The Hsp40 chaperone Jjj1 is required for the nucleo-cytoplasmic recycling of preribosomal factors in *Saccharomyces cerevisiae*

EMILIE DEMOINET,[1,2] ALAIN JACQUIER,[2] GEORGES LUTFALLA,[1] and MICHELINE FROMONT-RACINE[2]

[1]Défenses Antivirales et Antitumorales, cc86 CNRS-UMR5235, Université Montpellier II, 34095 Montpellier Cedex 5, France
[2]Unité de Génétique des Interactions Macromoléculaires, 2171CNRS-URA, Institut Pasteur, F-75724 Paris Cedex 15, France

ABSTRACT

Ribosome biogenesis is a major conserved cellular pathway that requires both ribosomal proteins and many preribosomal factors. Most of the pre-60S factors are recycled into the nucleus; some of them shuttle between the nucleus and the cytoplasm while a few others, like Rei1, are strictly cytoplasmic and are mostly involved in the dissociation/recycling of the pre-60S shuttling factors. Here, we investigated the role of the Jjj1 Hsp40 chaperone in ribosome biogenesis. The absence of Jjj1 leads to a cold sensitive phenotype, a defect in the relative amount of the large ribosomal subunit with the appearance of halfmers, and to cytoplasmic accumulation of shuttling factors such as Arx1 and Alb1, which stay bound to the pre-60S particles. Jjj1 is, thus, a novel pre-60S factor involved in the last cytoplasmic steps of the large ribosomal subunit biogenesis. We report the biochemical association of Jjj1 and Rei1 to similar pre-60S complexes, their two-hybrid interactions, and their functional links. Altogether, these results indicate that Rei1 and Jjj1 share many common features. However, while the functions of Jjj1 and Rei1 partially overlap, we could distinguish specific role of the two proteins in Arx1/Alb1 and Tif6 recycling. We propose that Jjj1 is preferentially required for the release of Arx1 and Alb1 shuttling factors from the cytoplasmic pre-60S particles while Rei1 is preferentially involved in their recycling.

Keywords: ribosome biogenesis; pre-60S maturation; chaperone; nucleocytoplasmic transport; pre-ribosomal factors recycling; yeast *Saccharomyces cerevisiae*

INTRODUCTION

The ribosome is one of the most important cellular macromolecular structures in terms of function, size, and the energy that a cell consumes for its biogenesis (Warner 1999). Ribosome biosynthesis begins with the transcription of the 35S and 5S rRNA precursors by RNA pol I and III, respectively. The processing of the 35S rRNA precursor generates the 18S rRNA (backbone of the small ribosomal subunit) and the 5.8S and 25S rRNAs (backbone of the large ribosomal subunit). The rRNAs are embedded in non-coding spacer regions, the external transcribed sequences, 5′- and 3′-ETS, and the internal transcribed sequences, ITS1 and ITS2 (Venema and Tollervey 1999). Association of the 35S rRNA precursor with ribosomal and preribosomal factors generates the large 90S preribosomal complex that undergoes various steps of maturation such as chemical modifications (Decatur and Fournier 2002) and exo- and endonucleolytic cleavages (Venema and Tollervey 1999) to remove the ETS and ITS regions. Among a well-orchestrated series of cleavage events, the A2 processing generates the large 60S precursor particles containing the 27SA2 intermediate rRNA and the small 40S precursor particles containing the 20S intermediate rRNA (Fatica and Tollervey 2002; Fromont-Racine et al. 2003; Tschochner and Hurt 2003). Then, both pre-ribosomal precursor particles follow independent routes; the pre-40S particle is mostly processed in the cytoplasm, whereas the maturation of the pre-60S particle is mostly achieved in the nucleus before export to the cytoplasm where final maturation takes place.

In addition to the large ribosomal protein themselves, around 80 factors have now been predicted or shown to

Reprint requests to: Micheline Fromont-Racine, Génétique des Interactions Macromoléculaires, Institut Pasteur, 25 rue du docteur Roux, 75724 Paris Cedex 15, France; e-mail: mfromont@pasteur.fr; fax: +33 14568790; or Georges Lutfalla, Défenses Antivirales et Antitumorales, cc86 CNRS-UMR5235, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France; e-mail: lutfalla@univ-montp2.fr; fax: +33 467 14 92 01.

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participate in the biogenesis of the large ribosomal subunit (Fatica and Tollervey 2002; Fromont-Racine et al. 2003; Tschochner and Hurt 2003). In contrast to the ribosomal proteins, pre-ribosomal factors associate temporarily with the maturing subunits. Most of them associate with and dissociate from the subunits into the nucleus. A few other factors like Arx1, Alb1, Tif6, Rlp24, and Nmd3 are shuttling factors (Senger et al. 2001; Nissan et al. 2002; Saveanu et al. 2003; Hedges et al. 2005; Lebreton et al. 2006b). They bind the pre-60S in the nucleus and dissociate in the cytoplasm. Finally, other factors like Lsg1 (Hedges et al. 2005), Rei1 (Hung and Johnson 2006; Lebreton et al. 2006b), Efl1 (Senger et al. 2001), and Drg1 (H. Bergler and M. Fromont-Racine, unpubl. data) are strictly cytoplasmic and required for the release and recycling of shuttling factors. These final cytoplasmic steps involve structural rearrangements but no rRNA cleavage. GTPase or ATPase proteins appear to be involved in this process. Indeed, Lsg1 GTPase activity is required for Nmd3 release (Hedges et al. 2005) and in the absence of the Efl1 GTPase, Tif6 accumulates into the cytoplasm (Senger et al. 2001). The Drg1 ATPase protein (Zakalskiy et al. 2002) is also required for the recycling of shuttling pre-60S factors (H. Bergler and M. Fromont-Racine, unpubl. data). Thus, the ribosomal cleavage and maturation events are tightly coupled to nucleocytoplasmic transport. Recycling requires transport machineries involving karyopherin Kap121, Kap123, and Kap104, which are used for the import of shuttling factors (Rout et al. 1997; Leslie et al. 2004) and exportin (Crm1/Xpo1) for the translocation of the pre-60S particles through the nuclear pore (Ho et al. 2000; Gadal et al. 2001).

In this study, we investigate the role of Jjj1, an Hsp40 chaperone (Meyer et al. 2007) that would be specifically involved in the biogenesis/maturation of the large ribosomal subunit. In eukaryotes, two chaperone networks have been described, a stress inducible network that protects the cellular proteome from stress and another stress repressed chaperone network that is dedicated to protein biogenesis (Albanese et al. 2006). Besides these ''general'' chaperones, some chaperones are specifically involved in structural transitions for polypeptides in large molecular complexes, as, for example, auxilin/Hsc70 required for clathrin uncoating (Ungewickell et al. 1995; Pishvaee et al. 2000; Fotin et al. 2004). Hsp40s function to specify the cellular targets of Hsp70 chaperones. In the classical view of the function of an Hsp40 chaperone, the protein forms a complex with an unfolded or nonnative protein to prevent its aggregation or misfolding. Hsp70 proteins recognize short hydrophobic polypeptide in this complex, but the polypeptide binding is regulated by the nucleotide bound state. Conformational changes generated upon ATP hydrolysis stabilize Hsp70's interaction with its polypeptide substrate, whereas the exchange of ADP for ATP promotes their releases (Fan et al. 2003; Hennessy et al. 2005).

Apart from Jjj1, no chaperone has been found to be specifically involved in the biogenesis of the large ribosomal subunit. We show here that Jjj1 is involved in the last steps of the biogenesis of the ribosomal large subunit and interacts with Rei1, another late pre-60S factor cytoplasmic involved in the recycling of the shuttling factors (Arx1, Alb1). Even if Jjj1 and Rei1 share several common characteristics, they are required for different maturation events.

RESULTS

Jjj1 is involved in ribosome biogenesis

We recently demonstrated that Rei1 is involved in the last cytoplasmic steps of ribosome biogenesis. To gain more insight into potential partners of Rei1, a two-hybrid screen was performed. One of the identified candidates was *JJJ1/YNL227W*. In Figure 1A, left panel, the inset coding the Jjj1 protein encompassed a region from amino acid 162 to the end of the protein. In addition to the two conserved C_2H_2 Zn fingers from amino acids 338 to 362 and from 549 to 573, Jjj1 contains a N-terminal DNA J domain (up to amino acid 67) that assigns Jjj1 to the Hsp40 chaperone family. The DNA J domain was excluded from the selected Rei1 interacting domain. The physical interaction between Jjj1 and Rei1 was confirmed by a two-hybrid matrix approach. To further investigate additional interactions, we tested physical interactions between Jjj1 and several pre-60S factors previously described as partners of Rei1, such as Arx1, Alb1, and the ribosomal protein Rpl24. A two-hybrid interaction was observed between Jjj1 and Rei1 (Fig. 1A, right panel). No other physical interaction was found with Jjj1 whereas Rei1 interacted with Rpl24, and Arx1 interacted with itself and with Alb1 (Lebreton et al. 2006b). We noted that Alb1 was an autoactivating bait. The strongest two-hybrid signals were obtained between Arx1 and itself and between Arx1 and Alb1, as demonstrated by the dark blue color obtained by X-Gal overlay assay.

JJJ1 is not essential in yeast; however, its deletion leads to a cold sensitive phenotype. Apart from the DNA J motif, suggesting a chaperone activity, the function of this protein was unknown. However, in addition to the physical two-hybrid links between Rei1 and Jjj1, several lines of evidence suggested that Jjj1 could be involved in ribosome biogenesis. For example, the *JJJ1* mRNA level fluctuates similarly to the levels of mRNAs coding for preribosomal factors in response to various environmental stresses (Gasch et al. 2000). Moreover, Jjj1 has been identified in several preribosomal complexes using the TAP purification approach (Gavin et al. 2006).

To investigate the involvement of Jjj1 in ribosome biogenesis, we looked at the polysome profile of a *jjj1Δ* cellular extract from the ED10 (*jjj1Δ*) strain after ultracentrifugation in a sucrose gradient. The relative amount of

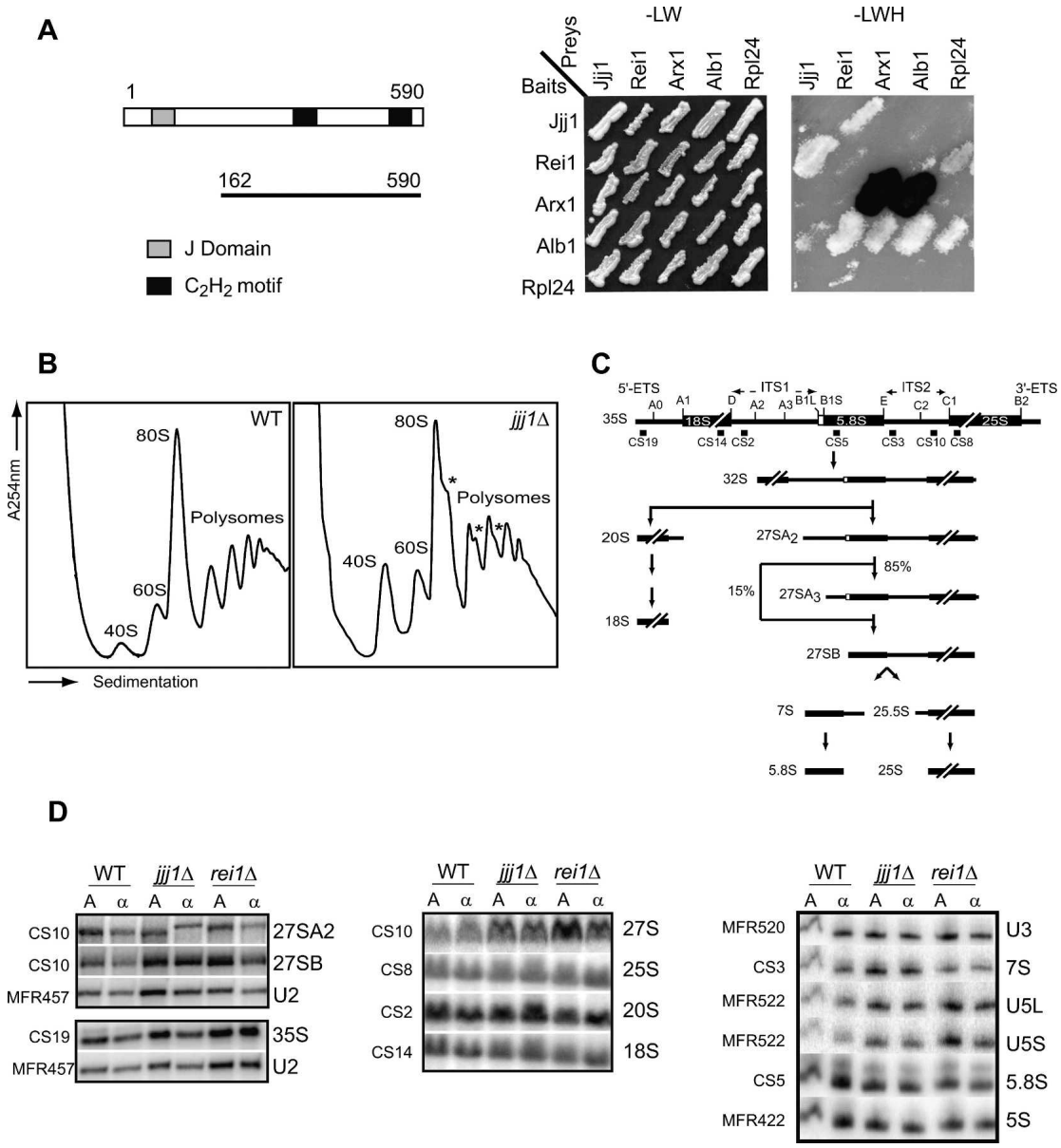

FIGURE 1. Jjj1 is involved in the 60S subunit biogenesis. (*A*) Two-hybrid screen identifies a physical link between Jjj1 and Rei1. (*Right* panel) Jjj1 was selected as a partner of Rei1 in a two-hybrid genomic screen. The domain of Jjj1 interacting with Rei1 corresponds to amino acids 162–590. The two putative C_2H_2 Zn-fingers motifs and the J domain are depicted by black and gray boxes, respectively. (*Left* panel) Various entire ORF were cloned into pAS2ΔΔ as bait and into pACTIIst as prey vectors, respectively. The bait and the prey vectors were transformed into the CG1945 and Y187 strains, respectively. Both strains were mated and the diploids were selected on minimal medium plates without leucine and tryptophan (−LW). The diploids that display a two-hybrid positive interaction were selected on minimal medium plates without leucine, tryptophan and histidine (−LWH). An X-Gal overlay was performed on the −LWH plate. (*B*) In the absence of Jjj1, the relative amount of 60S subunit is affected. A wild-type (BY4741) or a *jjj1Δ* strain (ED10) was grown in YPGlu and shifted at 23°C for 2 h. The whole-cell extracts from these strains were separated by ultracentrifugation on a 10%–50% sucrose gradient. Peaks corresponding to the various subunits and polysomes are indicated. Asterisks note halfmers. (*C*) Schematic representation of the ribosomal RNAs maturation. The relative position of the oligonucleotides used to detect specific rRNA by primer extension is indicated. (*D*) In the absence of Jjj1, the 27SB processing is very weakly affected. Total RNAs were extracted from culture cells of wild-type strain (BY4741), *jjj1Δ* (ED10), and *rei1Δ* mutant strains (ED70) in YPGlu medium after a shift to 23°C for 2 h. Mature and intermediate rRNA were detected either by primer extension (*left* panel) or by Northern blot on agarose gel (*middle* panel) or on acrylamide gel (*right* panel). The oligonucleotides used are noted. The U2 snRNA was used as a loading control.

60S subunit was clearly affected in the absence of Jjj1 (Fig. 1B, right panel) as compared to the wild-type strain (Fig. 1B, left panel). Moreover, we observed a decrease of the total amount of polysomes and the presence of half-mers, which reflects the relative deficit in 60S as compared to 40S during translation initiation. This phenotype was obvious at 30°C (data not shown) but was even more pronounced after a shift to 23°C for 2 h (Fig. 1B). To define

which step of ribosome biogenesis is affected by the absence of Jjj1, we tested the relative amounts of the different mature and intermediate rRNA in *jjj1Δ* strains (ED10 mat α, ED11 mat a) in comparison with a wild-type (BY4742, BY4741) or a *rei1Δ* (ED70 mat α, ED71 mat a) strain (Fig. 1D). Steady-state rRNA levels were analyzed by primer extension (Fig. 1D, left panel) and by Northern blotting either on agarose (Fig. 1D, middle panel) or acrylamide gels (Fig. 1D, right panel) from wild-type, Jjj1, and Rei1-depleted cells after a shift to 23°C for 2 h. With the exception of the 27SB/A2 pre-rRNA ratio, which very slightly increased (2.9 for the *jjj1Δ* strains compared to 1.5 for the WT strains and 3.2 to *rei1Δ* strains based on two experiments, one on each mating type), none of the other ratios were significantly affected. Similar results were obtained after a 15-min shift at 23°C with *rei1Δ*, *jjj1Δ*, and WT strains (mat a, mat α) (data not shown). These results are in agreement with the decrease of the relative amount of the 60S subunit in the absence of Jjj1 but suggest that Jjj1 has no direct influence on nuclear pre-rRNA processing.

Since ribosome maturation mutations are usually correlated with a ribosome export defect, we tested the localization of the ribosomal Rpl25-GFP fusion protein in the *jjj1Δ* strain. We observed that, like in a *rei1Δ* strain or in a wild-type strain, the fluorescence was mainly located in the cytoplasm (data not shown), suggesting that the export of Rpl25-GFP was not affected. In addition, like Rei1, Jjj1 is a cytoplasmic protein (Huh et al. 2003).

Altogether, these results show that Jjj1 is a cytoplasmic factor affecting nuclear ribosomal large subunit maturation. This is reminiscent of the effect described for *rei1Δ*, which participates in the recycling of shuttling pre-60S factors (Hung and Johnson 2006; Lebreton et al. 2006b).

Jjj1 physically interacts with the pre-60S particles

The physical two-hybrid interaction between Jjj1 and Rei1 correlates with similar defects of the 60S biogenesis in the absence of either of these proteins. To check whether Jjj1, like Rei1, is biochemically associated with the 60S particles, we performed a sucrose gradient experiment using a chromosomal Jjj1-TAP fusion protein (OT52) (Open Biosystem). After sedimentation of the whole cellular extracts on sucrose gradient, we observed that Jjj1-TAP fusion protein was present in the fractions corresponding to the 60S ribosomal particle but not in the polysome fractions (Fig. 2A) suggesting that Jjj1, like Rei1, is a pre-60S-associated factor. Since Jjj1 and Rei1 interact in a two-hybrid assay and since they are both associated to pre-60S ribosomal particles, we attempted to determine if both proteins were present in the same particles. We purified the complex associated with Jjj1-TAP using a strain producing a Rei1–13myc fusion protein and also performed the reverse experiment. Jjj1–13myc was only present in the complexes associated with Rei1 but not with Rlp24 (Fig. 2B) and Rei1–13myc was copurified with Jjj1-associated complexes but not with Rlp24. As a control, we observed that Rlp24 was copurified neither with Jjj1-TAP nor with Rei1-TAP while it copurified with Mak11-TAP. These results suggest that (1) Jjj1 and Rei1 are present in the same complexes and (2) like Rei1, Jjj1 binds to the particle after the release of Rlp24.

We checked for the dependence of the presence of Jjj1 for the binding of Rei1 to the particle and inversely by using strains depleted for the first one and producing a TAP fusion of the second one. The association of the proteins to the pre-60S complexes was tested by Western blotting on fractions of a sucrose gradient. Surprisingly, neither Jjj1 nor Rei1 was required for the recruitment of the other protein to the pre-60S particle (data not shown).

Jjj1 and Rei1 are functionally linked

Several lines of evidence indicate that there is a strong similarity between Jjj1 and Rei1: (1) Both proteins are

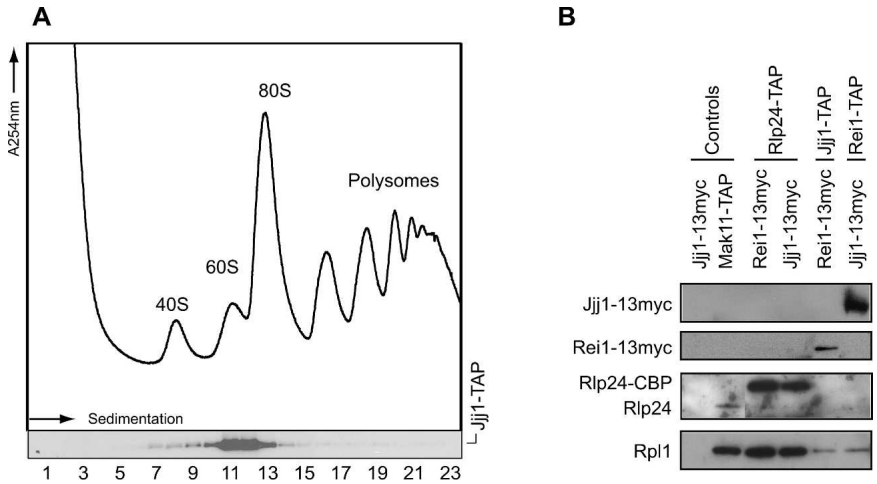

FIGURE 2. JjjI and Rei1 are involved in the same complexes. (*A*) Jjj1 is associated with the 60S particles. The whole-cell extract of a strain expressing a Jjj1-TAP fusion protein (OT52) was separated by ultracentrifugation on a 10%–50% sucrose gradient. Fractions of 0.5 mL were collected. Proteins were precipitated by TCA and separated on a SDS–4%–12% polyacrylamide gel. The presence of Jjj1-TAP fusion protein was revealed by Western blotting using PAP antibodies (Sigma). (*B*) Jjj1 and Rei1 are present in the same pre-60S complexes. TAP purifications were performed on strains expressing Rei1-TAP, Jjj1-13myc (ED46); Jjj1-TAP, Rei1–13myc (ED19); Rlp24-TAP, Jjj1–13myc (ED87); and Rlp24-TAP, Rei1–13myc (LMA352) fusion proteins. Mak11-TAP (LMA375) and Jjj1–13myc (ED82) strains were used as control. The presence of Jjj1–13myc, Rei1–13myc, Rlp24, and Rpl1 was tested using antibodies against myc, Rlp24, and Rpl1, respectively.

involved in the pre-60S biogenesis, (2) they are located in the cytoplasm, (3) they physically interact, (4) both proteins have Zn finger motifs, and (5) deletion of either *JJJ1* or *REI1* leads to a cold sensitive phenotype. To determine if these proteins would have redundant functions, we tested the effect of the double deletion (Fig. 3A). In comparison to a generation time of 2 h 41 min for a wild-type strain, we observe a generation time of 5 h 20 min for *jjj1Δ rei1Δ* double mutant strain (ED21) similar to the generation time of 5 h 37 min of the *rei1Δ* strain (LMA523), whereas the generation time for *jjj1Δ* is of 4 h 5 min, suggesting that both *JJJ1* and *REI1* genes are epistatic.

To further investigate the role of Jjj1, we performed a high-copy suppressor genetic screen with the *JJJ1*-deleted strain (ED10) at 20°C. Interestingly, in addition to *JJJ1* containing plasmids, one family of the rescued plasmids containing the *REI1* gene was able to partially complements the cold sensitivity of the *jjj1Δ* mutant strain. Figure 3B (left panel) shows the complementation using a 2 μm plasmid derived from pFL44L containing *JJJ1*. As expected, overexpression of *REI1* was also able to partially restore the cold-sensitive phenotype of *jjj1Δ* (Fig. 3B, left panel). We tested the reverse situation by overexpressing *JJJ1* into a *rei1Δ* mutant strain (ED72). While pFL44L-REI1 complements the absence of Rei1, the overexpression of *JJJ1* had no effect on the *rei1Δ* mutant (same as empty vector; data not shown). Overexpression of either *JJJ1* or *REI1* was tested on the *jjj1Δ rei1Δ* double mutant strain (ED21) (Fig. 3B, right panel). While the overexpression of *JJJ1* had no effect, we observed a partial rescue of the growth of *jjj1Δ rei1Δ* double mutant strain by overexpression of *REI1*.

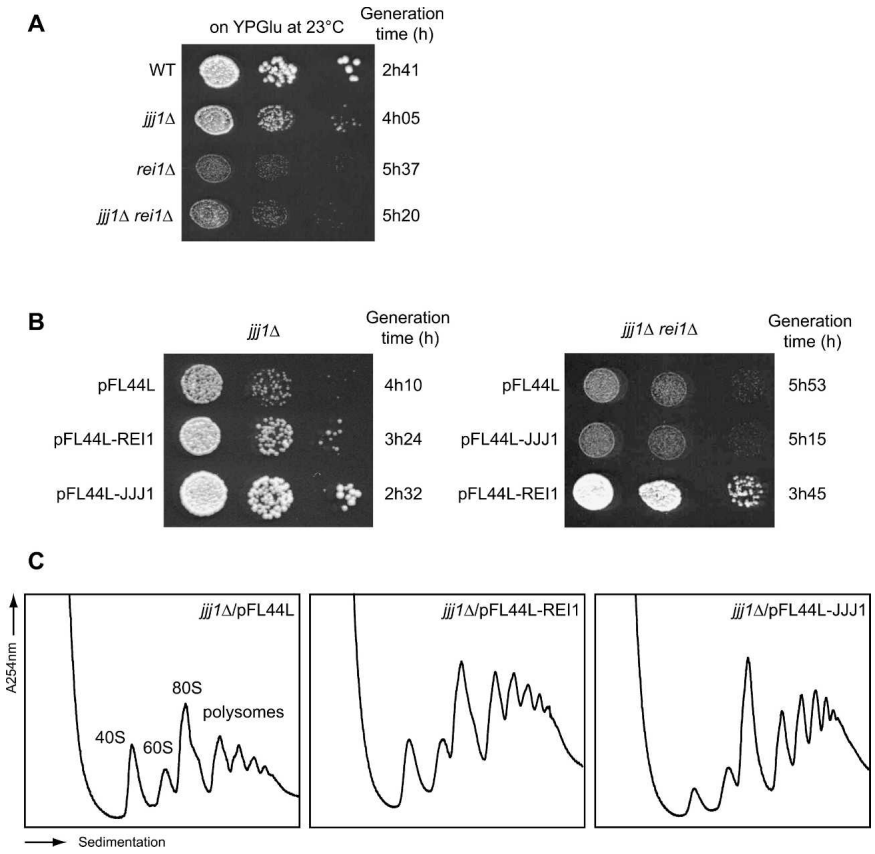

FIGURE 3. Rei1 and Jjj1 are genetically linked. (*A*) Deletion of both *JJJ1* and *REI1* genes is epistatic. Ten times serial dilutions of wild-type (BY4741), *rei1Δ* (LMA523), *jjj1Δ* (ED10), and *rei1Δ jjj1Δ* (ED21) culture were spotted on solid rich medium. Their growth on YPGlu at 23°C was compared. Generation times are indicated. (*B*) *REI1* overexpression partially complements the *jjj1Δ* growth defect. A *jjj1Δ* (ED10) strain (*left* panel) and a *rei1Δ jjj1Δ* (ED21) strain (*right* panel) were transformed with either the empty vector pFL44L or pFL44L-REI1 or pFL44L-JJJ1. Growth differences were illustrated by 10 times serial dilutions on solid minimal medium without uracil at 23°C for 2 d. Generation times are indicated. (*C*) *REI1* overexpression partially restores 60S amount of *jjj1Δ* strain. *jjj1Δ* strain (ED10) was transformed with either pFL44L (*left* panel) or pFL44L-REI1 (*middle* panel) or pFL44L-JJJ1 (*right* panel). The strains were grown in minimal media without uracil at 30°C and shifted to 23°C for 2 h. The whole-cell extracts were separated by ultracentrifugation on a 10%–50% sucrose gradient. Absorbance at 254 nm was measured. Peaks corresponding to the various subunits and polysomes are indicated.

Polysome profiles of the *jjj1Δ* strains transformed with different vectors were analyzed after a shift of temperature for 2 h at 23°C from 30°C. The amount of 60S subunit was clearly affected in the absence of Jjj1 as compared to the *jjj1Δ* strain complemented by the overexpression of *JJJ1*. Interestingly, *REI1* overexpression partially restored the relative amount of 60S subunit (Fig. 3C), in agreement with the partial growth rescue.

We conclude that overexpression of *REI1* partially complements the absence of Jjj1 but the reverse is not true. Therefore, while Jjj1 and Rei1 proteins display some similarities and are involved in the same pathway, their functions are not identical.

The absence of either Arx1 or Alb1 is able to complement the growth defect of the *rei1Δ* strain (Lebreton et al. 2006b; Meyer et al. 2007). To further investigate the similarities and the differences between Jjj1 and Rei1, we checked whether the presence of Arx1 or Alb1 was responsible for the cold sensitivity of the *jjj1Δ* strain. In fact the *jjj1Δ arx1Δ* and *jjj1Δ alb1Δ* double mutant strains grew as well as *arx1Δ* or *alb1Δ* strains at 23°C (Fig. 4A), showing that the absence of Arx1 (or Alb1) rescued the cold sensitivity of *jjj1Δ*. Moreover, using polysome profiles, we observed that the relative amount of 60S was restored to the

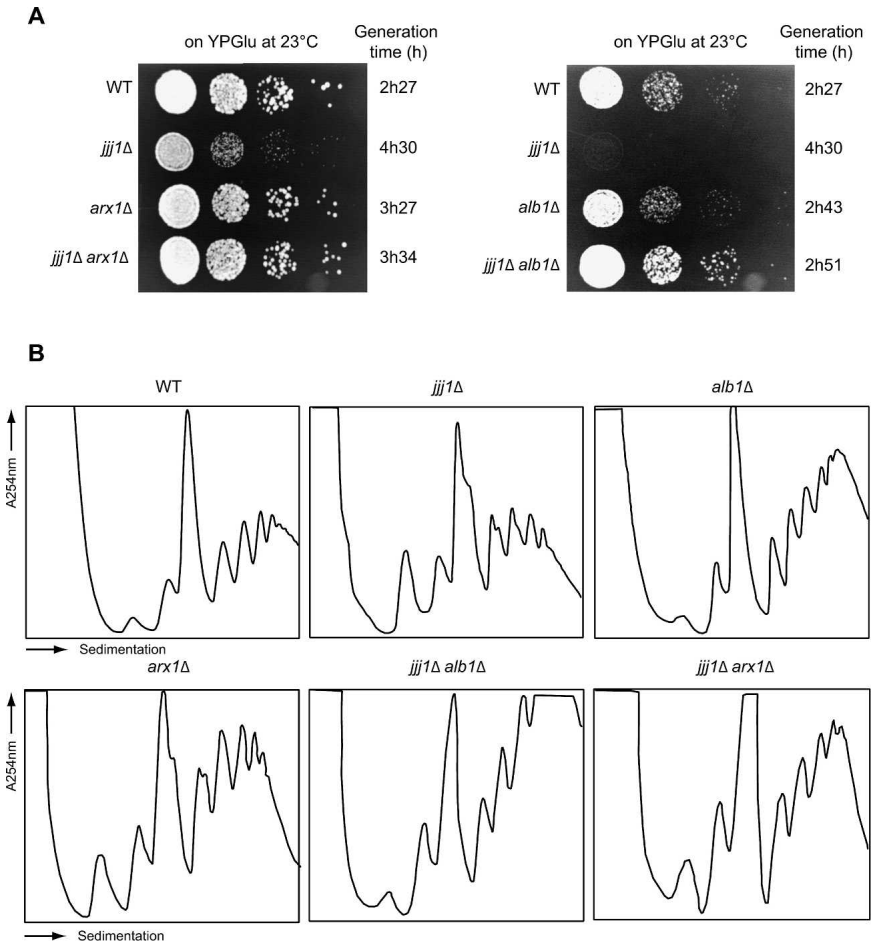

FIGURE 4. In the absence of Arx1/Alb1, deletion of *JJJ1* is not toxic. (*A*) Deletion of either *ARX1* or *ALB1* rescues the cold sensitivity effect of *jjj1Δ* strain. Ten times serial dilutions of wild-type (BY4741), *jjj1Δ* (ED10), *arx1Δ* (LMA539), *alb1Δ* (LMA525), *arx1Δ jjj1Δ* (ED52), and *alb1Δ jjj1Δ* (ED65) cultures were spotted on solid rich medium. Their growth on YPGlu at 23°C was compared. (*B*) Deletion of either *ARX1* or *ALB1* restores a wild-type polysome profile. The whole-cell extracts from the strains used in *A* were separated on a sucrose gradient as described in Figure 1B.

wild-type level in the *jjj1Δ arx1Δ* and *jjj1Δ alb1Δ* double mutant strains when compared to *jjj1Δ* alone (Fig. 4B). This observation is consistent with the recent hypothesis that the accumulation of Arx1 and Alb1 as a small cytoplasmic complex (Lebreton et al. 2006b) could be responsible for the slow growth phenotype of the *rei1Δ* strain.

Jjj1 and Rei1 have distinct functions in the Arx1/Alb1 recycling

Since the cold sensitivity of the two deleted *jjj1Δ* or *rei1Δ* strains was abolished by the deletion of *ARX1* or *ALB1*, we hypothesized that Jjj1 could participate with Rei1 in the recycling of shuttling pre-60S factors. We predicted that the proteins affected in their recycling in the absence of Rei1 would also be affected by the absence of Jjj1. We observed that Arx1-GFP and Alb1-GFP accumulated in the cytoplasm in the absence of Jjj1 as well as in the absence of Rei1 (Fig. 5A, left and middle panels). To check if Arx1 and Alb1 are blocked on the cytoplasmic pre-60S particles or present in small cytoplasmic complexes in the absence of Jjj1, whole-cell extracts from strains expressing Alb1-GFP in the absence of Jjj1 were separated by sedimentation on a sucrose gradient. In the absence of Jjj1, endogenous Arx1 and Alb1-GFP fusion protein sedimented in the 60S peak whereas in the absence of Rei1, both Arx1 and Alb1-GFP accumulated as small complexes (Fig. 5B) as previously described (Lebreton et al. 2006b). Similar results were obtained in a strain expressing an Arx1-GFP fusion protein (data not shown). These results are in agreement with the genetic links described above. Even if Jjj1 and Rei1 are involved in the same pathway, their functions are not fully equivalent. Previous results have suggested that the recycling of these factors involved the Kap121 karyopherin pathway. This hypothesis was supported by the fact that overexpression of *KAP121* was able to recycle Arx1 and Alb1 into the nucleus and partially restore the slow growth phenotype of the *rei1Δ* strain (Fig. 6A, left panel; Lebreton et al. 2006b). If in *jjj1Δ*, Arx1 and Alb1 are not released from the particle, overexpression of *KAP121* should not result in relocalization of Arx1/Alb1 in the nucleus. Indeed, in contrast to *rei1Δ*, *KAP121* overexpression (Fig. 6A, middle panel) had no effect on the *jjj1Δ* strain. It was able to restore neither the growth phenotype nor the polysome profile (data not shown) nor the normal localization of Arx1-GFP (Fig. 6B). As a control, we observed that, in a *rei1Δ* strain, *KAP121* overexpression allowed Arx1/Alb1 recycling (Fig. 6B). Interestingly, *KAP121* overexpression led to a partial rescue of the slow growth phenotype in the *jjj1Δ rei1Δ* double mutant strain as in *rei1Δ* strain (Fig. 6A, cf. right and left panels).

These results confirm that in the absence of Jjj1, Arx1 and Alb1 are mostly blocked on pre-60S cytoplasmic particles, where they are probably not available to the import machinery, whereas in the absence of Rei1, Arx1 and Alb1 accumulate as small cytoplasmic complexes, where they still can be caught by an overwhelming amount of Kap121 karyopherin.

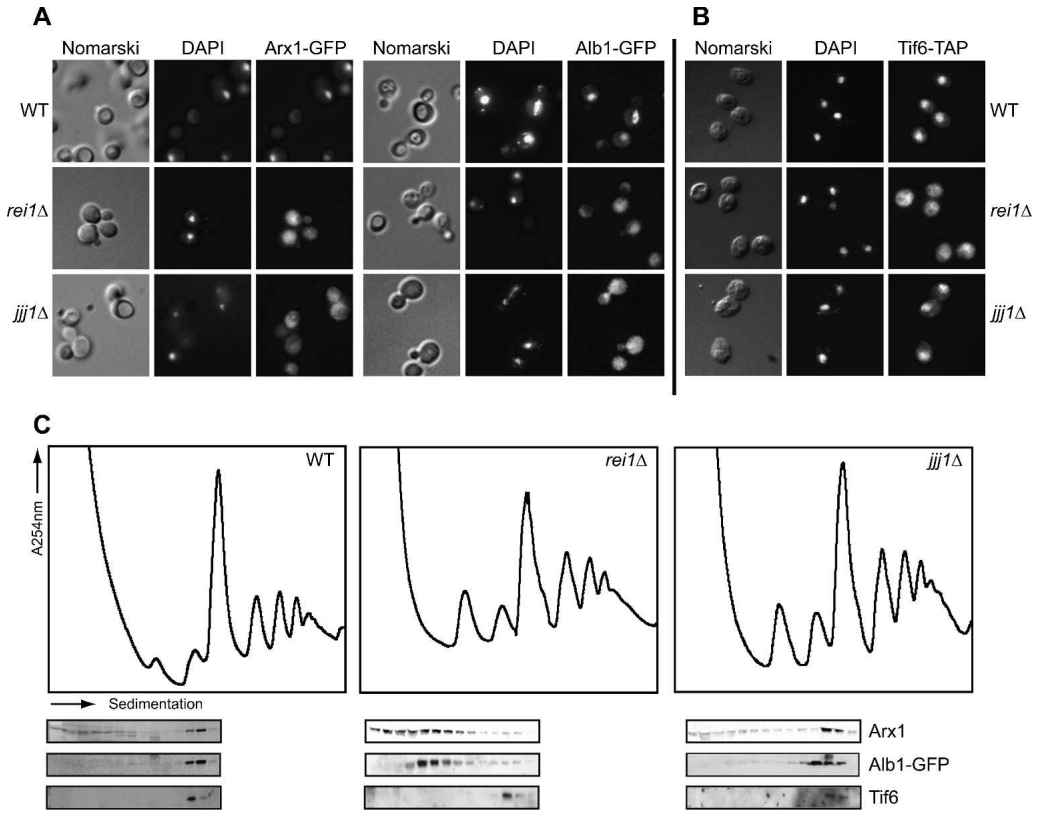

FIGURE 5. In the absence of Jjj1, Arx1/Alb1 are blocked on cytoplasmic 60S particles. (*A*) Arx1 and Alb1 but not Tif6 (*B*) accumulate into the cytoplasm in the absence of Jjj1. Arx1-GFP (LMA401), Alb1-GFP (LMA545-B), Arx1-GFP, *jjj1Δ* (ED49), Arx1-GFP, *rei1Δ* (LMA411), Alb1-GFP, *jjj1Δ* (ED66), Alb1-GFP, *rei1Δ* (LMA545-A), Tif6-TAP (ED72), Tif6-TAP, *rei1Δ* (ED92), and Tif6-TAP, *jjj1Δ* (ED89) strains were grown in minimal media at 30°C and shifted to 23°C for 2 h. The cells were observed by fluorescence microscopy. Tif6-TAP localization was observed on fixed cells using PAP antibodies (Sigma) at 1/5000 dilution followed by Cy3-conjugated secondary antibodies (Jackson Immunoresearch) at 1/250 dilution. (*C*) Arx1 and Alb1 are blocked on the 60S particle in the absence of Jjj1. The whole-cell extracts from the strains used in *A* were separated on a sucrose gradient and the fractions were analyzed as described in Figure 2A. The Western blots were performed using the fractions of the polysome profiles from 2 to 15. The presence Arx1, Tif6, and Alb1-GFP fusion proteins were checked using anti-Arx1, anti-Tif6, and anti-GFP antibodies (Santa Cruz Biotechnology) at 1/700, 1/5000 and 1/700 dilutions, respectively.

The absence of Jjj1 or Rei1 differently affects Tif6 recycling

We recently proposed that, in a *rei1Δ* strain, the cytoplasmic accumulation of small Arx1-associated complexes could be responsible for blocking Tif6 on the pre-60S particle (Lebreton et al. 2006b). In the absence of Jjj1, no such small cytoplasmic Arx1-associated complexes were formed; we therefore expected Tif6 to be released from the pre-60S particle and correctly recycled. Interestingly, while Tif6 accumulated in the cytoplasm in the absence of Rei1, it was localized in the nucleus in the absence of Jjj1 (Fig. 5A, right panel). We also checked the sedimentation of Tif6-TAP in the absence of Jjj1; it sedimented in fractions corresponding to the 60S peak (Fig. 5B). This suggests that in the absence of Jjj1, Tif6 is correctly released from the pre-60S cytoplasmic particles and recycles to the nucleus.

In conclusion, Tif6 does not seem to be affected by the deletion of *JJJ1* while it requires *REI1* for dissociation from the cytoplasmic pre-60S particle.

DISCUSSION

The first precursor particle involved in ribosome biogenesis, the 90S, generates the pre-40S and pre-60S intermediate ribosomal particles after the A2 cleavage. The large ribosomal subunit maturation requires about 80 pre-ribosomal factors. This pre-60S particle goes through successive maturation steps, including rRNA processing, conformational changes, and transport events through the nucleolus, the nucleoplasm, and the nucleopore complex. Thus, it is not surprising that most of these 80 or so pre-60S factors are nuclear factors whereas few are strictly cytoplasmic. When the pre-60S particles reach the cytoplasm, the mature ribosomal RNAs are formed, and many pre-ribosomal factors have already left the particles. Only a few shuttling factors, such as Arx1, Alb1, Tif6, Rlp24, and Nmd3, are still present. Then, some strictly cytoplasmic pre-60S factors act on the pre-60S particles to finalize their maturation.

We report here the role of a strictly cytoplasmic pre-60S factor, Jjj1, in the biogenesis of the large ribosomal subunit.

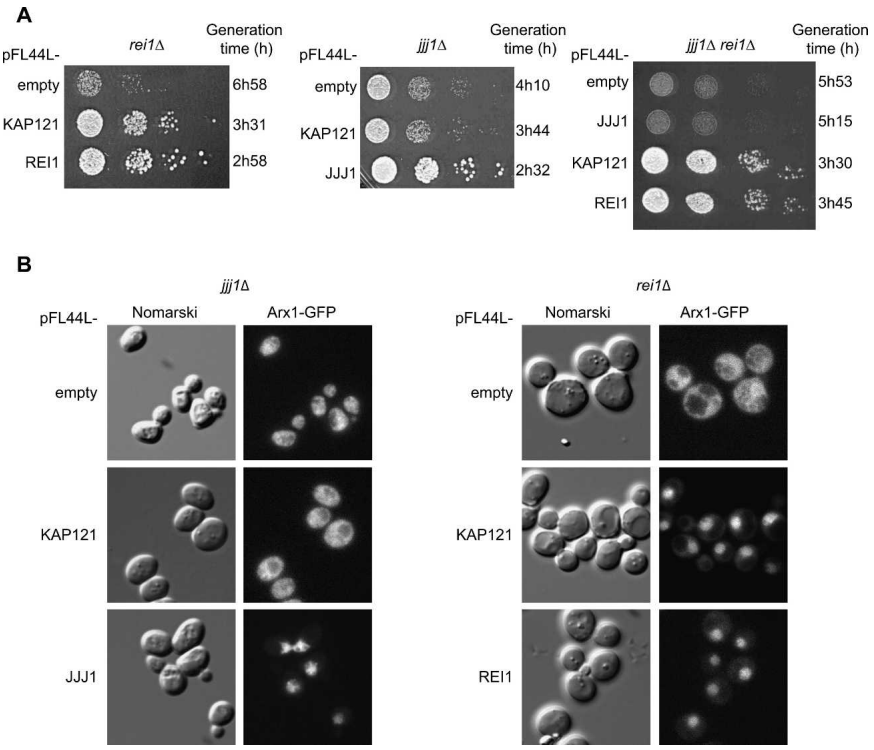

FIGURE 6. *KAP121* overexpression has distinct effects on *rei1Δ* and *jjj1Δ* strains. (*A*) *KAP121* overexpression does not restore the growth of *jjj1Δ*. Ten times serial dilutions of *reiΔ* (LMA523), *jjj1Δ* (ED10), and *rei1Δ jjj1Δ* (ED21) transformed with either the empty vector pFL44L or pFL44L-REI1 or pFL44L-JJJ1 or pFL44L-KAP121 were spotted on solid minimal medium without uracil at 23°C for 2 d. Generations times are indicated. (*B*) *KAP121* overexpression does not restore nuclear localization in the absence of Jjj1. Arx1-GFP, *jjj1Δ* (ED49) and Arx1-GFP, *rei1Δ* (LMA411) strains were transformed with either the empty vector pFL44L or pFL44L-REI1 or pFL44L-JJJ1 or pFL44L-KAP121. The cells were grown in minimal media at 30°C and shifted at 23°C for 2 h. Arx1-GFP localization was observed by fluorescence microscopy.

While this manuscript was being prepared, Meyer et al. (2007) reported a functional analysis of Jjj1. In agreement with our data, Meyer et al. report that Jjj1 is involved in the biogenesis of the 60S large ribosomal subunit and shares similar features with the Rei1 pre-60S factor. Additional experiments presented here revealed that, while the functions of Jjj1 and Rei1 proteins show similarities, they are nevertheless clearly distinct.

While Rei1 and Jjj1 share similar features, they have distinct functions

In addition to the fact that Jjj1 and Rei1 have zinc-finger motifs, both proteins share several similarities. They are late strictly cytoplasmic pre-60S associated factors. Their absence leads to a cold sensitivity and a slow growth phenotype correlated with a relative decrease of the amount of 60S subunit and a weak defect in rRNA maturation (Figs. 1, 3; Hung and Johnson 2006; Lebreton et al. 2006b; Meyer et al. 2007). Both proteins interact together not only in a two-hybrid assay (Fig. 1) but also in co-immunoprecipitation experiments (Fig. 2), revealing that both proteins are biochemically associated to similar pre-60S complexes. Since both Jjj1 and Rei1 are physically associated to the same cytoplasmic late particles, we tried to define the order of assembly of these two factors to the pre-60S particles. Surprisingly, sucrose gradient analysis of strains deleted for *REI1* or *JJJ1* revealed that the binding of each one of these proteins to the pre-60S particles is independent from the presence of the other (data not shown). Here, we observed that, as for the recruitment of Rei1 onto the particle, the binding of Jjj1 is one of the last steps in the ribosome biogenesis. Jjj1, like Rei1, binds to the particle after the release of Rlp24 pre-60S factor from the particles. Thus, the transient presence of Jjj1 on pre-60S particles correlates very well with the chaperone activity of Jjj1.

Besides the biochemical features of Jjj1 and Rei1, several data indicate that the roles of these proteins are intertwined. Both Jjj1 and Rei1 are involved in the recycling of the pre-60S shuttling factors Arx1 and Alb1 (Fig. 5A). The slow growth phenotype of *jjj1Δ* or *rei1Δ* strains can be rescued by the deletion of *ARX1* or *ALB1* (Fig. 4; Hung and Johnson 2006; Lebreton et al. 2006b), suggesting that the fate of Arx1 and Alb1 is responsible for the cold sensitivity of *jjj1Δ* and *rei1Δ* strains. As reported by Meyer et al. (2007), we observe that Arx1 and Alb1 also accumulate in the cytoplasm in the absence of Jjj1.

However, while Jjj1 and Rei1 proteins have strong similarities, we describe here key differences revealing distinct functions of these proteins in 60S formation. Our previous model proposed that, in the absence of Rei1, it is the cytoplasmic accumulation of the small complexes including Arx1 and Alb1 that prevents the release of Tif6 from the pre-60S particle. We show here that, in the absence of Jjj1, Arx1 and Alb1 do not accumulate in small cytoplasmic complexes but remain associated with the pre-60S particle and that Tif6 is correctly recycled to the nucleus (Fig. 5).

Three karyopherins, Kap121/Pse1, Kap123, and Kap104 (Rout et al. 1997; Leslie et al. 2004) are involved in the import of ribosomal components into the nucleus. In a *rei1Δ* strain, overexpression of *KAP121* partially restores the growth defect and allows the recycling of Arx1/Alb1 and Tif6 into the nucleus (Lebreton et al. 2006b) while in the

absence of Jjj1, overexpression of *KAP121* has no effect on Arx1 and Alb1 recycling (Fig. 6). This is correlated with the fact that Arx1 and Alb1 are not released from pre-60S particles in this context.

We conclude that Jjj1 would be preferentially involved in the dissociation of Arx1 and Alb1 from the pre-60S particle, whereas Rei1 would rather be preferentially involved in the recycling of these factors by the karyopherins pathway.

Why is the presence of Arx1 toxic when *JJJ1* or *REI1* are deleted?

Arx1 deficient cells have almost no growth phenotype. Surprisingly, the absence of Arx1 leads to halfmers formation, whereas the amount of 60S does not seem strongly affected (Fig. 4) suggesting that this factor is not directly required for the production of the pre-60S subunit but could rather be important for the quality of the produced 60S subunit. The data presented here and by Hung and Johnson (2006), Lebreton et al. (2006b), and Meyer et al. (2007) suggest that a failure in its recycling may be more deleterious to the cells than its absence. Indeed, we have described two mutants (*rei1Δ* and *jjj1Δ*) whose deleterious phenotypes are correlated with the cytoplasmic accumulation of Arx1 and Alb1 and are rescued by deletion of the *ARX1* or *ALB1* gene. Therefore, it appears that the cytoplasmic accumulation of Arx1 is mainly responsible for the growth defects observed in the absence of either Jjj1 or Rei1. But we now show that the deleterious effect occurs both when Arx1 and Alb1 are released from the pre-60S (*rei1Δ*) or stalled on the pre-60S particle (*jjj1Δ*). The recycling of Tif6 is also affected in the absence of Rei1 as Arx1 and Alb1 accumulate in small cytoplasmic complexes.

Surprisingly, nuclear Arx1 recycling is not necessarily correlated with a rescue of a normal growth of the cells. Indeed, in the absence of Rei1, *JJJ1* (data not shown) and *KAP121* overexpressions allow the recycling of Arx1 into the nucleus (Lebreton et al. 2006b). However, only *KAP121* overexpression is able to partially rescue the wild-type phenotype. In all cases, Arx1 is not anymore associated to the pre-60S particle and the polysome profile is not rescued. When the correct Arx1 process is interrupted, whatever the gene overexpressions tested to rescue the wild-type phenotype, only partial function of Arx1 is recovered, suggesting that the moment and the conditions in which Arx1 and Alb1 are released from the particles and reimported are crucial. Jjj1, as a Hsp40 chaperone catalyzing structural transitions, could play a key role in allowing the commitment of the ribosomal large subunit toward translation initiation.

The role of Jjj1 as a chaperone

The role of Hsp40, characterized by their J domain, is to stimulate the ATPase activity of their Hsp70 partner. Recently, Meyer et al. (2007) have shown that the Hsp70 cochaperone of Jjj1 could be Ssa1. In eukaryotic cells, there are two major cytosolic classes of Hsp70: the *SSA* family composed of fourgenes, *SSA1–4*, and the *SSB* family, encoded by *SSB1* and *SSB2*. Whereas Ssb chaperones are ribosome associated and bind nascent polypeptides to prevent their misfolding at the ribosome exit channel, Ssa chaperones have pleiotropic functions: translocation of the newly synthetized polypeptides to the reticulum endoplasmic or mitochondria and other folding process.

Apart from Jjj1, no chaperones have been found to be specifically involved in the biogenesis of the ribosomal large subunit. Indeed, the RAC (ribosome associated complex), composed of the Hsp40, Zuo1, and its two Hsp70 partners, Ssz1 and Ssb1/2, binds the NAC (nascent chain associated complex), composed of three factors, Egd2, Egd1 and Btt1, to prevent misfolding of the newly synthesized polypeptide at the exit ribosome channel; it is not involved in the maturation of the ribosomal subunits. The specific role of Jjj1 in ribosome biogenesis could be compared to the specific function of the auxilin Hsp40. Indeed, Swa2/auxilin Hsp40 is specifically involved in clathrin uncoating by inducing transconformational changes of clathrin (Ungewickell et al. 1995; Fan et al. 2003; Fotin et al. 2004; Hennessy et al. 2005). Jjj1 seems to be specifically involved in Arx1 release from the particle. Indeed, when the very conserved histidine–proline–aspartic acid (HPD) motif of the J domain is mutated, Arx1 cannot be recycled and accumulates in the cytoplasm (Meyer et al. 2007). This suggests that the function of Jjj1 relies on its chaperone activity.

CONCLUSION

When the pre-60S particles arrive in the cytoplasm, they are not yet competent for translation and a few final steps are required. A strong cytoplasmic control is important because commitment of pre-60S particles into translation initiation should be extremely deleterious for the cells. A coordinated pre-60S particles export and pre-60S factors import should be an important level of regulation to control the entry in translation.

MATERIALS AND METHODS

Plasmids, oligonucleotides, strains, and growth conditions

The strains used in this study are listed in Table 1. Chromosomal insertions were obtained by homologous recombination using PCR fragments in MGD13-353D or BY strains (Baudin et al. 1993). Standard yeast genetic methods and selective growth media were used. The plasmids encoded *JJJ1* as two-hybrid bait (pED1) or prey (pED4) were obtained by Gateway cloning *JJJ1* in pAS2ΔΔ and pACTIIst destination vectors. The other two-hybrid plasmids

TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference
MGD353-13D	*MATa, trp1-289, ura3-52, ade2, leu2,-3-112, arg4*	Rigaut et al. (1999)
BY4741	*MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0*	Brachmann et al. (1998)
BY4742	*MATα, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0*	Brachmann et al. (1998)
ED10	*MATα, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, jjj1Δ::KanMX4*	Winzeler et al. (1999)
ED11	*MATa, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, jjj1Δ::KanMX4*	Winzeler et al. (1999)
ED70	*MATα, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, rei1Δ::KanMX4*	Winzeler et al. (1999)
ED71	*MAT a, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, rei1Δ::KanMX4*	Winzeler et al. (1999)
ED21	*MATa, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, met15?, rei1Δ::NatMX4, jjj1Δ::KanMX4*	This study
ED52	*MATα, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, arx1Δ::Hygro, jjj1Δ::KanMX4*	This study
ED65	*MATα, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, alb1Δ::NATMX4, jjj1Δ::KanMX4*	This study
LMA523	*MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, rei1Δ::NATMX4*	Lebreton et al. (2006b)
LMA539	*MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, arx1Δ::Hygro*	Lebreton et al. (2006b)
LMA525	*MATα, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, alb1Δ:: NATMX4*	Lebreton et al. (2006b)
LMA401	*MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, ARX1–GFP/His3MX6*	Lebreton et al. (2006b)
LMA411	*MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, ARX1–GFP/His3MX6, rei1Δ::KanMX4*	Lebreton et al. (2006b)
ED49	*MATα, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, ARX1–GFP/His3MX6, jjj1Δ::KanMX4*	This study
LMA545-B	*MATa, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, met15Δ0, ALB1-GFP/His3MX6*	Lebreton et al. (2006b)
LMA545-A	*MATa, ura3Δ0, his3Δ1, leu2Δ0, lys2?, met15?, ALB1-GFP/His3MX6, rei1Δ::KanMX4*	Lebreton et al. (2006b)
ED66	*MATa, ura3Δ0, his3Δ1, leu2Δ0, lys2?, met15?, ALB1-GFP/His3MX6, jjj1Δ::KanMX4*	This study
OT52	*MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, JJJ1-TAP/His3MX6*	Winzeler et al. (1999)
ED19	*MATa, trp1-289, ura3-52, ade2, leu2,-3-112, arg4, JJJ1-TAP/klTRP1, REI1-13Myc/KanMX6*	This study
ED46	*MATa, trp1-289, ura3-52, ade2, leu2,-3-112, arg4, REI1-TAP/URA3, JJJ1-13Myc/klTRP1*	This study
ED87	*MATa, trp1-289, ura3-52, ade2, leu2,-3-112, arg4, RLP24-TAP/ klTRP1, JJJ1-13Myc/KanMX6*	This study
LMA352	*MATa, trp1-289, ura3-52, ade2, leu2,-3-112, arg4, RLP24-TAP/klTRP1, REI1-13Myc/KanMX6*	Lebreton et al. (2006b)
ED82	*MATa, trp1-289, ura3-52, ade2, leu2,-3-112, arg4, JJJ1-13Myc/KanMX6*	This study

(*continued*)

were indicated by Lebreton et al. (2006a,b). We constructed an empty vector pED13, which was derived from pFL44L plasmid, by elimination of an insert of the library by Kpn1/Sal1 digestion. The pED12 plasmid was generated by cloning *JJJ1* extending from 350 base pairs upstream from the initiation codon to 640 bp downstream of the stop codon at the Not1 site. Generation times were calculated from growth curves in liquid culture at 23°C during a period of 32 h.

Sucrose gradient sedimentation and Western blotting

The strains were grown at 30°C in appropriate medium to an A_{600} of 0.3–0.6, and the mutant strains were shifted at 23°C for 2 h before cycloheximide treatment. Extracts were prepared in 10 mM Tris-HCl (pH 7.4), 30 mM $MgCl_2$, 100 mM NaCl, and 50 μg/mL cycloheximide using glass beads vortexing. They were fractioned by ultracentrifugation in 10%–50% sucrose gradients for 3 h at 39,000 rpm at 4°C in an SW41 Ti rotor. The fractions were recovered with an ISCO fractionator, and the absorbance at 254 nm was measured. The proteins were precipitated with 10% TCA and separated on polyacrylamide-SDS gels.

Nog1 and Rlp24 (Saveanu et al. 2003), Nog2 (Saveanu et al. 2001), Tif6 (Senger et al. 2001), Rpl1 (a gift of F. Lacroute, Gif sur Yvette, France), and Nmd3 (a gift of A.W. Johnson, Austin, TX, USA) native proteins were detected by indirect immunoblotting, using specific polyclonal rabbit antibodies at a 1:5000 dilution (except at 1:10,000 for Rpl1). Specific polyclonal rabbit antibodies against Arx1 and Rei1 were used at 1:700 and 1:2000, respectively (Lebreton et al. 2006b). TAP-tagged proteins were revealed with a 1:10,000 dilution of peroxidase-antiperoxidase complex (PAP; Sigma). GFP-tagged and 13Myc-tagged proteins were detected using anti-GFP 1:700 (rabbit polyclonal antibody, Santa Cruz) and anti-myc 1:1000 (mouse monoclonal antibody, Santa Cruz), respectively. Secondary antibodies (Goat Anti-Rabbit- or Goat Anti-Mouse-HRP Conjugate from Bio-Rad) were used at a 1:10,000 dilution. Peroxidase activity of the secondary antibodies was revealed using Millipore chemiluminescence HRP substrate system.

High copy number suppressor genetic screen

The *jjj1Δ* strain (ED11) was transformed with a yeast genomic library cloned into a

TABLE 1. *Continued*

Strain	Genotype	Reference
LMA375	*MATa, ura3Δ0; his3Δ1; leu2Δ0; met15Δ0; lys2Δ0, Mak11-TAP/His3MX6*	Saveanu et al. (2007)
ED72	*MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, TIF6-TAP/His3MX6*	Winzeler et al. (1999)
ED89	*MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, TIF6-TAP/His3MX6, jjj1Δ::KanMX4*	This study
ED92	*MATa, ura3Δ0, his3Δ1, leu2Δ0, met15Δ0, TIF6-TAP/His3MX6, rei1Δ::KanMX4*	This study

Question marks indicate that the genotype was not determined for this auxotrophy marker in this strain.

(*URA3*) pFL44L plasmid. The transformants were isolated on minimum medium plate without uracil (−URA). The plates were incubated at 30°C overnight and then shifted at 20°C for several days. The clones that display a slow growth rescue at 20°C were selected by comparing the size of the transformants with those transformed with an empty vector. Their plasmidic DNAs were extracted and DNA inserts were sequenced using M13 forward and reverse universal primers. The suppressor plasmids were tested by retransformation of the *jjj1Δ* strain (ED11).

Fluorescence microscopy

Cells were grown in minimal medium overnight at 30°C to an A_{600} of 0.5. The mutant strains were shifted to 23°C for 2 h. Indirect immunofluorescence of the TAP-tagged fusion proteins was detected using anti-protein A antibodies and Cy3 secondary antibodies as described (Pringle et al. 1991). Fluorescence was visualized using an epifluorescence microscope (model DMRB; Leica) as described by Lebreton et al. (2006b).

RNA extraction, Northern blotting, and primer extension

After growth of each yeast strain in rich media at 30°C up to an A_{600} of 0.5, the cultures were shifted at 23°C for 15 min or 2 h. The cultures were centrifuged, and yeast cells were broken using glass beads. RNA extractions were performed with phenol/chloroform. Mature and intermediate large species were separated on 1% agarose gel and small species were separated on 5% polyacrylamide–urea gels. RNAs were transferred on Hybond+ membranes, and their identification was performed by hybridization with various ^{32}P-labeled oligonucleotides. Primer extensions were performed using ^{32}P-labeled oligonucleotides, and the products were separated on 5% polyacrylamide–urea gels. The sequences of the oligonucleotides were previously described by Saveanu et al. (2001).

Two-hybrid matrix

The strain CG1945 transformed with the bait Gateway plasmid pAS2ΔΔ-*JJJ1*, *REI1*, *ARX1*, *ALB1*, and *RPL24B* was mated with the strain Y187 transformed with the prey Gateway plasmid pActII containing the same open reading frame (ORF). Diploids were selected on minimal medium without leucine and tryptophan (−LW) plates, and the diploids displaying a positive two-hybrid interaction were selected on minimal medium without leucine, tryptophan, and histine (−LWH) plates. An X-Gal overlay was performed according to Fromont-Racine et al. (1997) to select the positive two-hybrid clones on the second reporter gene, the LacZ gene.

Tandem affinity purification

Purifications were performed from 4 L of yeast culture as described by Rigaut et al. (1999) using classical buffers containing 0.1 M NaCl. The TEV eluates were precipitated with 10% TCA, and the final TAP purifications were precipitated with methanol/chloroform. The purified complexes were separated on a 4%–12% polyacrylamide gradient–SDS gel and analyzed by Western blotting as described above.

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