomyces cerevisiae, the 40S and 60S subunits are assembled from a large primary 35S rRNA transcribed by RNA polymerase I. Processing of this rRNA yields 5.8S, 18S, and 25S rRNAs. The 5S rRNA is transcribed by RNA polymerase III separately (see reviews in Refs. 1–4). In yeast, 5S, 5.8S, and 25S (28S in higher eukaryotes) rRNA assemble with 39 ribosomal proteins to make up the 60S subunit, whereas 18S rRNA and 33 ribosomal proteins constitute the 40S subunit (5–7).

Ribosome biogenesis is necessary for cell growth and proliferation. The synthesis of ribosome is a complex pathway in which over 200 trans-acting factors are involved. Many of the RNA folding, protein assembly, and maturation events are hierarchical, requiring the correct completion of one event to progress to the next event. This is seen in the assembly of ribosomal proteins onto the RNA in bacterial ribosome assembly (8) as well as in cytoplasmic maturation of the large subunit in yeast (9, 10). In addition, trans-acting factors also perform quality control steps for the assembly of the protein synthesis machinery. Syo1 facilitates the synchronized import of the two 5S rRNA-binding proteins, Rpl5 and Rpl11, to ensure stoichiometric incorporation of these two proteins into the pre-60S subunits and also works as an assembly platform for the 5S ribonucleoprotein (RNP) (11, 12). Release of the anti-association factor Tif6 requires elongation factor-like Efl1 and tRNA-like Sdo1 to confirm the integrity of the P-site (13–15). Trans-acting factors on cytoplasmic pre-40S subunits not only prevent them from joining translation (16) but also perform a translation-like cycle to ensure the quality of the 40S subunits prior to actual translation of mRNA (17).

In this study, we addressed the functional connections among Puf6, Loc1, and ribosomal protein 43 (Rpl43 or L43e in newer nomenclature (18)). Whereas previous studies of Puf6 and Loc1 functions have been focused on their roles in the asymmetric distribution of ASH1 mRNA in yeast, their roles in 60S biogenesis have not been clearly elucidated.

The cytoplasmic transport and asymmetric distribution of mRNA are critical for eukaryotic cells to restrict protein expression to a subcellular region for determination of cell fate, polarity, and development. In budding yeast, Ash1 protein (19) is asymmetrically localized in the daughter cells to repress mating type switching. ASH1 mRNA is transcribed in the mother cells and translationally repressed by association with She2, Puf6, Loc1, and Khd1 (20–22) (reviewed in Ref. 23). Upon export from the nucleus, Loc1 is released and the ASH1 mRNA is transported by myosin (Myo4) to the daughter cells (24).

Puf6 belongs to the Pumilio/fem-3 mRNA-binding factor (PUF) family of RNA-binding proteins, which are post-transcriptional regulators of gene expression. They recognize 3′-UTRs and control the stability and translation of mRNA (reviewed in Ref. 15). All PUF proteins contain a feature known as a PUF domain with eight conserved Pumilio repeats (26–28). A recent report shows that the Pumilio repeats of Puf6 and its human homologue, Puf-A, form a unique "L-like" shape (29) and interact with DNA and RNA in a sequence-indepen-

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Dent manner, which might be an important characteristic of rRNA processing in 60S biogenesis (29). Puf6 has been connected to 60S biogenesis in a network-guided genetic screen (30, 31) and a global proteomic analysis (32). Puf6 show co-sedimentation with 60S subunits in sucrose gradients and is immunoprecipitated by 60S-associated trans-acting factors (31, 32). Upon Puf6 deletion, the synthesis and export of large ribosomal subunits is reduced in yeast (31).

Loc1, an RNA-binding protein, is also required for asymmetric localization of ASH1 mRNA in yeast (20). It is not an essential gene, but loc1Δ cells show a severe, slow growing phenotype. Loc1 is distributed predominantly in the nucleolus, consistent with a role in the biogenesis of 60S subunits. Loc1 associates with 60S subunits (33, 34), and loc1Δ mutant cells show significantly reduced levels of mature 25S rRNA and increased levels of 35S pre-RNA and aberrant 23S RNA (35). Additionally, 60S export is reduced in loc1Δ cells.

Here, we have described a mechanistic study of Puf6 and Loc1 function in 60S biogenesis in yeast. Direct protein-protein interaction was found among Puf6, Loc1, and Rpl43. In the absence of Puf6 or Loc1, Rpl43 could not be loaded efficiently into pre-60S subunits. Furthermore, depletion of Rpl43 in yeast blocked Puf6 and Loc1 release from 60S.

Results

**Rpl43 Is a High-copy Suppressor of puf6Δ**—PuF6 is nonessential, as yeast cells from which PuF6 has been deleted are viable and do not show a slow growth phenotype at 30 °C; they have only a slight reduction in growth rate at 37 °C and a somewhat greater defect at 20 °C (Fig. 1A). A sucrose gradient analysis was carried out in puf6Δ cells at different temperatures. Despite the fact that puf6Δ cells did not show a significant growth defect at 30 °C, there was a modest deficiency in 60S subunits, evident as a reduced ratio of free 60S to free 40S. These defects became more pronounced at 20 and 37 °C (Fig. 1B). In summary, the growth rate of puf6Δ is correlated with 60S levels.

To gain insight into the function of PuF6 in 60S biogenesis, we first carried out a high-copy suppressor screen in a puF6Δ mutant. Because puf6Δ cells showed only a modest slow growth defect at low temperature, we considered ways to enhance the growth defect in puf6Δ cells. We found that the puF6 mutant with a truncated PuF6 domain (ΔPuF) was slower growing than puf6Δ (Fig. 1C). Therefore, a plasmid containing the ΔPuF mutant was transformed into puf6Δ to enhance the severity of the growth phenotype (Fig. 1C). A ΔPuF strain was transformed with a high-copy number (2μ) yeast genomic library. Compared with empty vector control, transformants showing a larger colony size should contain either a wild-type PuF6 clone or genes that suppress the growth defects of ΔPuF when expressed in a higher amount. In addition to PuF6, RPL43B was identified as a suppressor of both ΔPuF and the puf6Δ mutant (Fig. 1C). Yeast has two homologues of RPL43, RPL43A and RPL43B. Each gene was cloned into a high-copy number (2μ) plasmid, and each was found able to suppress the growth defects of ΔPuF and the puf6Δ mutant (Fig. 1C and data not shown).

To determine whether the suppression of puf6Δ by RPL43 reversed the ribosome biogenesis defects of puf6Δ, Pu11-GFP was used to monitor the localization of 60S subunits in the cells. Although Rpl11 was localized predominantly in the nucleus in puf6Δ at 25 °C, with Rpl43 overexpression the cytoplasmic localization of Rpl11 was significantly restored in puf6Δ cells (Fig. 1D). We concluded that Rpl43 is a high-copy suppressor of puf6Δ.

The Functions of PuF6 and Loc1 in 60S Biogenesis Are Connected—PuF6 and Loc1 function together in the asymmetric distribution and translational repression of ASH1 mRNA (20, 36). They are both RNA-binding proteins, localized predominantly to the nucleolus, and required for the efficient assembly and export of 60S subunits (31, 35). We asked whether they also work together in 60S biogenesis.

Compared with puf6Δ, loc1Δ showed more severe defects in growth (Fig. 2A) and 60S biogenesis (Fig. 2, B–D). We first examined the potential genetic interaction between puf6Δ and loc1Δ. Interestingly, the deletion of PuF6 in loc1Δ partially rescued the growth rate at 25 and 30 °C and slightly rescued it at 37 °C. But at the lower temperature of 16 °C, the puf6Δloc1Δ double mutant displayed a worsened growth compared with either the puf6Δ or loc1Δ mutant (Fig. 2A). To determine whether the slow growing phenotype was correlated with its function in 60S biogenesis, we assayed the export of 60S subunits with Pu11-GFP as a reporter. Although wild-type cells did not show nuclear accumulation of Pu11-GFP, 30% of the puf6Δ cells showed nuclear accumulation of Pu11-GFP at 30 °C (Fig. 2C). Pu11 accumulated more strongly in the nucleus in loc1Δ cells, in which 86% of the cells showed nuclear accumulation of Pu11-GFP (Fig. 2C). Consistent with the growth rate, the nuclear fraction of Pu11 was reduced (45%) in puf6Δloc1Δ cells compared with loc1Δ cells (Fig. 2C).

We further analyzed the ribosomal subunit levels in these strains. Interestingly, loc1Δ showed reduced amounts of both the 40S and 60S subunits. The additional deletion of PuF6 slightly enhanced the levels of both subunits above those of loc1Δ alone (Fig. 2D). The ability of the deletion of PuF6 to partially suppress the loc1Δ mutant implies a functional connection between PuF6 and Loc1 in 60S biogenesis.

PuF6 and Loc1 Are Present on the Pre-60S Subunit Concurrently—PuF6 and Loc1 interact directly (21) and are constituents of the ASH1 RNP complex. We asked whether PuF6 and Loc1 also exist concurrently on nascent 60S. We selected several 60S-associated trans-acting factors and immunoprecipitated nascent 60S subunits with the tandem affinity purification (TAP) tag. Brxl1 assembles into pre-60S at an early stage (37); Tif6 joins the nascent 60S from the nucleolus until almost the end of the maturation pathway (10, 38–40); Arxl is localized predominantly in the nucleus and released in late rRNA processing and nuclear export of 60S subunits (43). PuF6 and Loc1 most strongly co-purified with Brxl1, and the levels of PuF6 and Loc1 decreased in order from the Brxl1-, Tif6-, Arxl1-, and Rix1-containing nascent 60S subunits (Fig. 3A). This observation is consistent with the nuclear localization of PuF6 and Loc1. To further demonstrate that PuF6 and Loc1 coexist on nascent 60S subunits, ribosomal subunits were pelleted by ultracentrifugation to separate them from free pro-
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Deletion of PUF6 Results in Abnormal Accumulation of Loc1 on Pre-60S Subunits and Vice Versa—To further dissect the functional connection between Puf6 and Loc1, the cellular localization of Puf6 was monitored in the loc1Δ condition and vice versa. Sik1-mRFP was used as a nucleolar marker. Puf6 and Loc1 are localized predominantly in the nucleolus in wild-type cell extracts were prepared from wild-type and puf6Δ cells cultured at different temperatures and fractionated by sedimentation through 7–47% sucrose density gradients by ultracentrifugation as described under "Materials and Methods." Half-mers are indicated by arrows. C, wild-type, puf6Δ, and ΔPUF (PKL55) were transformed with vector (pRS426) and RPL43B (PKL308). Plates were incubated at 20 °C. D, Rpl11-GFP (PKL228) was transformed into wild-type and puf6Δ with vector or 2μ RPL43B (PKL308). Cells were subcultured in fresh medium and incubated at 25 °C for 4–5 h before GFP monitoring. DIC, differential interference contrast; N%, the percentage of cells with nuclear Rpl11-GFP was counted and is shown here.

FIGURE 1. RPL43 is a high-copy suppressor of puf6Δ. A, wild-type and puf6Δ (KLY67) cells were normalized and spotted in a 10-fold dilution on YPD plates. Plates were incubated at different temperatures as indicated here. B, protein extracts were prepared from wild-type and puf6Δ cells cultured at different temperatures and fractionated by sedimentation through 7–47% sucrose density gradients by ultracentrifugation as described under "Materials and Methods." Half-mers are indicated by arrows. C, wild-type, puf6Δ, and ΔPUF (PKL55) were transformed with vector (pRS426) and RPL43B (PKL308). Plates were incubated at 20 °C. D, Rpl11-GFP (PKL228) was transformed into wild-type and puf6Δ with vector or 2μ RPL43B (PKL308). Cells were subcultured in fresh medium and incubated at 25 °C for 4–5 h before GFP monitoring. DIC, differential interference contrast; N%, the percentage of cells with nuclear Rpl11-GFP was counted and is shown here.

Deletion of PUF6 Results in Abnormal Accumulation of Loc1 on Pre-60S Subunits and Vice Versa—To further dissect the functional connection between Puf6 and Loc1, the cellular localization of Puf6 was monitored in the loc1Δ condition and vice versa. Sik1-mRFP was used as a nucleolar marker. Puf6 and Loc1 are localized predominantly in the nucleolus in wild-type cell extracts were prepared from wild-type and puf6Δ cells cultured at different temperatures and fractionated by sedimentation through 7–47% sucrose density gradients by ultracentrifugation as described under "Materials and Methods." Half-mers are indicated by arrows. C, wild-type, puf6Δ, and ΔPUF (PKL55) were transformed with vector (pRS426) and RPL43B (PKL308). Plates were incubated at 20 °C. D, Rpl11-GFP (PKL228) was transformed into wild-type and puf6Δ with vector or 2μ RPL43B (PKL308). Cells were subcultured in fresh medium and incubated at 25 °C for 4–5 h before GFP monitoring. DIC, differential interference contrast; N%, the percentage of cells with nuclear Rpl11-GFP was counted and is shown here.
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Figure 2. Deletion of PUF6 partially rescues the defects of loc1Δ cells. A, wild-type, puf6Δ (KLY67), loc1Δ (KLY218), and puf6Δloc1Δ (KLY312) cells were normalized and spotted in 10-fold dilution on YPD plates. The plates were incubated at different temperatures as indicated here. Plates were incubated for shorter times (see Fig. 1A) and longer times (as shown here) to make the growth differences more prominent. B, wild-type and loc1Δ cell lysates were fractioned through a 7–47% sucrose gradient. Half-mers are indicated by arrows. C, cells carrying Rpl11-GFP (PKL228) were grown at 30 °C. The localization of Rpl11-GFP was visualized by fluorescence microscopy. DIC, differential interference contrast. The percentage of cells with nuclear Rpl11-GFP was counted and is shown here. D, ribosomal subunits were prepared under dissociation conditions and fractioned by sucrose gradient sedimentation. The levels of ribosomal subunits, quantified using ImageJ, were calculated as follows: relative amount of ribosomal subunits = (area of 40S (or 60S) peak of mutant)/(area of 40S (or 60S) peak of wild type); 60S to 40S ratio of individual strain = (area of 60S peak)/(area of 40S peak).

To test whether these nuclear diffused proteins are free or 60S-bound, we immunoprecipitated nascent 60S subunits using the trans-acting factors Ssf1, Brx1, Nug1, and Nog2. Although Ssf1 and Brx1 immunoprecipitated comparable amounts of Loc1 in wild-type and puf6Δ cells, Puf6 was enriched 1.5–2-fold in loc1Δ cells compared with the level in the wild type (Fig. 3D). Nug1 and Nog2 immunoprecipitated low levels of Loc1 in wild-type cells but had a 3–4-fold increased amount of Loc1 in puf6Δ (Fig. 3E, left panel). The accumulation of Puf6 increased ~1.3–1.7-fold in loc1Δ cells in Nug1- and Nog2-containing 60S subunits (Fig. 3E, right panel). These data demonstrate that Puf6 and Loc1 do not depend on each other for binding to pre-60S subunits but that deletion of either protein causes the other to accumulate on pre-60S subunits. The protein accumulation on intermediates may suggest that transitions between steps in biogenesis are slowed.

Rpl43 Interacts Directly with Puf6 and Loc1—We next asked whether direct interaction could be detected among these factors. Recombinant His₆-tagged Puf6 and GST-tagged Loc1 were overexpressed in Escherichia coli. Glutathione beads were first incubated with extracts containing GST or GST-Loc1 and then incubated with protein extracts containing His₆-tagged Puf6. Puf6 was pulled down with GST-Loc1 but not with GST alone (Fig. 4A, right panel, compare lanes 1 and 2) and the interaction was not disrupted by RNase treatment (Fig. 4A, right panel, compare lanes 2 and 3), suggesting direct physical interaction between Puf6 and Loc1. This result is consistent with the data published by Shahbadian et al. (21).

We then tested whether Rpl43 would interact directly with either Puf6 or Loc1. The interactions between GST-Rpl43 and His₆-tagged Puf6 or Loc1 were assessed in vitro. Surprisingly, both Puf6 and Loc1 co-eluted with GST-Rpl43 but not with GST alone (Fig. 4B, compare lanes 1 and 2 and lanes 2 and 4). The above data suggest a direct protein–protein interaction between Puf6 and Loc1, Loc1 and Rpl43, and Rpl43 and Puf6.
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Puf6 and Loc1 Are Required for Proper Loading of Rpl43—Rpl43 had a direct physical interaction with Puf6 and Loc1 in in vitro assays (Fig. 4B). Consequently, we wondered whether they coexisted on the same pre-60S particle in vivo. Puf6-myc and Loc1-myc were immunoprecipitated, and the presence of Rpl43 was detected by Western blotting. Rpl43 was present in both Puf6- and Loc1-containing nascent 60S subunits (Fig. 5A).

During the course of this work, we noticed that although the Rpl43 protein levels were comparable in wild-type and puf6Δ mutant cells, they decreased significantly in loc1Δ cells (Fig. 5B), suggesting that Loc1 may play a role in loading Rpl43. The association of Rpl43 with nascent 60S subunits was further examined in puf6Δ and loc1Δ strains. Pre-60S subunits were immunoprecipitated with Nog2-myc and Arx1-myc. Surpris-
ingly, the level of Rpl43 decreased dramatically in pre-60S subunits in \textit{loc1}/H9004 but not the level of Rpl23 (Fig. 5C, compare lanes 2 and 4 and lanes 5 and 7), which suggests that Loc1 is required to accommodate Rpl43.

In contrast, the level of Rpl43 on pre-60S subunits was slightly decreased in Nog2 containing pre-60S in \textit{puf6}/H9004 cells compared with the WT cells (Fig. 5C, compare lanes 2 and 3) but not in Arx1-containing subunits (Fig. 5C, compare lanes 5 and 6) or Nmd3-containing subunits (data not shown). Arx1 and Nmd3 are required for the efficient export of 60S subunits from the nucleus. These results suggest that the decrease of Rpl43 on pre-60S particles in \textit{puf6}/H9004 cells is more pronounced in the earlier nuclear steps but not in the later nuclear or cytoplasmic steps.

As Rpl43 levels were much lower in \textit{loc1}/H9004 cells, perhaps the decrease of Rpl43 levels accounted for the more severe defects in the growth and ribosomal levels of \textit{loc1}/H9004 versus \textit{puf6}/H9004 (Fig. 2). To address this possibility, we tested whether overexpression of \textit{RPL43} could suppress the growth defect of \textit{loc1}/H9004 (data not shown). We further tested the suppression in the \textit{puf6}/H9004 \textit{loc1}/H9004 double mutant, and again no suppression was detected (data not shown). This implies that the suppression of the growth defect of \textit{puf6}/H9004 by Rpl43 is Loc1-dependent.

Previous studies show that Puf6 and Loc1 are RNA-binding proteins (20, 36), indicating the possibility that Puf6 and Loc1 are required to maintain the stability of \textit{RPL43} mRNA. The levels of \textit{RPL43} transcripts from wild-type, \textit{puf6}/H9004, and \textit{loc1}/H9004 cells were checked with semiquantitative RT-PCR; however no significant differences in the mRNA levels were detected (data not shown). We also found that Puf6 and Loc1 did not interact with \textit{RPL43} mRNA (data not shown).

\textbf{Rpl43 Is Required for Release of Puf6}—Because Rpl43, Puf6, and Loc1 may interact independently of the 60S subunits, Rpl43 may serve as a binding site of Puf6 or Loc1 on the pre-60S particle. We expressed \textit{RPL43B} under the control of the inducible GAL promoter in an \textit{rpl43A}/\textit{rpl43B\Delta} strain to make a conditional GAL::\textit{RPL43} strain. Deletion of either \textit{RPL43A} or \textit{RPL43B} barely showed a phenotype (data not shown), but...
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FIGURE 5. Puf6 and Loc1 are required for proper loading of Rpl43.

We observed the cellular distribution of Puf6 and Loc1 under Rpl43 depletion. Most of the Puf6 and Loc1 was found in the nucleolus in wild-type and GAL::RPL43 cells in galactose medium (data not shown). After the addition of glucose to repress RPL43, Puf6 and Loc1 remained in the nucleolus in the wild-type cells but showed increased mislocalization to the nucleoplasm in the Rpl43-repressed strain (Fig. 6C).

We further analyzed how depletion of Rpl43 impacted the abundance of Puf6 and Loc1 on the 60S subunits. Pre-60S subunits were immunoprecipitated with various trans-acting factors marking different stages of assembly and export. Nog2 and Arx1 are localized predominantly in the nucleolus and nucleus, respectively. Under the wild-type situation, Puf6 and Loc1 were low on pre-60S subunits (Fig. 6D). After repression of Rpl43, Puf6 and Loc1 accumulated dramatically on the 60S subunits, but their protein levels remained unchanged (Fig. 6D, WCE). Thus, Puf6 and Loc1 do not require Rpl43 for binding to the pre-60S subunit and may be loaded independently of Rpl43.

Pre-60S subunits were also immunoprecipitated from Rpl43-depleted cells using tagged Puf6 and Loc1. Although the Rpl23 levels did not change in the Puf6- and Loc1-containing 60S subunits after depletion of Rpl43, Tif6 increased highly (Fig. 6E). This result suggests that Loc1 and Puf6 were retained on the pre-60S subunits, which also contain Tif6 when Rpl43 is depleted. Thus, Puf6 and Loc1 depend on Rpl43 for their proper release from nascent ribosomal subunits.

In conclusion, our results reveal that Puf6 and Loc1 are required for the stability and efficient loading of Rpl43 into the nascent 60S subunit. Furthermore, the assembly of Rpl43 is required for the subsequent release of Puf6 and Loc1.

Discussion

The Roles of Puf6 and Loc1 in 60S Biogenesis Are Interdependent—A physical interaction between Puf6 and Loc1 is required for their role in the asymmetric distribution and translational inhibition of ASH1 mRNA (20, 21, 36). Their co-involvement in 60S biogenesis (31, 32, 35) raises the question of whether Puf6 and Loc1 also function interdependently in the ribosome synthesis pathway. Here, we performed a functional study of the roles of Puf6 and Loc1 in 60S biogenesis. We showed that both proteins were present at the same stage of nascent 60S subunits. Puf6 and Loc1 do not depend on each other for 60S interaction but for accurate release (Fig. 7). Furthermore, we showed that Puf6 and Loc1 interact with Rpl43 and that efficient accommodation of Rpl43 into nascent 60S subunits depends on the Puf6 and Loc1 (Fig. 7).

Efficient Assembly of Rpl43 Depends on Puf6 and Loc1—Although Loc1 and Puf6 interacted directly with Rpl43, the protein levels of Rpl43 decreased in loc1Δ but not in puf6Δ. Many RNA-binding proteins are important for post-transcriptional regulation of the turnover and translation of associated mRNA (44–46). As Loc1 is a known RNA-binding protein, one possibility is that Loc1 alters the half-life or expression of RPL43 transcripts. However, we think this is an unlikely explanation for Loc1 function, because neither Loc1 nor Puf6 interacted with RPL43 mRNA. In addition, the level of RPL43 mRNA was not decreased in puf6Δ or loc1Δ cells. Finally, we expressed the open reading frame of RPL43 under control of the Nmd3 promoter, and a similar lower protein level of Rpl43 was observed in loc1Δ cells (unpublished data).

Puf6 and Loc1 interact directly with Rpl43, and both are required for the accommodation of Rpl43 into pre-60S subunits, supporting the idea that they may work like chaperones of Rpl43. Because ribosomal proteins have many positive charges, tend to denature easily, and must be transported to the nucleus, many unique chaperones have been reported to accommodate specific ribosomal proteins. Sqt1 is a chaperone for Rpl10 (ul16) (47, 48); Rbr1 protects Rpl3 (ul3) (49); Yar1 interacts with Rps3 (S3) and prevents Rps3 from aggregation (50, 51); Sso1 imports Rpl5 (ul18) and Rpl11 (ul5) concurrently and chaperones 5S RNP assembly (11, 12); Acl4 binds free Rpl4

repression of RPL43B in the double deletion mutant led to lethality (Fig. 6A) and loss of 60S subunits (Fig. 6B).
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(uL4), and they are imported to the nucleus together (52, 53); and Tsr2 works as an escortin of Rps26 (eS26) (54). Here, we reported that Puf6 and Loc1 work together to ensure the efficient accommodation of Rpl43 into the ribosome.

In a recent study, several chaperones of ribosomal proteins were shown to interact with the nascent polypeptide of their client proteins as they emerged from the exit tunnel of the ribosome (52, 55). We tested whether Puf6 and Loc1 could similarly be recruited to nascent Rpl43. Puf6-TAP- and Loc1-TAP-containing complexes were purified by IgG-Sepharose in the presence of cycloheximide to preserve mRNA on translating ribosomes. The interacting mRNA were assayed by real-time quantitative RT-PCR. However, we did not observe a significant enrichment of RPL43 mRNA with either Puf6 or Loc1 (data not shown). Therefore, we propose that Puf6 and Loc1 bind nascent Rpl43 after it has been translated.

FIGURE 6. Rpl43 is required for proper release of Puf6 and Loc1. A, wild-type and GAL::RPL43 (KLY628) cells were spotted on YPGal and YPD plates. B, wild-type and GAL::RPL43 cells were cultured in galactose-containing medium, and 2% glucose was added for 4 h. Cell extracts were prepared and separated through 7–47% sucrose density gradients. C, the localization of Puf6-GFP was monitored in wild-type (KLY135) and GAL::RPL43 (KLY829) cells, and the localization of Loc1-GFP (PKL337) was monitored in wild-type and GAL::RPL43 (KLY628) cells. Sik1-mRFP (PKL32) was used as nucleolar marker. Overnight culture was subcultured in galactose medium for 2 h, and 2% glucose was added and incubated for 4 h. The cells with GFP signals in the nucleoplasm are indicated by white arrows. Cells enlarged for greater detail are indicated by yellow arrowheads. D and E, Nog2-myc (PAJ1014), Arx1-myc (PAJ1026), Puf6-myc (PKL85), and Loc1-myc (PKL334) containing complexes were immunoprecipitated from wild-type and GAL::RPL43 cells and analyzed by Western blotting. WCE, whole cell extracts. vec, vector control; W, wildtype.
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Correct Puf6 Function in 60S Biogenesis Requires Loc1—In this study, an abnormal retention of Puf6 on 60S subunits was observed in loc1Δ cells. Although overexpression of Rpl43 could not rescue the growth defects of loc1Δ, deletion of Puf6 partially suppressed the growth defects of loc1Δ cells. In previous studies, similar genetic relationships have been observed. Re11 is a release factor of Arx1. Deletion of ARX1 does not cause significant growth defects. However, deletion of RE11 makes cells grow very slowly, and deletion of ARX1 relieves the growth defect of re11Δ cells (42). Mutations in the Tif6 release factors, Ef1 and Sdo1, also cause severe growth retardation, and mutant Tif6, having lower affinity for the ribosome, bypasses the requirement of Ef1 and Sdo1 and restores the growth rate (14, 56). The genetic and cellular evidence from this study suggests that abnormal retention of Puf6 is more deleterious than loss of Puf6 from the ribosome synthesis pathway. Moreover, although puf6Δloc1Δ shows a better growth rate than loc1Δ, the level of Rpl43 in puf6Δloc1Δ is comparable with that of loc1Δ (data not shown). This further supports the idea that Loc1 is required for the maintenance of Rpl43 levels.

Loading of Rpl43 Is Required for Proper Release of Puf6 and Loc1—In the absence of Rpl43, Puf6 and Loc1 accumulated on nascent 60S subunits, suggesting that the proper assembly of Rpl43 triggers the release of Puf6 and Loc1 downstream (Fig. 7). One possibility is that a complex constituted of Puf6, Loc1, and Rpl43 was loaded onto 60S subunits and the conformation change upon loading triggered the release of Puf6 and Loc1. Alternatively, another trans-acting factor may require Rpl43 to trigger the subsequent release of Puf6 and Loc1. Therefore, the accommodation of Rpl43 may directly or indirectly trigger the release of Puf6 and Loc1 downstream.

Materials and Methods

Strains, Plasmids, and Reagents—All S. cerevisiae strains used in this study are listed in Table 1. Unless otherwise indicated, all strains were grown at 30 °C in rich medium (yeast extract, peptone) or synthetic dropout medium containing 2% glucose. KLY628 (GAL::RPL43) was derived from transforming the PCR product containing rpl43BΔ::NAT (KLY561) to KLY219 (rpl43AΔ) with PKL350 (RPL43::HA-URA3). The cells with double mutants were selected with resistance to both G418 and CloNAT. PKL381 (GAL::RPL43B CEN HIS3) was transformed into the double mutant. The cells were selected on 5FOA-galactose plates. The GAL::RPL43 mutant strain was identified by lethality on YPD (yeast extract, peptone, glucose) plates. The plasmids used in this study are listed in Table 2.

Anti-Loc1, anti-Puf6, anti-Tif6, anti-Rpl23, and anti-Rpl8 antibodies were generated in our laboratory. Briefly, the individual gene was cloned into the pET28a vector and overexpressed in E. coli BL21(DE3). The purified proteins were injected into rabbit for antibody generation. Anti-Myc antibody was obtained from MYC 1–9E10.2 (9E10) (ATCC CRL1729™). Anti-Arx1 was obtained from the laboratory of Dr. Arlen Johnson. Anti-HA (MMS-101P), anti-TAP (CAB1001), and anti-GST (GST001M) were purchased from Covance, Thermo, and Bioman, respectively. Signals were detected using Clarity™ Western ECL substrate (Bio-Rad) and scanned by MultiGel-21 (Top Bio, Taipei, Taiwan). The signal intensity was measured by ImageJ for quantification.

High-copy Suppressor Screen—A high-copy number (2μ UR3) (gift from Dr. Phil Hieter) yeast genomic library was transformed into puf6Δ cells carrying the ΔPIF mutant (PKL55) on the plasmid, and colonies were screened for better growth at 20 °C. Compared with empty vector control, cells with better growth must contain PUF6 or genes that can suppress the growth defects of puf6Δ. The plasmids were isolated and checked with enzymatic digestion to eliminate PUF6. Those plasmids containing potential dosage-dependent suppressors were retransformed to the ΔPIF strain to eliminate false positives. The suppressors were sequenced and searched in the Saccharomyces genome database.

Polysome Profile Analysis—For sucrose density gradients, cultures were collected at an A600 of 0.2–0.3. Cycloheximide was added to a final concentration of 50 μg/ml, and the mixture was incubated continuously for another 15 min. Cells were poured onto ice and harvested by centrifugation. Extracts were prepared by vortexing (four times for 30 s with 1-min cooling intervals on ice) with glass beads in polysome lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl2, 6 mM β-mercaptoethanol, and 200 μg/ml cycloheximide). 10.5 A260 units of protein extracts were loaded onto linear 7–47% sucrose gradients in polysome lysis buffer. After 2.5 h of centrifugation at 284,000 × g in a P40ST rotor (Hitachi), gradient fractions were collected on a density gradient fractionator (BR-188, Brandel) continuously measuring absorbance at 254 nm. For detection of protein, fractions were precipitated with 10% trichloroacetic acid. Pellets were suspended in 50 μl of I × Laemml
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TABLE 1
Strains used in this study

| Strain        | Genotype          | Source       |
|---------------|-------------------|--------------|
| BY4741        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0              | This study   |
| KLY67         | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 puf6Δ::KanMX | This study   |
| KLY134        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PUFE-TAP::HIS3MX | This study   |
| KLY135        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PUFE-GFP::HIS3MX | This study   |
| KLY218        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 loc1Δ::KanMX | This study   |
| KLY219        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpl43Δ::KanMX | This study   |
| KLY12         | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 puf6Δ::KanMX loc1Δ::KanMX | This study   |
| KLY317        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ARX1-TAP::HIS3MX | This study   |
| KLY405        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 loc1Δ::KanMX PUFE-GFP::HIS3MX | This study   |
| KLY471        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BRX1-TAP::HIS3MX | This study   |
| KLY228        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BRX1-TAP::HIS3MX | This study   |
| KLY558        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BRX1-TAP::HIS3MX | This study   |
| KLY561        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpl43Δ::CloNAT | This study   |
| KLY583        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPL11B-GFP CEN LEU2 | This study   |
| KLY596        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPL43B-HA CEN HIS3 | This study   |
| KLY598        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPL43B-HA CEN HIS3 | This study   |
| KLY628        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpl43Δ::KanMX PUFE-GFP::HIS3MX | This study   |
| KLY674        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPL43B-HA CEN HIS3 | This study   |
| KLY818        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SSF1-TAP::HIS3MX | This study   |
| KLY820        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SSF1-TAP::HIS3MX | This study   |
| KLY829        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PUFE-GFP::HIS3MX rpl43Δ::CloNAT + GAL::RPL43B (Pkl381) | This study   |

Buffers

buffer, separated on SDS-PAGE, and detected by Western blotting.

For analyses of total 40S and 60S subunits, cells were collected without cycloheximide treatment and lysed in dissociation buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 1 mM DTT). The cell extracts were separated in sucrose density gradient as described above.

Fluorescence Microscopy—GFP fluorescence was visualized on a fluorescent microscope. Overnight cultures of wild-type, puf6Δ, loc1Δ, and puf6Δloc1Δ cells that had plasmid-expressed or genomically tagged GFP protein were diluted with fresh TEN100 buffer five times. Protein extracts containing GST or recombinant proteins. Whole cell extracts containing GST or tagged recombinant proteins. Whole cell containing GST or GST-tagged proteins were first incubated with 25 μl of glutathione beads for 1 h at 4°C and then washed with chilled TEN100 buffer five times. Protein extracts containing prey protein were incubated with glutathione beads for 1 h at 4°C subsequently. Beads were washed with buffer five times and eluted with 1× SDS sample buffer. Samples were separated on SDS-PAGE and stained with Coomassie Blue. The interaction proteins were further confirmed with anti-Puf6, anti-Loc1, and anti-GST antibodies by Western blotting. To further detect whether the potential interaction between these two proteins is a direct protein-protein interaction or an RNA-dependent interaction, 100 μg/ml RNase A was included at the incubation stage.

Immunoprecipitation— Cultures were grown to an A600 of ~0.5 in selective medium. For the preparation of protein extracts, cells were resuspended in immunoprecipitation buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 6 mM MgCl2, 10% glycerol, 1 mM PMSF, and 1 mM leupeptin), lysed by vortexing with glass beads, and clarified by centrifugation. α-MyC antibody (9E10) and protein A-agarose beads were added to the normalized samples and rocked at 4°C. The beads were washed three times with immunoprecipitation buffer and the proteins were eluted in 1× Laemmli sample buffer. Subsequently, proteins were separated on SDS-PAGE and observed by Western blotting.

Sedimentation through Sucrose Cushions— Cultures were grown to an A600 of 0.4–0.5. Protein extracts were prepared by vortexing with glass beads in extraction buffer (50 mM NaCl, 20 mM Tris, pH 7.5, 6 mM MgCl2, 10% glycerol, 0.1% Nonidet P-40, 1 mM PMSF, 1 μM leupeptin, and 1 μM pepstatin A). Normal-
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ized protein extracts were centrifuged at 385,900 × g in a rotor (MLA130, Beckman Coulter) at 4 °C for 60 min. The top layer (free protein) and the pellet (ribosome-containing pool) were recovered. Pellets were fully resuspended with the same volume of extraction buffer. The target proteins in free and ribosomal-bound pools were further immunoprecipitated.

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