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Nuclear Recycling of the Pre-60S Ribosomal Subunit-Associated Factor Arx1 Depends on Rei1 in *Saccharomyces cerevisiae*

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Arx1 and Rei1 are found on late pre-60S ribosomal particles containing the export adaptor Nmd3. Arx1 is related to methionine aminopeptidases (MetAPs), and Rei1 is a C_2H_2 zinc finger protein whose function in ribosome biogenesis has not been previously characterized. Arx1 and Rei1 localized predominately to the nucleus and cytoplasm, respectively, but could be coimmunoprecipitated, suggesting that they are transiently in the same 60S complex. *arx1*Δ mutants showed a modest accumulation of 60S subunits in the nucleus, suggesting that Arx1 enhances 60S export. Deletion of *REI1* led to cold sensitivity and redistribution of Arx1 to the cytoplasm, where it remained bound to free 60S subunits. However, deletion of *ARX1* or the fusion of enhanced GFP (eGFP) to Rpl25 suppressed the cold sensitivity of an *rei1*Δ mutant. The presence of eGFP on Rpl25 or its neighboring protein Rpl35 reduced the binding of Arx1 to 60S subunits, suggesting that Arx1 binds to 60S subunits in the vicinity of the exit tunnel. Mutations in Arx1 that disrupted its binding to 60S also suppressed an *rei1*Δ mutant and restored the normal nuclear localization of Arx1. These results indicate that the cold sensitivity of *rei1*Δ cells is due to the persistence of Arx1 on 60S subunits in the cytoplasm. Furthermore, these results suggest that Rei1 is needed for release of Arx1 from nascent 60S subunits after export to the cytoplasm but not for the subsequent nuclear import of Arx1.

In eukaryotic cells, ribosomes are assembled in the nucleolus and must be exported to the cytoplasm for their use in translation. Assembly of ribosomal subunits is a complex and energy-intensive process that requires more than 150 *trans*-acting factors (8, 13, 27, 49). In yeast cells, the two ribosomal subunits, the small (40S) and large (60S) subunits, are assembled from a large primary 35S rRNA transcribed by RNA polymerase I. Processing of this RNA during subunit assembly yields 18S, 25S, and 5.8S RNAs. The 5S RNA is transcribed separately by RNA polymerase III. Recent evidence from yeast suggests that the small subunit is assembled first and largely independently of the large subunit (6, 12). This is consistent with the 18S rRNA of the small subunit being encoded in the 5′ portion of the primary 35S transcript and thus being transcribed first. The subunits that are released from the nucleus are largely preassembled but not yet competent for translation, requiring further processing and maturation in the cytoplasm (8, 23, 49). Although the two subunits are exported independently of each other, both subunits depend on the export receptor Crm1 and RanGTP to facilitate translocation through the nuclear pore complex (24, 34, 47, 48).

Considering the large size of ribosomal subunits (>2.5 MDa), they may be close to the upper limit for the diameter of cargo that can be accommodated by the nuclear pore complex (NPC) (diameter of the central channel, ~35 nm [40]). Because of this, it may be important to minimize steric hindrance during export by preventing interactions of the subunit with other macromolecules. The large subunit has several sites for engaging other factors. These include the entire joining face that must bind to the small subunit, the tRNA binding sites, the GTPase center, and the exit tunnel, the aperture through which the nascent polypeptide emerges. Presently, little is known about packaging the subunit for export and whether specific factors "cap" these interaction sites during export. One potential example of such a packaging factor is Tif6, whose presence on the large subunit prevents joining with the 40S subunit (4, 42).

Although many *trans*-acting factors are required for subunit biogenesis in the nucleolus, most of these proteins appear to be retained in the nucleolus when subunits are released into the nucleoplasm for transport. During export, subunits contain only a few nonribosomal proteins. For the large subunit, these include the export adapter Nmd3 that provides the nuclear export signal (18), Tif6 (36), the uncharacterized protein Arx1 (encoded by YDR101c) (36), and Rlp24, related to Rpl24 found in mature subunits (41). As none of these proteins remains associated with subunits during translation, they are removed prior to subunit joining and must be recycled to the nucleus for subsequent rounds of export. For example, recycling the export adapter Nmd3 requires the essential G protein Lsg1, which may sense the correct assembly of Rpl10 into the subunit (17). In addition, some factors, such as Tif6, must be removed to activate the subunit for translation competence (4).

Here we provide evidence that Arx1, related to methionine aminopeptidases, binds to the large subunit in the vicinity of the exit tunnel. We show that Arx1 functionally interacts with the zinc finger protein Rei1 (encoded by YBR267w), a cytoplasmic 60S binding protein. Furthermore, we show that Rei1 is required for recycling Arx1 back to the nucleus, possibly by facilitating its release from nascent 60S subunits.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
W303	*MAT***a** *his3–11 leu2–3,112 trp1-1 ura3-1*	
AJY1395	*MAT*α *ade2 his3 leu2 tp1 ura3 rpl25*Δ*::HIS3 pASZ11-Rpl25-eGFP*	1
AJY1539	*MAT***a** *his3 leu2 ura3 CRM1T539C*	17
AJY1901	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 arx1*Δ*::KanMX4*	This study
AJY1902	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 rei1*Δ*::KanMX4*	This study
AJY1903	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 arx1*Δ*::KanMX4 rei1*Δ*::KanMX4*	This study
AJY1904	*MAT*α *his3 leu2 trp1 ura3 rei1*Δ*::KanMX4 rpl25*Δ*::HIS3* pAJ908	This study
AJY1905	*MAT*α *his3-11 leu2-3,112 trp1-1 ura3-1 ARX1-3HA::His3MX6*	This study
AJY1906	*MAT*α *his3 leu2 ura3 rpl25*Δ*::HIS3 ARX1–3HA::His3MX6* pAJ908	This study
AJY1907	*MAT*α *his3 leu2 ura3 rei1*Δ*::KanMX4 ARX1-3HA::His3Mx6*	This study
AJY1908	*MAT*α *his3 leu2 ura3 rei1*Δ*::KanMX4 rpl25*Δ*::HIS3 ARX1-3HA::His3MX6* pAJ908	This study
AJY1909	*MAT***a** *his3-11 leu2-3,112 trp1-1 ura3-1 ARX1-GFP::His3MX6*	This study
AJY1910	*MAT***a** *his3-11 leu2-3,112 trp1-1 ura3-1 REI1-13myc::TRP1*	This study
AJY1911	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0*	This study
AJY2125	*MAT***a** *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 Rpl35A-GFP::HIS3 RPL35B-GFP::HIS3*	This study
AJY2128	*MAT***a** *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 Rpl19A-GFP::HIS3 RPL19B-GFP::HIS3*	This study

MATERIALS AND METHODS

Strains, media, and growth conditions. The strains used in this study are listed in Table 1. Rich medium (yeast extract-peptone-glucose) and dropout medium containing 2% glucose as the carbon source were as described previously (25).

Strains. Strains AJY1901 (*arx1*Δ) and AJY1902 (*rei1*Δ) were haploid spore clones obtained by sporulating heterozygous deletion mutants (Research Genetics). AJY1903 (*arx1*Δ *rei1*Δ) was obtained by sporulating the diploid made by crossing AJY1901 and AJY1902. Strains with genomic *ARX1* tagged with three tandem copies of the hemagglutinin epitope (HA) or with green fluorescent protein (GFP) (AJY1905 and AJY1909, respectively) were made by homologous recombination (31) in the wild-type strain W303. Strain AJY1904 (*rei1*Δ *rpl25*Δ pAJ908 [Rpl25-eGFP]) was obtained from a cross of AJY1395 (*rpl25*Δ pAJ908 [Rpl25-eGFP]) and AJY1902 (*rei1*Δ). AJY1904 was then crossed to Arx1-HA-expressing strain AJY1905 to make AJY1906 (*ARX1-HA rpl25*Δ pAJ908 [Rpl25-eGFP]), AJY1907 (*ARX1-HA rei1*Δ), and AJY1908 (*ARX1-HA rei1*Δ *rpl25*Δ pAJ908 [Rpl25-eGFP]). The 13myc genomically tagged *REI1* strain (AJY1910) was made similarly in W303. Strain AJY2125 (*RPL35A-GFP RPL35B-GFP*) was obtained from a cross of genomic GFP-tagged strains *RPL35A-GFP* and *RPL35B-GFP* (Research Genetics) (20). Strain AJY2128 (*RPL19A-GFP RPL19 B-GFP*) was made similarly from a cross of genomic tagged strains *RPL19A-GFP* and *RPL19B-GFP*.

Plasmids. Plasmids used in this study are listed in Table 2. pAJ1017 (*REI1-GFP*) and pAJ1018 (*REI1-13myc*) were made by PCR amplification of *REI1* from wild-type yeast genomic DNA with the primers AJO567 (5′-CTGAAGCT TCGCCCGCATTATTACCACGGCGATAT) and AJO568 (5′-GCGCCCGGG CTTAATTAACTGCAGAAGTTGGTCTCT). The PCR product was digested with EagI and PacI and ligated into the same sites of pAJ755 (*NMD3-GFP*) and pAJ901 (*LSG1-13myc*). pAJ1016 was made by PCR amplification of *ARX1* from wild-type yeast genomic DNA with the primers AJO563 (5′-CTGGGTACCCG GCCGTCATGCCTCTGTGAAGCT) and AJO564 (5′-GCGCCCGGGCTTAA TTAACATTTTCATGGTTTCTTCAACTC). pAJ1026 (*ARX1-13myc LEU2*) was made by EagI and PacI digestion of pAJ1016 (*ARX1-13myc URA3*). The *ARX1* B6 mutant (pAJ1463) was obtained by PCR mutagenesis. pAJ1015 plasmid was used as template for PCR using *Taq* and primers AJO319 (5′-GCGC CATGGATTTGTATAGTTCATCCAT) and AJO563 (5′-CTGGGTACCCGG CCGTCATGCCTCTGTGAAGCT). The PCR product was cotransformed with BclI- and PacI-digested pAJ1015 into AJY1903 (*arx1*Δ *rei1*Δ). Cells were plated onto Ura$^-$ dropout plates, and the screen was performed at room temperature; *arx1* mutants were identified by their suppression of the cold sensitivity of *rei1*Δ.

TABLE 2. Plasmids used in this study

Plasmid	Relevant marker	Reference or source
pAJ538	*LEU2 CEN NMD3-13myc*	18
pAJ543	*LEU2 CEN GAL10::NMD3*	18
pAJ545	*LEU2 CEN GAL10::nmd3*Δ*100*	18
pAJ903	*LEU2 CEN LSG1-13myc*	26
pAJ908	*URA3 CEN RPL25-eGFP*	26
pAJ1015	*URA3 CEN ARX1-GFP*	This study
pAJ1016	*URA3 CEN ARX1-13myc*	This study
pAJ1017	*URA3 CEN REI1-GFP*	This study
pAJ1018	*URA3 CEN REI1-13myc*	This study
pAJ1026	*LEU2 CEN ARX1-13myc*	This study
pAJ1463	*URA3 CEN ARX1*(*B6*)*-GFP*	This study
pAJ1464	*URA3 CEN ARX1*(*B6*)*-13myc*	This study

Microscopy. Overnight cultures were diluted into fresh medium to an optical density at 600 nm of 0.1 to ~0.2 and cultured at room temperature or 30°C to mid-log phase. Cultures were fixed with formaldehyde (3.7% final concentration) for 40 min at room temperature or 30°C, washed three times in cold 0.1 M potassium phosphate buffer, pH 6.6, and resuspended in 0.1 M potassium phosphate, pH 6.6, 1.2 M sorbitol. For 4′,6′-diamidino-2-phenylindole (DAPI) staining, Triton X-100 was added to fixed cells to a final concentration of 0.1% for 5 min, and DAPI was added subsequently to a final concentration of 1 μg/ml for 1 min. Cells were then washed three times with cold phosphate-buffered saline (PBS) and resuspended in PBS with 0.02% NaN_3. Fluorescence was visualized on a Nikon Eclipse E800 microscope fitted with a 100× objective and a SPOT cooled color digital camera controlled with the SPOT software package (version 1.0.02). Captured images were prepared using Adobe Photoshop 5.0. Indirect immunofluorescence was performed as described previously (18). Antibodies were the following: anti-HA, HA.11 (Covance) at 1:500 dilution; anti-myc, 9e10 (Covance) at 1:1,000 dilution; and Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch Labortories, Inc.) at 1:300 dilution.

Other methods. Leptomycin B (LMB) experiments and polysome analysis on sucrose gradients were performed as described previously (26). Briefly, extracts for polysome analysis were prepared in extract buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 30 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 50 μg/ml cycloheximide, 200 μg/ml heparin) and loaded onto 7% to 47% gradients (50 mM Tris-acetate, pH 7.0, 50 mM NH_4Cl, 12 mM $MgCl_2$, 1 mM DTT, 50 μg/ml cycloheximide). For Western blotting, proteins from sucrose gradient fractions were precipitated with trichloroacetic acid and separated by electrophoresis on 8% sodium dodecyl sulfate (SDS) polyacrylamide gels.

RESULTS

Arx1 and Rei1 are associated with the free 60S subunits. Late pre-60S particles that are copurified with the export adaptor Nmd3 contain several additional *trans*-acting factors (11, 26). Here we have focused on two of these proteins: Arx1 (encoded by YDR101c) and Rei1 (encoded by YBR267w). Sequence alignment revealed that Arx1 is related to methionine aminopeptidases (MetAP) (Fig. 1), a class of proteins that are required for processing the N terminus of many cellular proteins (19, 30). However, several of the residues essential for catalytic activity in this family of peptidases are not conserved

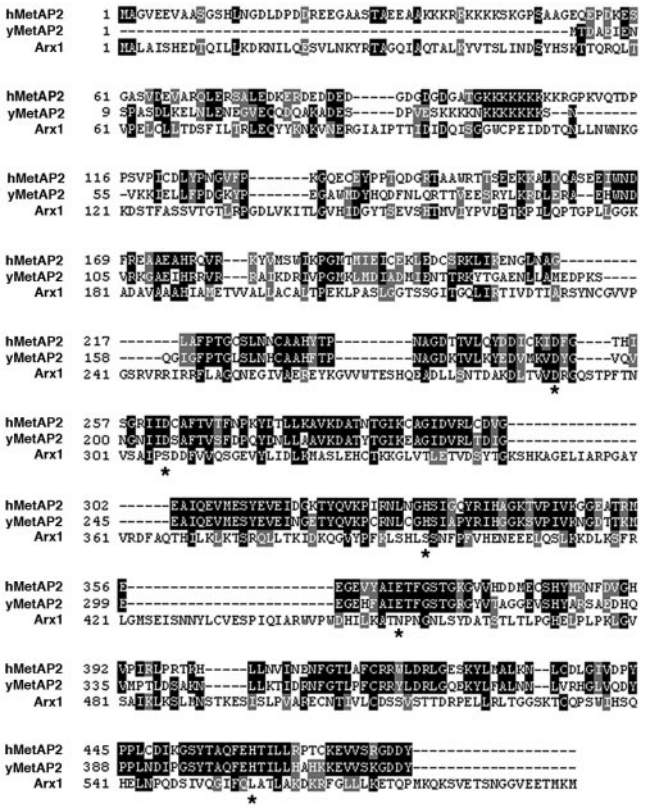

FIG. 1. Sequence alignment of Arx1 and MetAP2. Sequences of Arx1, yeast MetAP2 (yMetAP2), and human MetAP2 (hMetAP2) were aligned using ClustalW1.8 (http://serachlauncher.bcm.tmc.edu/). Identical residues in at least two sequences are shaded in black, and similar residues are shaded in gray. Residues responsible for MetAP2 enzymatic activity are indicated by asterisks. The insertions within the Arx1 sequence are indicative of the Arx1 protein family. Based on threading the Arx1 sequence onto the structure of MetAP, the insertions are predicted to lie predominately on one face of the protein.

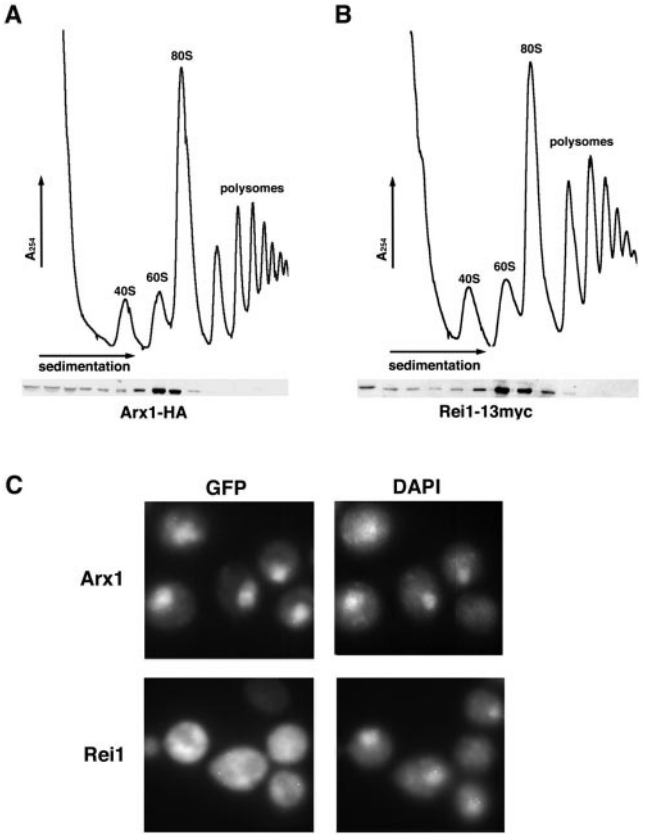

FIG. 2. Sucrose gradient sedimentation and cellular localization of Arx1 and Rei1. (A and B) Lysates were prepared in the presence of cycloheximide (50 μg/ml) from room-temperature cultures of strains AJY1905 (*ARX1-3HA*) (A) and AJY1910 (*REI1-13myc*) (B) and fractioned on 7% to 47% sucrose gradients by ultracentrifugation. Buffer conditions were as described in Materials and Methods, except that extracts for AJY1910 (*REI1-13myc*) were prepared in a low-ionic-strength buffer (20 mM HEPES, 10 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 50 μg/ml cycloheximide), because Rei1-60S association is salt sensitive. Fractions were collected, and the absorbance at 254 nm was monitored continuously. Proteins were precipitated with trichloroacetic acid, separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and immunoblotted for HA (Covance) or myc (Covance) for visualization of Arx1 or Rei1, respectively. (C) Cultures of AJY1909 (*ARX1-GFP*) and AJY1902 (*rei1*Δ) with pAJ1017 (*REI1-GFP*) were grown to mid-log phase at room temperature, and the in vivo localizations of Arx1-GFP and Rei1-GFP were monitored by fluorescence microscopy. DAPI staining was used to identify the position of nuclei.

in Arx1, suggesting that it is not an active peptidase (Fig. 1). Arx1 is also related to the human protein Ebp1, a member of the proliferation-associated PA2G4 family (52) that has recently been shown to be associated with nucleolar pre-60S particles in HeLa cells (46). Rei1 is a C_2H_2 zinc finger protein that was reported to be involved in the mitotic signaling network (22) and whose function in ribosome metabolism has not been analyzed previously.

To further examine the cellular localization and association of these two proteins with 60S subunits, we epitope tagged Arx1 and Rei1. Both tagged proteins were functional and were able to coimmunoprecipitate 60S subunits (data not shown). The association of Arx1 and Rei1 with ribosomes was further examined by sucrose gradient sedimentation. Both proteins cosedimented specifically with free 60S subunits (Fig. 2A and B), consistent with previously published results for Arx1 (36) and with the identification of Rei1 in late pre-60S particles (8). The Western signal trailing from free 60S to the top of the gradient (Fig. 2A and B) probably reflects partial dissociation of the proteins from the subunits during sucrose gradient sedimentation.

Nmd3 is a shuttling protein, and 60S complexes that are coimmunoprecipitated with Nmd3 represent a mixture of nuclear and cytoplasmic particles. Consequently, proteins associated with Nmd3-bound 60S subunits could be derived from the cytoplasm, the nucleus, or from both compartments. Genomically expressed Arx1-GFP localized primarily to the nucleus or nucleolus (Fig. 2C), consistent with previous results from Nissan et al. (36) and results from a recent genome-wide analysis of protein localization (20). The faint cytoplasmic signal is consistent with the idea that Arx1 shuttles. Similar results were obtained with HA-tagged Arx1 (data not shown). In contrast, Rei1-GFP was predominantly cytoplasmic (Fig. 2C).

The steady-state cytoplasmic localization of Rei1 does not address whether or not the protein shuttles. Export of nascent

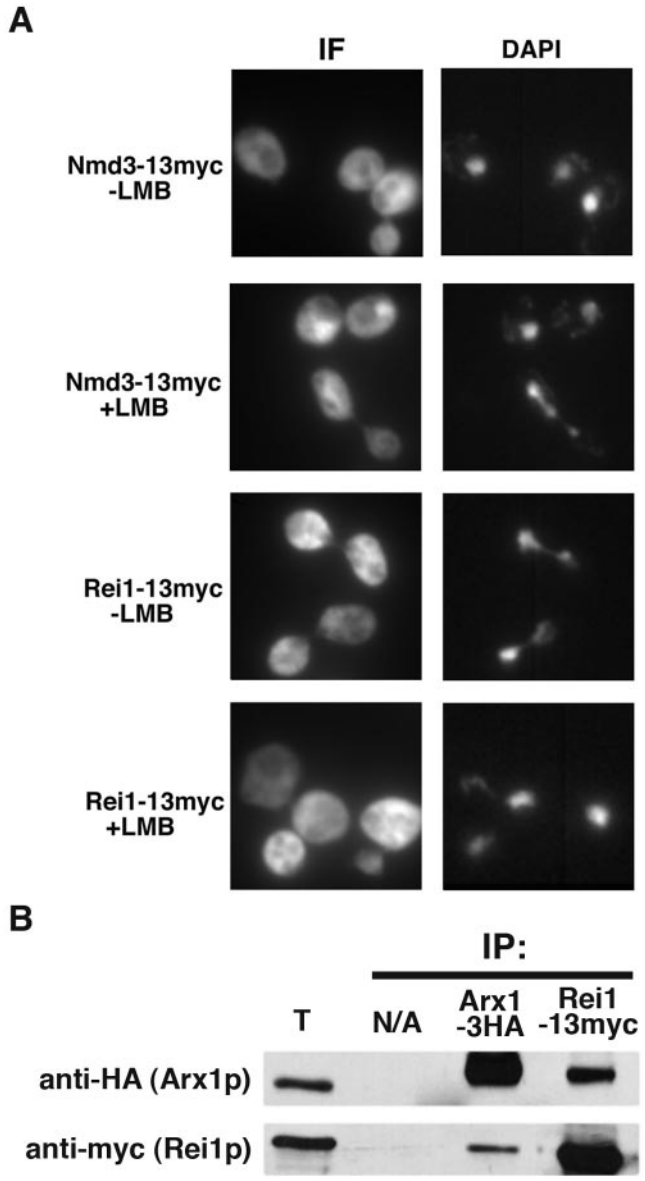

FIG. 3. Rei1 does not shuttle in a Crm1-dependent fashion but can be found in the Arx1 particle. (A) The LMB-sensitive strain AJY1539 (*CRM1T539C*) containing plasmid pAJ538 (*Nmd3-13myc*) or pAJ1018 (*REI1-13myc*) was cultured in selective media at room temperature. Cells were concentrated 20-fold in fresh media, LMB was added to a final concentration of 0.1 μg/ml, and cultures were incubated for 30 min. Cells were then fixed with 3.7% formaldehyde (final concentration) and subjected to indirect immunofluorescence microscopy (IF). (B) Extract from strain AJY1907 (*rei1*Δ *Arx1-HA*) with pAJ1018 (*REI1-13myc*) was incubated without addition of antibody (N/A) or with anti-HA or anti-myc antibody and protein A beads. Precipitated proteins were eluted from protein A beads in sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Total protein extract (T) was included as a loading control for the immunoprecipitations (IP). Western blotting was performed against the HA or c-*myc* epitopes.

60S subunits requires the adapter protein Nmd3 to provide the nuclear export signal that is recognized by the export receptor Crm1 (18). If Rei1 shuttles and binds to nascent subunits in the nucleus, its export would also be expected to be Crm1 dependent and, consequently, sensitive to leptomycin B (LMB) (28, 35). For this, we used myc-tagged Rei1, as we have observed that GFP can lend a nuclear bias in protein localizations (50). Functional myc-tagged Rei1 did not relocalize to the nucleus in the presence of LMB (Fig. 3A). As a positive control for Crm1-dependent shuttling, Nmd3-13myc was retained in the nucleus after addition of LMB, as we have shown previously (18). Rei1 also was not retained in the nucleus in the presence of a dominant-negative Nmd3 lacking its nuclear export sequence that blocks 60S export (data not shown). Thus, if Rei1 shuttles, its export is not dependent on Crm1 or export of the 60S subunit.

Arx1 has been reported to be a nucleolar protein that associates with relatively mature rRNA species (25S, 7S, 5.8S, and 5S) and may be involved in nuclear export of 60S subunits (36). Rei1 likely associates with 60S subunits only when they reach the cytoplasm. The sequential loading of Arx1 and Rei1 onto 60S ribosomes in the nucleus and cytoplasm, respectively, suggests that they identify different subpopulations of 60S subunits but does not preclude the possibility that they may transiently reside on the same 60S species. To test this idea, we performed immunoprecipitation experiments with Arx1 and Rei1. As shown in Fig. 3B, Arx1-HA could be coimmunoprecipitated with Rei1-13myc and vice versa, suggesting that after the Arx1-containing particle is exported to the cytoplasm, Rei1 binds and is present briefly before Arx1 is released.

Deletion of *REI1* leads to a cold-sensitive defect in 60S subunit levels. The association of Arx1 and Rei1 with 60S subunits raised the possibility that they are involved in 60S subunit biogenesis or export. Consequently, we analyzed polysome profiles by sucrose gradient sedimentation of extracts from strains deleted of *ARX1* or *REI1*, since the deletion mutants are viable. *arx1*Δ mutants showed a slight reduction in the free 60S peak and the appearance of half-mers (mRNAs containing a 40S subunit not joined with 60S), indicating a modest 60S biogenesis defect (Fig. 4B). This modest defect was consistent with the mild slow-growth phenotype of *arx1*Δ cells (see below). On the other hand, *rei1*Δ mutants displayed a more dramatic cold-sensitive defect in 60S levels (Fig. 4C), reflecting their cold-sensitive growth phenotype (see below). At room temperature (25°C), *rei1*Δ displayed a low free 60S peak relative to the high free 40S peak and severely reduced polysomes and half-mers (25°C) (Fig. 4C). The low 60S level and half-mer defects were almost completely suppressed at a higher temperature (37°C) (Fig. 4C), corresponding with the increased growth rate of *rei1*Δ cells at a higher temperature (see below).

We also monitored 60S subunit export using Rpl25-enhanced GFP (eGFP) as a reporter. A modest nuclear retention of Rpl25-eGFP was observed in *arx1*Δ strains at 25°C (Fig. 4B). Because Arx1 rides out of the nucleus on the pre-60S particle, the nuclear export defect of *arx1*Δ cells likely reflects a direct role of Arx1 in enhancing 60S export. *rei1*Δ mutants also showed a defect in 60S export (Fig. 4C), corresponding with the cold-sensitive defect in 60S levels observed by sucrose gradient sedimentation (Fig. 4C). Rpl25-eGFP showed a normal cytoplasmic distribution in *arx1*Δ and in *rei1*Δ cells at 37°C (Fig. 4C and data not shown), indicating that the export block was relieved at higher temperatures. Considering the interactions between Rpl25-eGFP, Arx1, and Rei1 (see below), we also monitored 60S export with Rpl8-YFP. Results with this

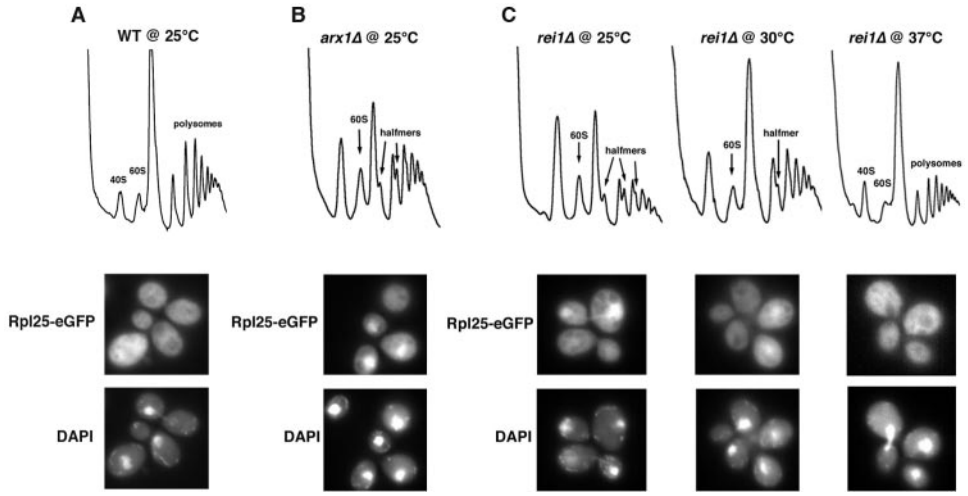

FIG. 4. Deletion of *ARX1* or *REI1* affects 60S subunit levels and export. Extracts, prepared from mid-log-phase cultures of strain AJY1911 (wild type [WT]) grown at 25°C (A), AJY1901 (*arx1*Δ) grown at room temperature (B), and strain AJY1902 (*rei1*Δ) grown at the temperatures indicated (C) were fractionated by sucrose gradient sedimentation as described in the legend to Fig. 2. Polysome profiles of the wild type grown at 30°C or 37°C were virtually indistinguishable from that shown in panel A (data not shown). Culture of AJY1911 (WT), AJY1901 (*arx1*Δ), or AJY1902 (*rei1*Δ) carrying plasmid pAJ908 (*RPL25-eGFP*) was incubated at the indicated temperature, and the in vivo localization of Rpl25-eGFP was monitored by fluorescence microscopy.

reporter were indistinguishable from those with Rpl25-eGFP (data not shown). We note that the ribosome export defects are similar in both *arx1*Δ and *rei1*Δ mutants at low temperature. In addition, the export defect in an *arx1*Δ *rei1*Δ double mutant is also similar to that of the single mutants (data not shown). Since Rei1 is a cytoplasmic protein, its effect on 60S export is likely indirect and may be due to the failure in recycling Arx1 (see below).

Genetic interactions among *REI1*, *ARX1*, and *RPL25-eGFP*. To test for possible functional interaction between Arx1 and Rei1, we asked if they displayed genetic interaction. Deletion of *ARX1* showed only a mild growth defect on rich media at room temperature (25°C) and no obvious defect at 30°C and 37°C (Fig. 5A, *arx1*Δ). However, deletion of *REI1* led to a severe slow-growth phenotype (cold sensitivity) at room temperature (25°C) but only a modest growth phenotype at 30°C, with no obvious defect at higher temperatures (Fig. 5A, *rei1*Δ). Interestingly, deletion of *ARX1* largely suppressed the cold sensitivity of *rei1*Δ (Fig. 5A, *arx1*Δ *rei1*Δ), indicating strong genetic interaction between *ARX1* and *REI1*. In addition, this genetic interaction suggests that the defect in an *rei1*Δ mutant is caused, at least in part, by the presence of Arx1. More surprisingly, the cold-sensitive phenotype of *rei1*Δ was also suppressed by the introduction of a plasmid expressing Rpl25 with a C-terminal fusion to eGFP (27.6 kDa) (Fig. 5A, *rei1*Δ + Rpl25-eGFP). The level of suppression was greater when the Rpl25-eGFP fusion was the sole source of Rpl25 in the cell (Fig. 5A, *rei1*Δ *rpl25*Δ + Rpl25-eGFP). The suppression of *rei1*Δ was specific to C-terminally tagged Rpl25 (untagged Rpl25 or Rpl8-YFP did not suppress *rei1*Δ; data not shown). We also observed suppression by Rpl25 containing 13 tandem copies of the c-*myc* epitope (20.5 kDa) but not by the smaller fusion of three tandem HA epitopes (4.8 kDa) (data not shown). These data suggested that the suppression of *rei1*Δ is specific to large fusions to the carboxyl terminus of Rpl25 that likely sterically block the association of other factors. Rpl25 is an integral ribosomal protein located near the polypeptide exit tunnel (3). Because expression of Rpl25-eGFP had an effect similar to that of deletion of *ARX1*, we reasoned that eGFP

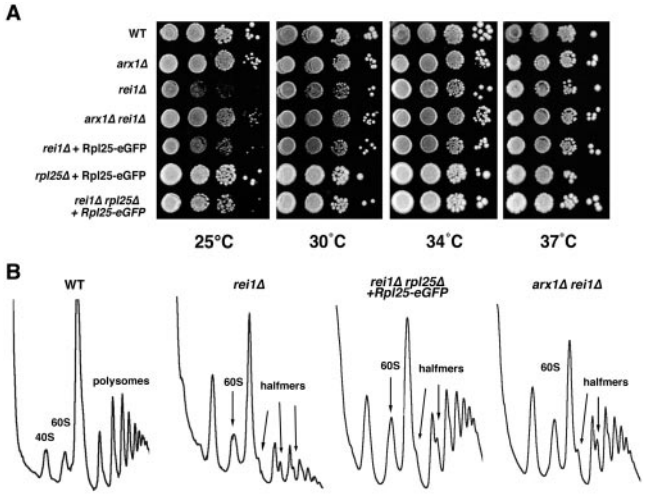

FIG. 5. Deletion of *ARX1* or the fusion of *eGFP* to *RPL25* suppresses the cold sensitivity and 60S subunit deficiency of *rei1*Δ mutant cells. (A) Tenfold serial dilutions of saturated cultures were spotted onto yeast extract-peptone-glucose plates and incubated for 3 days at the indicated temperatures. The strains used were the following: AJY1905 (wild-type [WT] *ARX1-HA*), AJY1901 (*arx1*Δ), AJY1907 (*rei1*Δ *ARX1-HA*), AJY1903 (*arx1*Δ *rei1*Δ), AJY1907 (*rei1*Δ *ARX1-HA*) carrying plasmid pAJ908 (*RPL25-eGFP*), AJY1906 (*rpl25*Δ *ARX1-HA* pAJ908), and AJY1908 (*rei1*Δ *rpl25*Δ *ARX1-HA* pAJ908). (B) Extracts were prepared from mid-log-phase cultures of strains AJY1911 (WT), AJY1907 (*rei1*Δ), AJY1903 (*arx1*Δ *rei1*Δ), and AJY1908 (*rei1*Δ *rpl25*Δ Rpl25-eGFP) at room temperature (25°C) and fractionated by sucrose gradient sedimentation as described in the legend to Fig. 2.

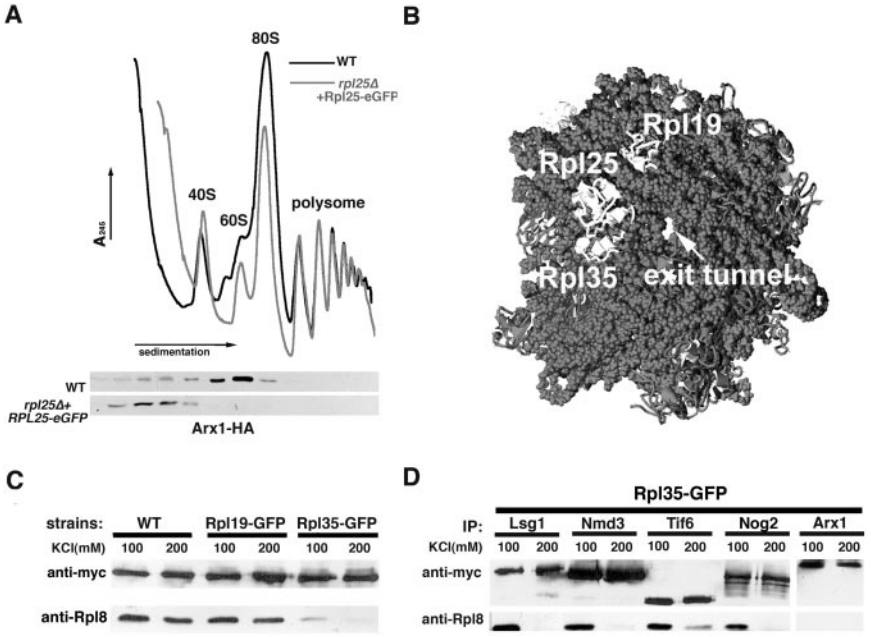

FIG. 6. Rpl25-eGFP and Rpl35A-GFP and Rpl35B-GFP alter Arx1-60S subunit association. (A) Lysates were prepared in the presence of cycloheximide from room-temperature cultures of strain AJY1905 (*ARX1-HA*) and AJY1906 (*rpl25Δ ARX1-HA Rpl25-eGFP*) and fractioned on sucrose gradients as described in the legend to Fig. 2. Fractions were collected, and proteins were precipitated with trichloroacetic acid, separated on SDS-polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membrane, and immunoblotted for HA. (B) Cartoon of ribosomal proteins surrounding the exit tunnel (adapted from reference 45). (C) Cell extracts from strains AJY2128 (Rpl19A-GFP Rpl19B-GFP), AJY2125 (Rpl35A-GFP Rpl35B-GFP), and an isogenic wild type carrying Arx1-13myc (pAJ1016) plasmids were prepared and incubated with anti-myc antibodies and protein A beads. Precipitated proteins were eluted from protein A beads in sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Western blotting was performed against myc or Rpl8. (D) Cell extracts were prepared from Rpl35A-GFP Rpl35B-GFP strain AJY2125 expressing c-*myc* tagged Lsg1 (pAJ901), Nmd3 (pAJ1001), Tif6 (pAJ1010), Nog2 (pAJ1014), and Arx1 (pAJ1026). The tagged proteins were immunoprecipitated, and their association with 60S subunits was monitored by blotting for Rpl8. IP, immunoprecipitation.

sterically blocks Arx1 binding to 60S subunits, suggesting that Arx1 binds in the vicinity of Rpl25.

We next asked if deletion of *ARX1* or the presence of Rpl25-eGFP restored 60S subunit levels in *rei1*Δ cells. As seen in Fig. 5B, these conditions partially restored the 60S levels of *rei1*Δ mutants at room temperature (25°C). In fact, the polysome profile of the *arx1*Δ *rei1*Δ double deletion mutant was virtually indistinguishable from that of the single *arx1*Δ deletion mutant (compare Fig. 5B to 4B). Although the growth rate of the *arx1*Δ *rei1*Δ strain was somewhat less than that of the *arx1*Δ strain, the defect observed by polysome profile analysis of an *rei1*Δ strain appears to be attributed almost entirely to the presence of wild-type *ARX1*.

Arx1 binding to 60S subunits is altered by the addition of eGFP to Rpl25. Arx1 is related to methionine aminopeptidases that work cotranslationally to remove methionine from newly synthesized polypeptides. Although the binding site of methionine aminopeptidases on the ribosome is not known, it is likely that they bind near the exit tunnel of the large subunit to act on nascent peptides as they emerge. By extension, Arx1 may utilize a similar binding site. Rpl25 is located close to the exit tunnel and is significant for its role as part of the docking site for signal recognition particles (SRP) (15, 38) as well as the translocon in the endoplasmic reticulum membrane (3). To ask if Rpl25-eGFP sterically blocks Arx1 association, we looked at Arx1 sedimentation in the presence of Rpl25-eGFP. Arx1 normally cosediments with free 60S subunits in sucrose gradients. However, in the presence of Rpl25-eGFP, Arx1 was largely absent from the free 60S peak and was present at the top of the gradient, indicating loss of binding to the 60S subunits (Fig. 6A). These sucrose gradients were carried out under relatively high ionic strength, which we found enhanced the difference in Arx1 binding. At low ionic strength, Arx1 cosedimented with 60S subunits, indicating that Rpl25-eGFP alters but does not completely block Arx1 binding (data not shown).

Several other ribosomal proteins are present around the exit tunnel. Among these, the two closest neighbors of Rpl25 are Rpl35 and Rpl19 (44) (Fig. 6B). We tested if GFP fusions to these proteins also affected Arx1 binding to the large subunit. Rpl19 and Rpl35 are each expressed from two loci (*RPL19A* and *RPL19B* as well as *RPL35A* and *RPL35B*, respectively). Consequently, we expressed epitope-tagged Arx1 in cells in which the genomic copy of both *RPL19A* and *RPL19B* or *RPL35A* and *RPL35B* were tagged with GFP (20). Cells expressing GFP-tagged Rpl19 grew at wild-type rates. In contrast, the GFP fusion to Rpl35 caused a severe slow-growth defect and low 60S subunit levels (data not shown), suggesting that this fusion affected assembly or stability of the subunit. Because the Rpl35A-GFP Rpl35B-GFP signal was cytoplasmic with no obvious nuclear accumulation (data not shown), the fusion protein may fail to be imported, or it may cause instability of 60S subunits in the cytoplasm.

To test the effect of the GFP fusions on Arx1's association

with the subunits, we immunoprecipitated Arx1 and assayed for coimmunoprecipitation of subunits by monitoring Rpl8. As shown in Fig. 6C, Arx1-13myc was able to coimmunoprecipitate similar levels of Rpl8 from either wild-type or Rpl19A-GFP Rpl19B-GFP strains. However, in the presence of Rpl35A-GFP Rpl35B-GFP, we observed a significantly reduced level of Rpl8. These results support the idea that Arx1 binds in the vicinity of Rpl25 and Rpl35 at the exit tunnel. Considering that the Rpl35A-GFP Rpl35B-GFP strain had a severe growth defect, we were concerned that the lack of 60S binding in the Rpl35A-GFP Rpl35B-GFP strain could be an artifact due to low 60S levels. To address this, we tested a panel of other *trans*-acting factors for their association with 60S subunits. These included Lsg1, Nmd3, Tif6, and Nog2, which localize to different cellular compartments and are required at different stages of 60S biogenesis (2, 8, 18, 26, 33, 43). We observed that all of these factors were able to coimmunoprecipitate Rpl35A-GFP Rpl35B-GFP subunits with similar efficiencies (Fig. 6D). Thus, the reduced binding of Arx1 was not an artifact of low subunit levels and likely reflects a specific loss of interaction caused by GFP. Due to the severe growth defect of the Rpl35A-GFP Rpl35B-GFP strain, we were not able to test if fusion of GFP to Rpl35 would suppress the cold sensitivity of an *rei1*Δ strain, as we observed for Rpl25-eGFP.

Arx1 remains associated with 60S subunits in the cytoplasm in the *rei1*Δ strain. As Arx1-containing particles enter the cytoplasm, Rei1 binds and Arx1 must be released shortly thereafter, as it is predominantly nuclear in wild-type cells. The genetic interaction between *ARX1* and *REI1* indicates that Arx1 is detrimental in the absence of Rei1. One possibility is that Rei1 is needed to release Arx1 from subunits in the cytoplasm. To investigate this, we examined the cellular localization of Arx1 in *rei1*Δ cells. As noted above, in wild-type cells Arx1-HA was concentrated in the nucleus but was also evident in the cytoplasm (Fig. 7A). However, in an *rei1*Δ strain, the bulk of Arx1-HA was cytoplasmic (Fig. 7A). Thus, the normal nuclear localization of Arx1 depends on Rei1. We then asked if Arx1 in an *rei1*Δ strain remained bound to 60S subunits. Indeed, Arx1 cosedimentated with 60S subunits on sucrose gradients (Fig. 7B). These results suggest that in the absence of Rei1, Arx1 persists on cytoplasmic 60S subunits and fails to recycle efficiently to the nucleus.

Considering that Arx1 accumulates on cytoplasmic subunits in an *rei1*Δ mutant and deletion of *ARX1* suppresses the growth defect of an *rei1*Δ mutant, we suggest that the retention of Arx1 on 60S subunits is the cause of the growth defect of *rei1*Δ mutant cells. To test this hypothesis, we mutagenized *ARX1* and identified a mutant (referred to as B6) that suppressed the growth defect of an *rei1*Δ mutant (Fig. 8A). Whereas wild-type Arx1-GFP was found throughout cells in an *rei1*Δ mutant, the B6 mutant was predominantly nuclear (Fig. 8B). We then assayed the B6 mutant for 60S binding by coimmunoprecipitation. As shown in Fig. 8C, the B6 mutant showed no detectable binding to 60S subunits compared to wild-type Arx1. Sequencing B6 revealed multiple mutations throughout the protein, precluding identification of any single residue or domain responsible for binding. Nevertheless, these results show that loss of Arx1 binding to 60S subunits suppresses the growth defect of *rei1*Δ cells by preventing the ac-

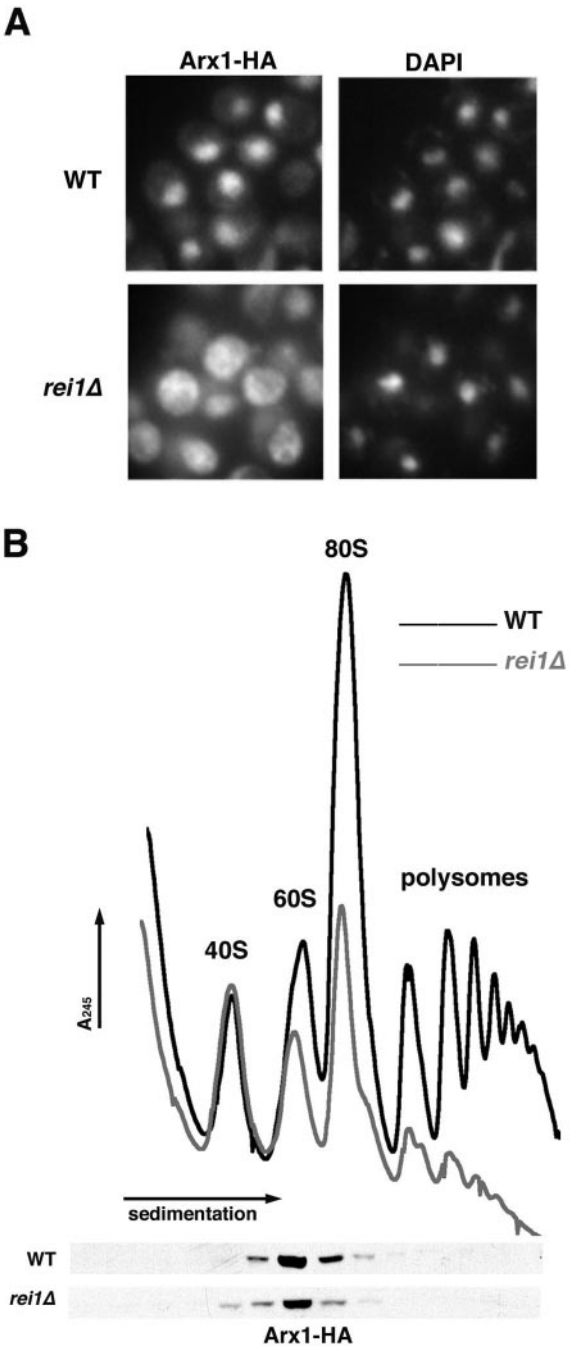

FIG. 7. Cellular localization and sucrose gradient sedimentation of Arx1 in an *rei1*Δ strain. (A) Cultures of strains AJY1905 (*ARX1-HA*) and AJY1907 (*rei1*Δ *ARX1-HA*) were grown to mid-log phase at room temperature, and the localization of Arx1-HA was monitored by indirect immunofluorescence microscopy as described in Materials and Methods. Cells were treated with 1 μg/ml DAPI to visualize nuclear DNA. (B) Lysates were prepared in the presence of cycloheximide from room-temperature cultures of strain AJY1907 (*rei1*Δ *ARX1-HA*) and fractioned on sucrose gradients as described in the legend to Fig. 2. Fractions were collected, and proteins were precipitated with trichloroacetic acid, separated on SDS-polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membrane, and immunoblotted for HA.

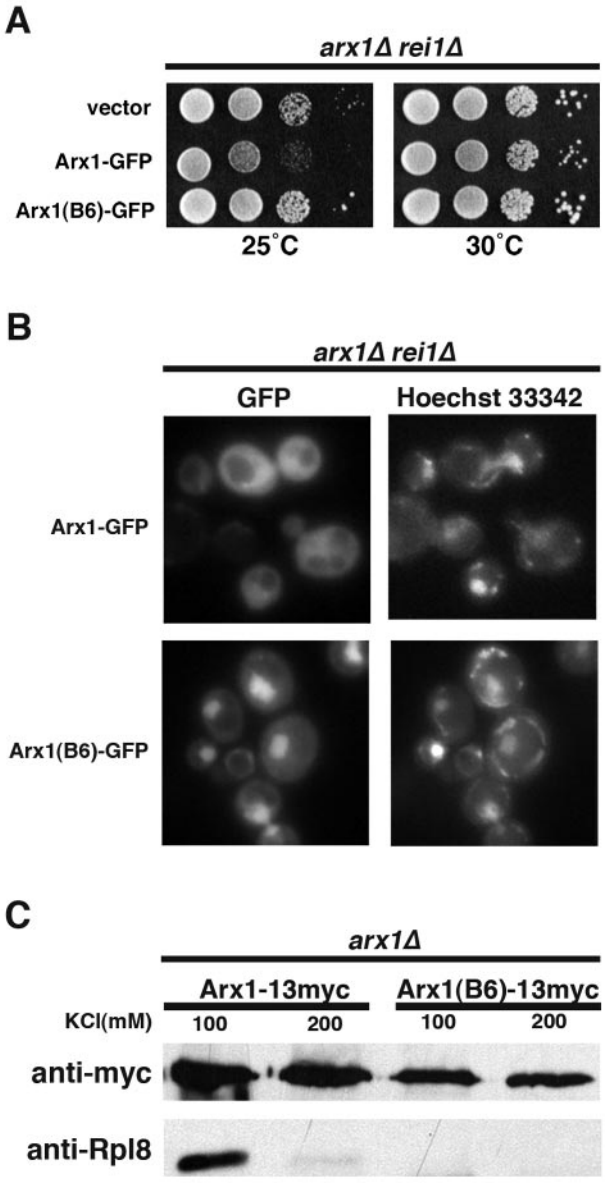

FIG. 8. An *arx1* mutant that suppresses *rei1*Δ exhibited reduced binding to 60S subunits. (A) Tenfold serial dilutions of saturated cultures were spotted onto Leu$^-$ dropout plates and incubated for 3 days at the indicated temperatures. The strains used were AJY1903 (*arx1*Δ *rei1*Δ) carrying empty vector (pRS416), Arx1-GFP (pAJ1015), and Arx1(B6)-GFP (pAJ1463). (B) Cultures from strain AJY1903 (*arx1*Δ *rei1*Δ) carrying Arx1-GFP (pAJ1015) or Arx1(B6)-GFP (pAJ1463) were grown to mid-log phase. Cultures were incubated for a further 30 min in the presence of 4 μM Hoechst 33342 dye to stain nuclei. Cells were then immediately taken to monitor in vivo localizations of Arx1-GFP by fluorescence microscopy. (C) Cell extracts from strain AJY1901 (*arx1*Δ) carrying Arx1-13myc (pAJ1916) or Arx1(B6)-13myc (pAJ1464) plasmids were prepared and incubated with anti-myc antibodies and protein A beads. Precipitated proteins were eluted from protein A beads in sample buffer and separated by SDS-polyacrylamide gel electrophoresis. Western blotting was performed against myc (Arx1) or Rpl8 as a marker for 60S subunits.

cumulation of Arx1 on cytoplasmic subunits. In addition, because mutant Arx1 that is defective for binding to 60S subunits accumulated in the nucleus in *rei1*Δ cells, we conclude that Rei1 is not needed for nuclear import of Arx1 per se but rather for the process of removing Arx1 from the subunit. Understanding the mechanism by which Rei1 removes Arx1, whether it involves additional factors, and whether it requires the nascent Arx1-containing subunit to engage in translation will require further work.

DISCUSSION

The combination of coimmunoprecipitation and mass spectrometry has become a powerful tool to characterize intermediates of ribosome assembly (1, 16, 26, 37). Although most of the *trans*-acting factors in the assembly pathway function in the nucleolus, some accompany the 60S subunits during export from the nucleus (18) or bind to 60S subunits only after reaching the cytoplasm, where the subunits become activated for translation (17, 26).

Here, we have characterized the two proteins Arx1 and Rei1, which transiently associate with the nascent 60S subunit at different stages in the biogenesis/export pathway. Arx1 is a shuttling protein that first binds to a pre-60S subunit in the nucleolus and remains associated with the subunit during export (36). Rei1, on the other hand, does not appear to shuttle and only binds to the subunit in the cytoplasm. Our results indicate that Rei1 is needed for release of Arx1 from subunits in the cytoplasm. While neither Arx1 nor Rei1 is essential, deletion of *REI1* leads to cold sensitivity for 60S biogenesis. Suppression of this cold-sensitive phenotype by deletion of *ARX1* or by conditions that inhibit Arx1 binding to the 60S subunit (mutations in Arx1 or large fusions to Rpl25) indicate that the primary defect in *rei1*Δ cells can be attributed to a failure to release Arx1 from cytoplasmic subunits.

We have shown that large fusions to Rpl25 or Rpl35 affect Arx1 binding to the 60S subunit. This is most easily explained if these fusions sterically hinder Arx1 binding, which would indicate that Arx1 binds to the subunit in the vicinity of the polypeptide exit tunnel. On the other hand, it is possible that the effect of fusions to Rpl25 or Rpl35 is indirect, mediated by long-range conformation changes within the subunit. However, considering that Arx1 is related to methionine aminopeptidases that act cotranslationally on nascent polypeptides as they emerge from the exit tunnel, we favor the idea that Arx1 also binds in this region.

What is the function of Arx1? Although *ARX1* is not essential, *arx1* deletion mutants show a mild 60S export defect, indicating that Arx1 is needed for efficient export of subunits from the nucleus. Arx1 may have evolved as a packaging factor that occupies a site near the exit tunnel to prevent functional interactions with additional factors during export. Multiple proteins bind near the exit tunnel during translation. Among these, SRP and nascent chain-associated complex are present in the nucleus (5, 7, 14). SRP is a 0.5-MDa ribonucleoprotein particle that is initially assembled in the nucleus. Although the export of SRP is independent of the ribosome, it has weak affinity for nontranslating ribosomes. Because ribosomes probably approach the upper limit for diameter of cargo that can be accommodated by the nuclear pore complex (NPC), the association of other large factors such as SRP could sterically hinder export. Thus, Arx1 could facilitate ribosome translocation through the NPC by preventing the premature interaction of additional factors. Alternatively, Arx1 could play a more

active role in promoting the correct assembly of factors on the pre-60S subunit required for export or recruitment of the nascent subunit to the nuclear pore complex to enhance its export.

A failure to release Arx1 is detrimental. It is intriguing that wild-type Arx1 is inhibitory in an *rei1*Δ mutant. Our results show that it is the persistence of Arx1 on cytoplasmic subunits and not the loss of Arx1 from the nucleus in an *rei1*Δ mutant that is detrimental. We do not yet understand why a failure to release Arx1 leads to cold sensitivity. The defect in 60S production could be due to enhanced degradation of subunits in the cytoplasm or inhibition of nuclear 60S production by a signaling event from the cytoplasm, possibly a failure in efficient recycling of another 60S-associated factor. Interestingly, the defects in 60S subunit and polysome levels in an *rei1*Δ strain are relieved by raising the temperature to 30°C or higher (data not shown). Thus, the presence of Arx1 is detrimental only at low temperature. Cold sensitivity can be caused by mutations in RNAs and RNA binding proteins that preferentially stabilize nonfunctional RNA conformations (53). By extension, it is possible that Arx1 stabilizes an unproductive conformation of the ribosome that inhibits its normal function or interaction with other factors. One candidate structure is expansion sequence 27, a highly dynamic RNA element near the exit tunnel (44). Although the functional significance of the dynamics of this RNA element is not known, Arx1 may lock it into a particular conformation at low temperature that inhibits ribosome function.

It is also possible that Arx1 inhibits the binding of a ribosome-associated factor at the exit tunnel. Such factors include RAC (ribosome-associated complex) (10), a dimeric complex of the protein chaperones Ssz1 and Zuo1 (9), the Hsp70 homolog Ssb1/Ssb2 (32), and NAC (nascent chain-associated complex), composed of Egd1 or Egd2 in association with Btt1 (39). Furthermore, nascent proteins destined for the secretory pathway are recognized by a signal recognition particle (SRP) whose binding site includes Rpl25 and Rpl35 (38). Ribosomes translating secretory proteins dock at the endoplasmic reticulum translocon through multiple interactions with the large-subunit RNA and proteins, including Rpl25 and Rpl35, surrounding the exit tunnel (3). However, none of these factors is known to affect ribosome biogenesis directly.

How does Rei1 release Arx1 from subunits in the cytoplasm? We have shown that Rei1 is required for release of Arx1 from nascent 60S subunits in the cytoplasm. It seems likely that the release of Arx1 requires Rei1 binding to the subunit. Rei1 binding could directly displace Arx1 from the subunit, or it could induce a conformational change in the binding site for Arx1 that reduces the affinity of Arx1 for the ribosome.

On the other hand, Rei1 could act in coordination with another factor. 60S subunits that are exported from the nucleus contain a small number of stably associated nonribosomal proteins, including Nmd3, Tif6, Arx1, and Rlp24 (8). We have recently shown that release of Nmd3 in the cytoplasm requires the GTPase Lsg1 and the ribosomal protein Rpl10 (17), and we have proposed that the loading of Rpl10 by the WD-repeat protein Sqt1 and Lsg1 is the event that triggers Nmd3 release (17). Tif6 has been shown to require Ria1/Efl1 for its release (42), but release of Tif6 is not known to be coupled with additional events. Whether or not release of Arx1 by Rei1 is coupled to these other events remains to be determined; however, the distribution of Arx1 is not affected by mutations in Lsg1 that prevent the release of Nmd3 from cytoplasmic subunits (M. West and A. Johnson, unpublished data).

Do Arx1 and Rei1 have additional roles? The human homolog of Arx1, Ebp1, is a cytoplasmic protein associated with the transmembrane protein ErbB-3, a member of the epidermal growth factor receptor family (52). In the presence of ErbB-3 ligand, Ebp1 translocates into the nucleus, where it is thought to act as a transcription factor (51, 54). Interestingly, ectopic expression of Ebp1 in human breast cancer cells inhibits cell proliferation (29). Ebp1 has also recently been identified as a nucleolar protein that is associated with pre-60S particles (46). These results from human cells raise the possibility that yeast Arx1 could also be involved in signaling from the cytoplasm to the nucleus, where it may play a role in transcriptional regulation of cell proliferation.

In addition to the identification of Rei1 as a pre-60S-associated protein (8), Rei1 was also identified in a two-hybrid screen using NIS1 as bait (21). Nis1 is a septin-binding protein that localizes to the bud neck in M phase (21), thus implicating Rei1 in the mitotic signaling network in *Saccharomyces cerevisiae*. Although Rei1 is distributed throughout the cytoplasm, its association with Nis1 could indicate a role in signaling between the cell cycle and ribosome biogenesis pathways or provide a mechanism for targeting nascent ribosomes to the site of active cell growth.

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