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The ribosomal protein Rps15p is required for nuclear exit of the 40S subunit precursors in yeast

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We have conducted a genetic screen in order to identify ribosomal proteins of *Saccharomyces cerevisiae* involved in nuclear export of the small subunit precursors. This has led us to distinguish Rps15p as a protein dispensable for maturation of the pre-40S particles, but whose assembly into the pre-ribosomes is a prerequisite to their nuclear exit. Upon depletion of Rps15p, 20S pre-rRNA is released from the nucleolus and retained in the nucleus, without alteration of the pre-rRNA early cleavages. In contrast, Rps18p, which contacts Rps15p in the small subunit, is required upstream for pre-rRNA processing at site A2. Most pre-40S specific factors are correctly associated with the intermediate particles accumulating in the nucleus upon Rps15p depletion, except the late-binding proteins Tsr1p and Rio2p. Here we show that these two proteins are dispensable for nuclear exit; instead, they participate in 20S pre-rRNA processing in the cytoplasm. We conclude that, during the final maturation steps in the nucleus, incorporation of the ribosomal protein Rps15p is specifically required to render the pre-40S particles competent for translocation to the cytoplasm.

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Introduction

Ribosomes are found in all living beings, but their production seems to have gained complexity during the course of evolution. Hence, although prokaryotic ribosome assembly was reconstituted *in vitro* more than 30 years ago (Traub and Nomura, 1968), eukaryotic ribosomes have as yet eluded similar attempts. In eukaryotes, ribosome biogenesis gives rise to a particular structure in the nucleus, the nucleolus, which reflects the high degree of spatial organization of this process. As translation occurs in the cytoplasm, the precursors of the two ribosomal subunits (also called pre-ribosomes) have to exit the nucleus to fulfil their function. This transport pathway is intimately linked to particle assembly and pre-rRNA maturation, and constitutes *de facto* a step unique to eukaryotes. How this highly integrated process has evolved to accommodate a spatial boundary between the site of synthesis and the site of activity of the ribosomal subunits is a central question to the understanding of ribosome biogenesis in eukaryotes.

Studies of the yeast *Saccharomyces cerevisiae* have recently given the first insights into pre-ribosome nuclear export (Johnson *et al*, 2002; Tschochner and Hurt, 2003). As for most cargoes exported from the nucleus, the function of the small GTPase Ran/Gsp1p is essential (Hurt *et al*, 1999; Moy and Silver, 1999; Gleizes *et al*, 2001), which regulates the association of cargoes with export receptors or exportins. In the case of pre-ribosomes, the exportin appears to be the karyopherin Crm1p, whose action has been mostly documented for the precursors to the large subunit (60 subunit). The current model states that Crm1p associates with the pre-60S particles in the nucleus through binding to a nonribosomal protein, Nmd3p, which in turn is linked to the ribosomal protein Rpl10p (Ho *et al*, 2000; Gadal *et al*, 2001). Nmd3p contains several leucine-rich nuclear export sequences (NESs) allowing docking of Crm1p. Recent results show that the requirement for Crm1p and Nmd3p for nuclear export of pre-ribosomes is conserved in vertebrates and that Crm1p binds to Nmd3p in a Ran-GTP-dependent manner, as expected for an exportin (Thomas and Kutay, 2003; Trotta *et al*, 2003). Nuclear export of the pre-40S particles is also affected if Crm1p function is altered (Moy and Silver, 1999), but the link between the karyopherin and these pre-ribosomes remains unclear. A subset of the 30 nucleoporins that compose the nuclear pore complex have also been shown to be involved in translocation of both subunits to the cytoplasm (Hurt *et al*, 1999; Moy and Silver, 1999, 2002; Stage-Zimmermann *et al*, 2000; Gleizes *et al*, 2001).

Maturation of the two ribosomal subunits starts with a common precursor, called the 90S pre-ribosome, and diverges after cleavage of the pre-rRNA within the internal transcribed spacer 1 (ITS1) at site A2 (Figure 1A). Recently, combined use of affinity purification of pre-ribosomes and mass spectrometry analysis has shown that the composition of pre-ribosomes is highly dynamic and involves over 150 nonribosomal proteins with various activities, including nucleases, RNA helicases, GTPases, AAA ATPases, kinases, etc. (for reviews, see Fatica and Tollervey, 2002; Milkereit *et al*, 2002; Fromont-Racine *et al*, 2003). Most striking was the discovery that the two subunits follow independent biogenesis pathways, with very little overlap between the factors involved in one or the other (Grandi *et al*, 2002). During rDNA transcription, a large

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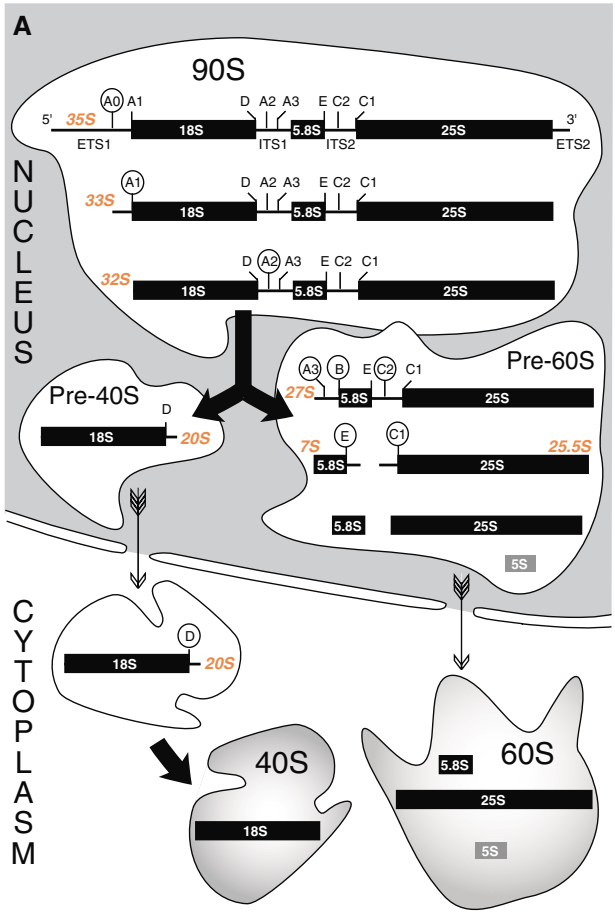

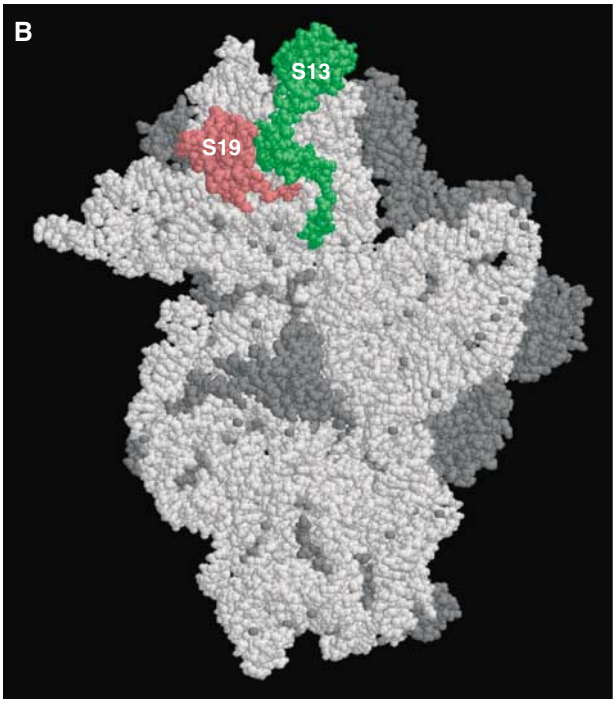

Figure 1 Ribosome biogenesis. (**A**) Pre-rRNA processing and nuclear export in *S. cerevisiae*. (**B**) Structure of the *T. thermophilus* 30S subunit. The ribosomal proteins S19 (red) and S13 (green), homologous to the eukaryotic proteins Rps15p and Rps18p, respectively, form a dimer on the verge of the interface domain. The other ribosomal proteins are shown in grey and the 16S rRNA in white. Figure derived from PDB file 1J5E (Wimberly *et al*, 2000) using RasMol.

complex containing the U3 snoRNP and proteins involved in the cleavage of the external transcribed spacer 1 (ETS1) binds to the 5′-end of the neo-synthesized pre-rRNA (Dragon *et al*, 2002). This complex, called 'the small subunit processome', seems to be released *en bloc* after cleavage of the pre-rRNA at site A2, from which point the fates of the two subunits become distinct. The pre-40S particles are rapidly exported to the cytoplasm, where their maturation is completed. In *S. cerevisiae*, these late cytoplasmic steps include processing of the 20S pre-rRNA to mature 18S rRNA. Only a few nonribosomal proteins have been found to associate with the late pre-40S particles (Schäfer *et al*, 2003). In contrast, the pre-60S particles are further matured in the nucleus: they bind with a large number of nonribosomal factors and undergo a complex maturation process. A proportion of these last biogenesis steps are thought to take place within the nucleoplasm (Kressler *et al*, 1999; Tschochner and Hurt, 2003), where pre-60S particles are much more abundant than 40S precursors (Gleizes *et al*, 2001).

Besides the numerous nonribosomal factors recently identified, the complete list of the ribosomal proteins has been known for many years in yeast. However, their role in ribosome biogenesis is still poorly understood. As mentioned above, studies of large subunit nuclear export have given hints regarding the role of the ribosomal protein Rpl10p in the recruitment of the export factor Nmd3p (Gadal *et al*, 2001). Assuming that this observation could be extended to the 40S particles, we have searched for proteins of the small ribosomal subunit required for, or just prior to, the nuclear export of the pre-40S particles in *S. cerevisiae*, as measured by intranuclear retention of the 20S rRNA precursor. We have screened a collection of mutants underexpressing one of the 32 Rps proteins, and have isolated Rps15p as an essential factor for late nuclear assembly and nuclear export of the small ribosomal subunit. Through a combination of biochemical and cytological approaches, we demonstrate that Rps15p assembly within the pre-ribosomes is dispensable for 35S pre-rRNA processing to 20S pre-rRNA and for release of the particle from the nucleolus, but is a prerequisite for nuclear exit of the pre-40S particles to the cytoplasm. Pre-ribosomes assembled in the absence of Rps15p contain most of the pre-ribosomal factors expected to be associated with pre-40S particles, indicating that Rps15p acts late in the small subunit biogenesis pathway. We propose that Rps15p participates in a late stage of the assembly of pre-40S particles within the nucleus and controls the export of these precursors to the cytoplasm.

Results

Identification of Rps15 as a putative nuclear export factor for the pre-40S particle

In order to identify small ribosomal subunit proteins (Rps proteins) required for nuclear export of the pre-40S particles, we screened all the strains from the EUROSCARF collection deleted for any one of the genes encoding these proteins. Out of the 32 Rps proteins in yeast, 24 are synthesized from duplicated genes. Single deletion of one gene copy is not lethal, but we found that it affects growth rate in all cases when compared to wild-type cells (data not shown). In the case of the eight Rps proteins encoded by a single gene, we looked at diploid strains bearing heterozygous deletions of

these genes. These 48 haploid and eight diploid strains were subjected to fluorescence *in situ* hybridization with a probe complementary to the 5′ part of the ITS1, which in *S. cerevisiae* is cleaved off in the cytoplasm. In wild-type cells, this probe yields intense labelling of the nucleolus and a moderate signal in the cytoplasm, whereas nucleoplasm is only faintly fluorescent due to rapid transfer of the pre-40S particles from the nucleolus to the cytoplasm (see Figure 2A).

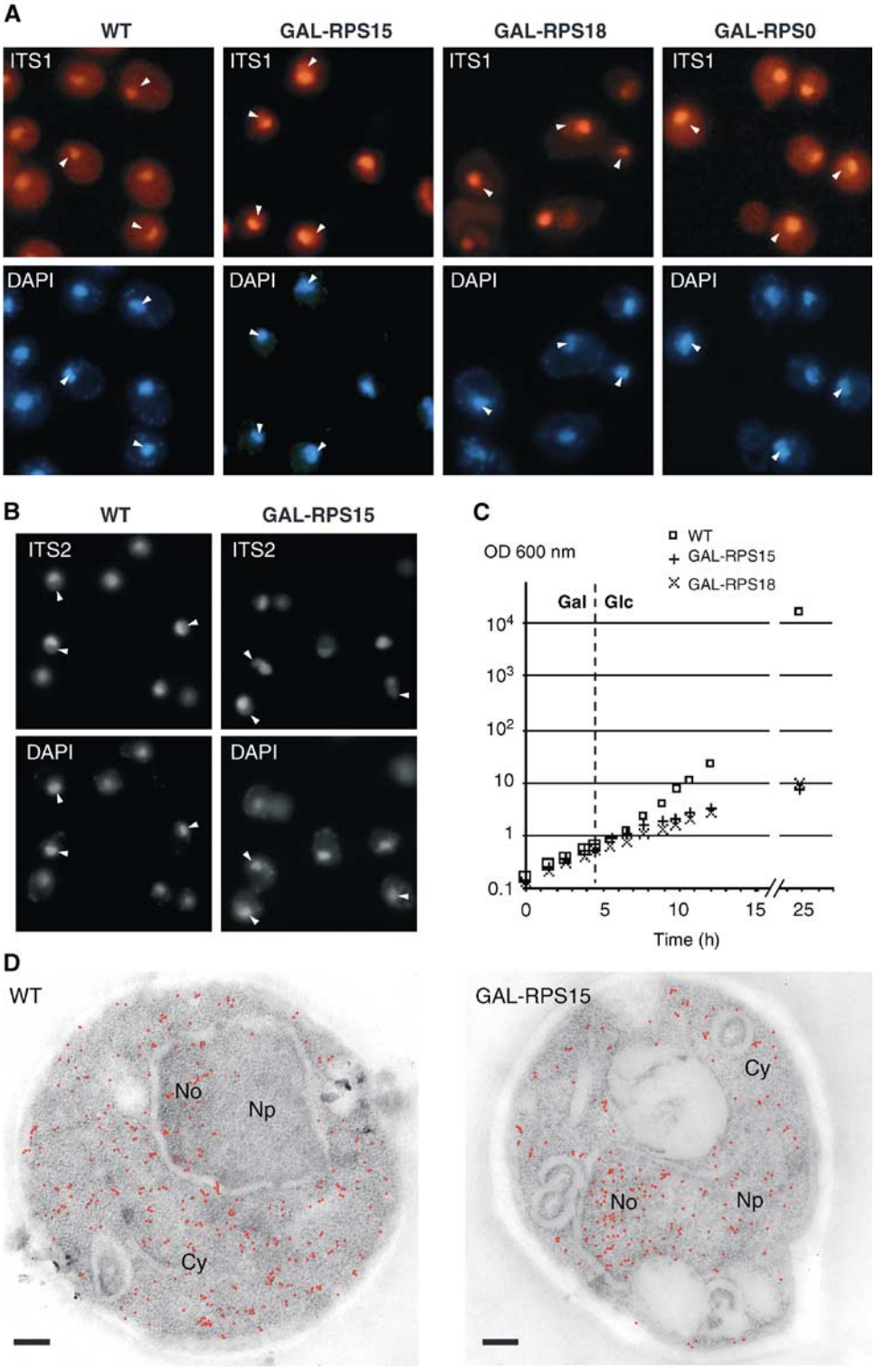

Figure 2 Rps15p-depleted cells accumulate precursors to the 18S rRNA in the nucleus. (**A**) Pre-18S rRNA FISH with a probe complementary to the D–A2 segment of the ITS1 in wild-type, GAL-RPS15, GAL-RPS18, and GAL-RPS0 strains grown for 4 h in glucose-containing medium. Arrowheads indicate the nucleoplasm as visualized by DNA staining with DAPI. (**B**) Pre-5.8/25S rRNA detected with a probe to the E-C2 domain in the ITS2. (**C**) Growth of wild-type, GAL-RPS15, and GAL RPS18 cells. At $t = 4.5$ h (dotted line), YP-galactose medium was replaced by YP-glucose. (**D**) Detection by electron microscopy of pre-18S RNAs with a riboprobe complementary to the D–A2 fragment of the ITS1. Cells were grown as in (A). Gold particles are highlighted in red. No: nucleolus; Np: nucleoplasm; Cy: cytoplasm. Bar = 200 nm.

We searched for strains displaying a very low level of ITS1 in the cytoplasm and accumulation of pre-18S RNA in the entire nucleus. As previously shown by us and others (Gleizes *et al*, 2001; Moy and Silver, 2002), such a phenotype is more likely to reflect a defect in nuclear export than in maturation of the pre-40S particles.

Only a few strains met these criteria to various degrees. The strain displaying the expected phenotype with the highest frequency and intensity was unambiguously the diploid strain bearing the heterozygous deletion of the *RPS15* gene. The group of positive strains also included *rps18AΔ* and *rps18BΔ*, which underexpress Rps18p, although labelling of the nucleoplasm was much weaker than in the *rps15+/−* strain. In crystal structures of bacterial 30S subunits, the bacterial counterparts of Rps15p and Rps18p, namely S19 and S13, form a dimer on the top of the 'head' and on the verge of the interface domain (Figure 1B). Owing to the structural relationship between the two proteins, we focused our attention on both Rps15p and Rps18p. We derived haploid strains expressing either of these proteins under the control of the *GAL1/GAL10* upstream activating sequence. These strains, called GAL-RPS15 and GAL-RPS18, grew as fast as the parental wild-type strain in galactose-containing medium, whereas their growth started to decrease 4 h after substitution of galactose by glucose (Figure 2C).

In the presence of galactose, the distribution of ITS1 in these strains was the same as in wild-type cells (not shown), indicating full complementation by the constructs under permissive conditions. However, culture in glucose-containing medium for 4 h was sufficient to induce a complete redistribution of the labelling to the entire nucleus in GAL-RPS15 cells, with hardly any signal persisting in the cytoplasm (Figure 2A). At this time point, Rps15p mRNA was reduced by at least 90% when compared to wild-type cells (data not shown). The change of ITS1 localization could be observed as soon as 1–2 h after the change of carbon source (not shown), indicating that it is very sensitive to shortage of newly synthesized Rps15p. FISH with a probe complementary to ITS2 did not reveal a similar accumulation of pre-25S/pre-5.8S rRNA in the nucleoplasm of Rps15p-depleted cells (Figure 2B), suggesting that the ITS1-containing pre-rRNAs detected in this compartment correspond to 18S rRNA late precursors after cleavage at site A2 or A3.

Upon depletion of Rps18p in glucose-containing medium, FISH also showed a dramatic drop of the ITS1 level in the cytoplasm (Figure 2A). However, unlike in Rps15p-depleted cells, detection of the probe in the nucleus was mostly restricted to the nucleolus, with only a faint nucleoplasmic signal. We observed yet another phenotype in cells depleted of Rps0p, which have previously been shown to have a defect in 20S pre-rRNA processing (Ford *et al*, 1999); in this case, the cells displayed a strong signal in both the nucleolus and the cytoplasm (Figure 2A).

Nuclear retention of pre-18S RNAs in Rps15p-depleted cells was confirmed at the ultrastructural level. Detection of ITS1 on ultrathin sections showed a marked redistribution of labelling from the cytoplasm to the entire nucleus, as compared to that seen in wild-type cells (Figure 2D). Gold particles were present over the nucleoplasm in regions whose dense appearance may correspond to an accumulation of ribonucleoprotein particles.

Rps15p depletion leads to retention of 20S pre-rRNA containing pre-ribosomes in the nucleus

The ribosomal RNA content of wild-type, GAL-RPS15, and GAL-RPS18 cells was extensively analysed by Northern blotting (Figure 3A). Whereas these strains displayed similar pre-rRNA patterns when grown in the presence of galactose, depletion of Rps15p in glucose-containing medium resulted in a three to four-fold decrease in the 18S rRNA level and a two-fold accumulation of 20S pre-rRNA, when compared to wild-type cells (Figure 3B). The 35S pre-rRNA was more abundant upon depletion of Rps15p, but 23S pre-rRNA was detected only after 8 h in glucose, indicating that altered ETS1 processing is not a primary consequence of loss of Rps15p function. Production of 25S rRNA appeared to be unaffected, as revealed by probes complementary to the mature 25S rRNA or to 25S precursors (A2–A3, E-C2). These results indicate that Rps15p depletion specifically induces a late defect in 18S rRNA synthesis. Consistent with these results, gradient analysis of ribosomal particles extracted under similar culture conditions showed a drop in 40S subunits and polysomes, and an accumulation of free 60S particles upon Rps15p depletion (not shown).

Loss of Rps18p also led to a strong drop in 18S rRNA, but 21S pre-rRNA was formed instead of 20S (Figure 3A and B). In parallel, the 27S-A2 pre-rRNA disappeared, revealing complete inhibition of cleavage at site A2. A slight accumulation of both 33S and 23S RNA indicates that early cleavage at sites A0/A1 is also partly deficient. The production of 25S rRNA remained unaffected, 27S pre-rRNA being produced by cleavage at site A3 or B. Thus, Rps18p is also necessary for the synthesis of the small subunit, but is involved in pre-rRNA processing at site A2, upstream of nuclear export.

We then performed nuclear-cytoplasmic fractionations to quantify the relative distribution of the accumulating 20S pre-rRNA in Rps15p-depleted cells. As shown in Figure 3C, 90% of the 20S pre-rRNA was located in the cytoplasm in wild-type cells. This is consistent with rapid nuclear exit of the pre-40S particles after pre-rRNA cleavage at site A2. It indicates that the rate-limiting step in 20S pre-rRNA processing is not nuclear export, but rather cleavage in the cytoplasm. It is noteworthy that, in wild-type cells, the more intense labelling of the nucleus with the ITS1-specific probe observed upon FISH analysis may give the misleading impression of a larger absolute amount of precursors in the nucleus, whereas it reflects rather the different concentrations of the pre-rRNAs in the nucleolus and the cytoplasm.

In contrast to wild-type cells, around 60% of the 20S pre-rRNA is retained in the nucleus of Rps15p-depleted cells (Figure 3C). This is fully consistent with the FISH experiment showing ITS1 nuclear accumulation under the same conditions. Thus, lack of Rps15p leads to nuclear retention of late pre-40S particles containing 20S pre-rRNA, but does not affect earlier processing steps.

Dynamic assay of pre-40S particle nuclear export upon loss of Crm1p or Rps15p function

From the experiments presented above, it appears that Rps15p is primarily involved in pre-ribosomal nuclear export, whereas Rps18p is required for pre-rRNA maturation. To complement the steady-state view of pre-ribosome status in Rps15-depleted cells, we used a more direct and dynamic assay of pre-rRNA maturation and nuclear export, and spe-

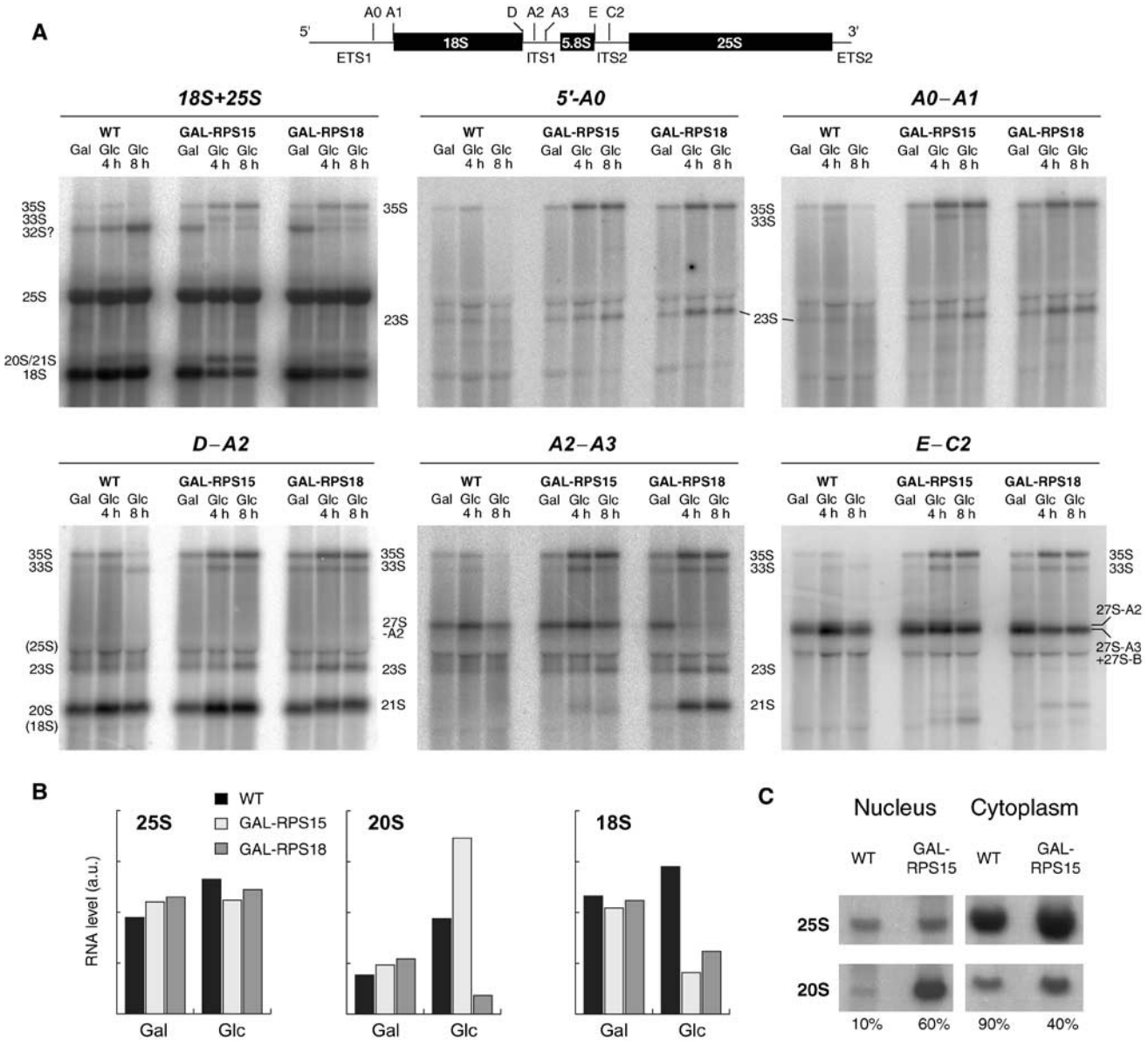

Figure 3 Northern blot analysis of pre-rRNA processing in Gal-RPS15 and GAL-RPS18 cells. (**A**) Northern blot of total RNA extracts from wild-type, GAL-RPS15, and GAL-RPS18 cells grown in YP-galactose medium (Gal), or transferred for 4 or 8 h to YP-glucose (Glc). (**B**) Levels of 25S, 20S, and 18S RNAs in the cells grown in YP-galactose medium (Gal) or shifted to YP-glucose medium for 4 h (Glc); quantitative analysis was performed on the Northern blots probed with the 25S, 18S, and D–A2 probes (a.u.: arbitrary units). (**C**) Detection of the 20S and 25S RNAs extracted from the nuclear and cytoplasmic fractions of wild-type and GAL-RPS15 cells grown for 4 h in YP-glucose medium. The distribution of 20S pre-rRNA in the two fractions was evaluated by phosphorimager quantification.

cifically analysed the transport of neo-synthesized rRNAs. For this purpose, yeast cells were first metabolically labelled with [^{3}H]uracil for 15 min before proceeding to nuclear-cytoplasmic fractionation. This approach was first tested on cells bearing the *CRM1* leptomycin B-sensitive allele *crm1-T539C* (Neville and Rosbash, 1999). FISH experiments have shown that these cells accumulate pre-40S particles in the nucleus when treated with leptomycin B (Moy and Silver, 2002; our unpublished results). As seen in Figure 4A, labelling cells expressing the wild-type *CRM1* allele with [^{3}H]uracil for 15 min at 30°C was sufficient to detect radioactive mature 18S and 25S rRNAs in the cytoplasm. This fraction also contained most of the neo-synthesized 20S pre-rRNA, confirming that this species is very rapidly exported from the nucleus and then processed in the cytoplasm with slower kinetics. As expected, all the other pre-rRNAs were found in the nucleus. Strikingly, incubation of MNY8 (*crm1-T539C*) cells for as little as 5 min with 100 nM LMB resulted in a dramatic drop in neo-synthesized 18S and 25S rRNAs in the cytoplasm, parallelled by nuclear retention of 20S and 25S rRNAs, directly showing the requirement of Crm1p for nuclear export of both subunits. Thus, this technique is sensitive enough to monitor in parallel transport disturbances and pre-rRNA processing defects, even after short times under non-permissive conditions.

When applied to GAL-RPS15 cells depleted of Rps15p (Figure 4B), pre-rRNA metabolic labelling showed the complete retention of neo-synthesized 20S pre-rRNA in the

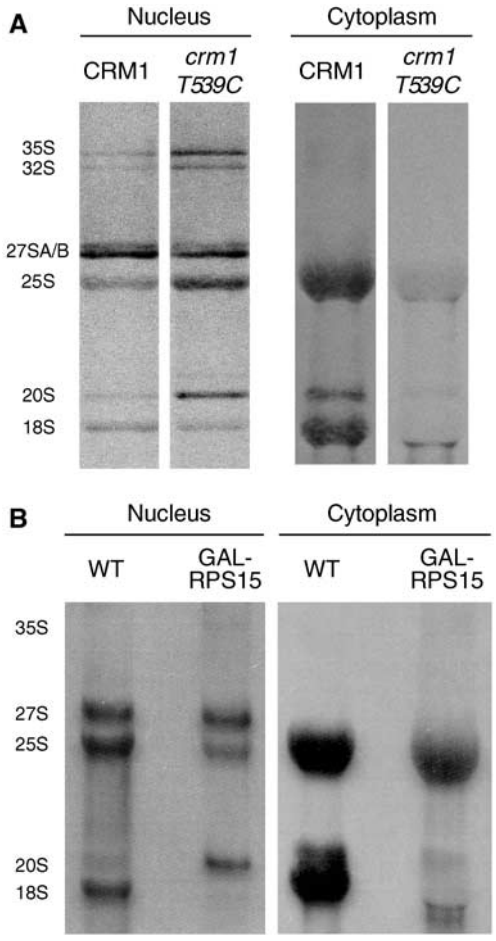

Figure 4 Analysis of newly synthesized pre-ribosome nuclear export by metabolic labelling and cell fractionation. Newly synthesized RNAs in spheroblasts were labelled for 15 min with [^{3}H]uracil prior to nuclear-cytoplasmic fractionation. RNAs were then isolated and analysed by gel electrophoresis and fluorography. Equivalent amounts of radioisotope were loaded in each lane. (**A**) Nuclear export of pre-ribosomes in *crm1*Δ cells expressing either the wild-type CRM1 gene (MNY7) or the leptomycin B (LMB)-sensitive *crm1T539C* mutant allele (MNY8) from a plasmid. LMB was added to the medium 5 min before the addition of [^{3}H]uracil. Note the nuclear retention of the 25S and 20S (pre-) rRNA in the LMB-sensitive cells. (**B**) Upon Rps15p depletion in GAL-RPS15 cells (4 h in the presence of glucose), 20S pre-rRNA, but not 25S rRNA, is blocked in the nucleus.

nucleus and the absence of 18S rRNA in the cytoplasm, as in LMB-treated MNY8 cells. Consistent with the Northern blot analysis, pre-18S rRNA processing upstream of cleavage at site D appeared unaffected. In contrast to Crm1p-deficient cells, the level of 25S rRNA in the cytoplasm was comparable to that of wild-type cells, indicating efficient nuclear exit of the pre-60 particles. We observed a moderate decrease in pre-rRNA synthesis; this is linked to the slower growth rate measured under these conditions, and is probably secondary to the unbalanced production of the two ribosomal subunits and a translation deficit. In both MNY8 and GAL-RPS15 cells, pre-20S RNA accumulating in the nucleus was less abundant than mature 18S rRNA in wild-type cells, suggesting faster turnover of the pre-40S particles blocked in the nucleus. This dynamic analysis concurs with the FISH and Northern blot studies, and points out towards a specific role of Rps15p in a very late nuclear step of 40S subunit biogenesis, just prior to translocation to the cytoplasm.

Dynamics of early and late pre-ribosomal factors upon Rps15p depletion

To more precisely define the stage along the biogenesis pathway at which pre-40S particles accumulate in Rps15p-depleted cells, we tagged pre-ribosomal factors involved in different steps: Noc4p is part of the small subunit processome in the 90S particle (Dragon *et al*, 2002; Grandi *et al*, 2002); Enp1p is found both in 90S and in pre-40S particles (Schäfer *et al*, 2003); Tsr1p and Rio2p are associated with late pre-40S particles (Schäfer *et al*, 2003; Vanrobays *et al*, 2003). The corresponding open reading frames (ORFs) were fused with a sequence encoding the TAP tag (Rigaut *et al*, 1999), both in wild-type and GAL-RPS15 cells. Immunodetection of these proteins with anti-protein A antibodies by fluorescence microscopy showed distinct localizations in wild-type cells (Figure 5A): Noc4p was found in the nucleolus, Enp1p was found both in the nucleolus and the cytoplasm, whereas Tsr1p and Rio2p were mainly cytoplasmic. Upon depletion of Rps15p, Noc4p remained nucleolar while, in contrast, Enp1p and Tsr1p distribution mimicked that of ITS1; these proteins, nucleolar and cytoplasmic in wild-type cells, relocalized to the entire nucleus after Rps15p depletion, suggesting that they are associated with the pre-40S particles retained in the nucleus. Interestingly, Rio2p localization was restricted to the cytoplasm both in the presence and the absence of Rps15p. Thus, Rio2p does not accumulate with the small subunit precursors retained in the nucleus under these conditions.

We next checked the nature of the pre-rRNAs bound to these proteins in cells lacking Rps15p. Noc4p-TAP, Rio2p-TAP, Enp1p-TAP, and Tsr1p-TAP were immunoprecipitated from whole-cell extracts using IgG sepharose, and co-purifying RNAs were analysed by Northern blot. As shown in Figure 5B, Enp1p-TAP and Tsr1p-TAP mostly co-precipitated with 20S pre-rRNA, which was significantly enriched when compared to the input, confirming that they are part of the late pre-40S particles accumulating in the nucleus under these conditions. In contrast, Noc4p-TAP was strongly associated with the 35S, 32S, and 23S pre-rRNAs, which are correlated with its nucleolar localization. The proportion of 20S pre-rRNA precipitated from the input was barely higher than that of 25S rRNA, which is here taken as background. Last, 20S pre-rRNA was also co-enriched with Rio2p-TAP under nonpermissive conditions, but two-fold less than when Rps15p was synthesized (Figure 5B), whereas the fraction of 20S pre-rRNA precipitated with Enp1p-TAP and Tsr1p-TAP did not change. Given the cytoplasmic localization of Rio2p in Rps15-depleted cells, this fraction of 20S pre-rRNA most probably corresponds to the remaining 40% found in this compartment (see Figure 3).

We conclude that the lack of Rps15p blocks the pre-40S particles in the nucleoplasm after dissociation of Noc4p, a bona fide component of the small subunit processome, and before the incorporation of Rio2p.

Correctly assembled pre-40S particles are blocked in the nucleus in the absence of Rps15p

The above results show that Enp1p-TAP remains associated with the pre-40S particles accumulating in the nucleus when

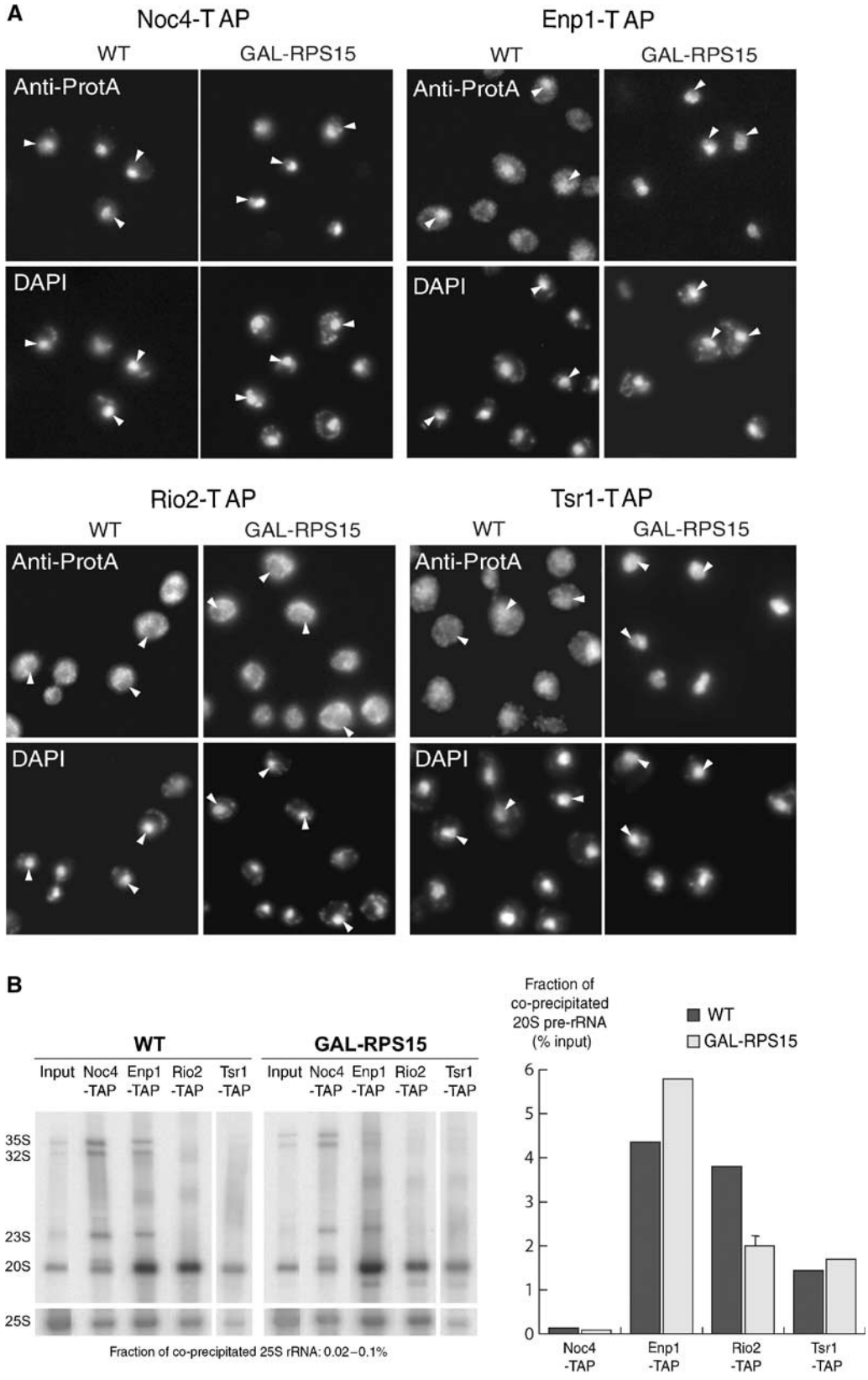

Figure 5 Behaviour of pre-ribosomal factors in Rps15p-depleted cells. (**A**) Immunolocalization of TAP-tagged Noc4p, Enp1p, Tsr1p, and Rio2p in wild-type and GAL-RPS15 strains cultured for 4 h in the presence of glucose. Immunofluorescence with anti-protein A antibodies. Arrowheads indicate the nucleoplasm as visualized by DNA staining with DAPI. (**B**) Detection of pre-18S rRNAs co-purifying with TAP-tagged Noc4p, Enp1p, Rio2p, and Tsr1p in Rps15p-depleted cells. GAL-RPS15 cells expressing one of the TAP-tagged proteins were grown for 4 h in YP-glucose medium and total cell extracts were submitted to immunoprecipitation with IgG-Sepharose. Co-precipitating RNAs were revealed after Northern blot with a probe hybridizing between points D and A2 in the ITS1. All samples, except Tsr1-TAP, were processed in parallel, starting with equivalent amounts of cell extract; one representative input lane (0.05% of input) is displayed. Semiquantification of the fraction of precipitated RNA was performed by phosphorimager analysis.

Rps15p is missing. This protein has been previously used to isolate pre-40S particles by TAP purification and identify pre-ribosomal factors (Schäfer *et al*, 2003). In order to characterize the composition of the pre-ribosomes blocked just prior to nuclear export, we TAP-purified Enp1p-TAP from Rps15p-depleted cells and examined the associated proteins by SDS–PAGE, using wild-type cells as a control (Figure 6). Partial analysis by mass spectrometry of the proteins from wild-type cells identified components of the 90S pre-ribosomes (Utp4p, Utp7p, Noc4p, Nop14p, Krr1p) and of 40S precursors (Ltv1p, Tsr1p, Rrp12p, Hrr25p, Dim1p), as expected for a protein previously shown to belong to both the 90S and pre-40S particles. After 3 h of Rps15p depletion by growth in glucose, the protein profile of the Enp1p-associated complex was similar to that obtained in a wild-type background and included the pre-40S-specific factors Rrp12p, Ltv1p, and Hrr25p. Interestingly, the amount of co-purifying Tsr1p appeared to be reduced when compared to wild-type cells. Thus, although Tsr1p is still associated with 20S pre-rRNA and is found in the nucleus under these conditions (Figure 5), we conclude that the binding of this protein to pre-40S pre-ribosomes is more labile when Rps15p is absent. However, these data indicate that most of the known pre-40S nonribosomal factors are present within the pre-40S particles accumulating in the nucleoplasm.

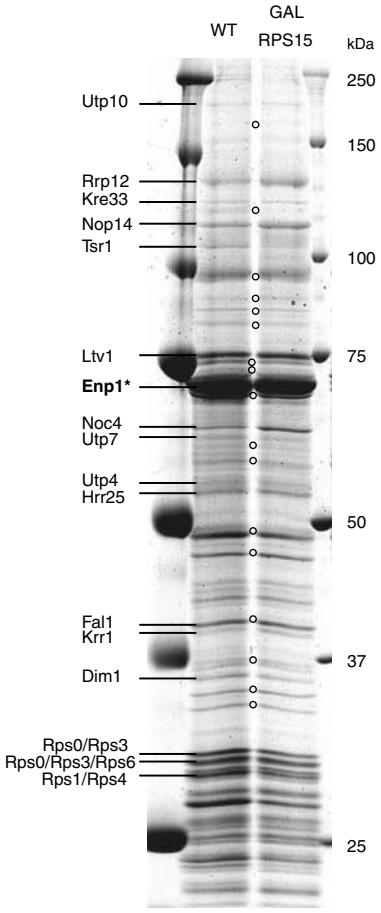

Figure 6 Composition of the pre-40S complexes in wild-type and Rps15p-depleted cells. Wild-type and GAL-RPS15 cells expressing Enp1-TAP were grown in the presence of galactose and then shifted to medium containing glucose for 3 h. After TAP purification, the Enp1p-associated proteins were separated by SDS–PAGE and identified by mass spectrometry. Rio2p could not be detected in these preparations, and may be masked by a degradation product of Enp1p-TAP.

The late associating proteins Tsr1p and Rio2p are necessary for 20S pre-rRNA processing in the cytoplasm

From the results presented in Figures 5 and 6, it can be hypothesized that nuclear retention of pre-ribosomes in the absence of Rps15p is directly linked to disturbance of the association of Rio2p and Tsr1p with the particles. Tsr1p, a putative GTP-binding protein, and Rio2p, an ATP-dependent kinase, were independently shown to be necessary for 20S pre-rRNA maturation to 18S rRNA (Gelperin *et al*, 2001; Vanrobays *et al*, 2003). Using an Rps2–GFP fusion protein as a reporter, Schäfer *et al* (2003) recently published data suggesting a role of these two proteins in pre-ribosomal export; in contrast, Vanrobays *et al* (2003) found that depleting cells of Rio2p did not hamper 20S pre-rRNA translocation to the cytoplasm. To gain further insight into the role of these proteins in the late maturation steps of the pre-40S particles, the precursors of the 18S rRNA were detected by FISH in strains either bearing a thermosensitive allele of RIO2 (*rio2-1*; Schäfer *et al*, 2003), or expressing TSR1 under the control of a GAL promoter (Gelperin *et al*, 2001). When grown under nonpermissive conditions, these strains displayed a brighter labelling of the cytoplasm compared to that of wild-type cells (Figure 7), indicating that 20S pre-rRNA is exported from the nucleus, but not converted to 18S rRNA. The data obtained here with the *rio2-1* thermosensitive allele are fully consistent with observations that we have previously made in a GAL-RIO2 strain (Vanrobays *et al*, 2003). To examine the effect of the combined loss of expression of both factors, we derived a haploid strain expressing both Rio2p and Tsr1p under the control of the GAL promoter. Depletion of both proteins after 12 h in the presence of glucose led again to a strong accumulation of 20S pre-rRNA in the cytoplasm. Thus, we conclude that Rio2p and Tsr1p do not participate in nuclear export of the pre-40S particles, but are necessary for 20S pre-rRNA processing at site D in the cytoplasm.

Discussion

The work presented here allows us to identify Rps15p as a factor essential for nuclear export of pre-40S particles. This conclusion is supported by a number of observations in Rps15p-depleted cells: (1) 18S rRNA synthesis and 40S subunit production are greatly impaired; (2) the pre-18S maturation pathway is specifically altered in its last step, namely 20S pre-rRNA processing at site D, whereas maturation of earlier 18S precursors is not affected; (3) 20S pre-rRNA is retained in the nucleus, as seen both by *in situ* hybridization and cell fractionation after *in vivo* pre-rRNA labelling; (4) the pre-40S particles accumulating in the nucleoplasm are associated with late pre-40S nonribosomal proteins, as verified by localizing some of these proteins and analysing the particle protein composition; (5) in parallel, the 60S subunit production pathway is not affected.

Accumulation of 20S pre-rRNA in the nucleoplasm has only been observed in cells mutated in genes encoding proteins involved in nucleocytoplasmic transport, including nucleoporins, Ran accessory factors, exportin Crm1p (Moy and Silver, 1999, 2002; Gleizes *et al*, 2001), as well as upon

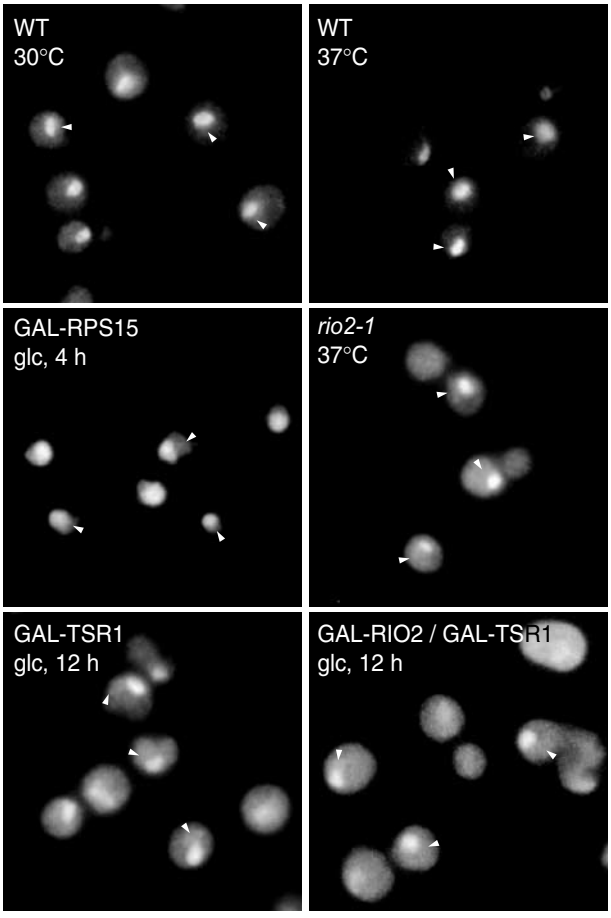

Figure 7 Rio2p and Tsr1p are required for 20S pre-rRNA cleavage in the cytoplasm. Cells bearing the thermosensitive allele *rio2-1* were grown in YP-glucose medium at 25°C and shifted to 37°C for 3 h. GAL-TSR1 cells were grown in glucose-containing medium for 12 h. ITS1 detection by FISH shows a clear increase of the cytoplasmic signal under nonpermissive conditions, compared to wild-type cells. This phenotype is also observed when Rio2p and Tsr1p are simultaneously affected in a GAL-RIO2/GAL-TSR1 strain. GAL-RPS15 cells grown under nonpermissive condition are presented here for comparison. Arrowheads indicate position of the nucleoplasm as detected in parallel by DAPI staining.

alteration of Rrp12p, a pre-ribosomal protein containing HEAT repeats (Oeffinger *et al*, 2004). Loss of function of early processing factors, like snoRNP proteins, or of the ribosomal proteins Rps18p (this study) and Rps27p blocks pre-40S particles in the nucleolus (Moy and Silver, 1999; Gleizes *et al*, 2001). These observations, when correlated with the composition of the accumulating particles, indicate that Rps15p is required for the biogenesis of 40S ribosomal subunits when the pre-40S particles are already competent for release from the nucleolus, but before nuclear export. This step corresponds to a short and precise time frame; quantification of 20S pre-rRNA distribution by cell fractionation points out that this species is very rapidly exported to the cytoplasm in wild-type *S. cerevisiae* cells. In addition, the *in vivo* labelling experiments presented here clearly show that 20S pre-rRNA nuclear export is much faster than processing at site D. A similar conclusion was reached 30 years ago by Planta and co-workers in *Saccharomyces carlsbergensis* (Trapman and Planta, 1976). Accordingly, only a small fraction of the nuclear pre-40S particles is detected in the nucleoplasm of wild-type yeast by electron microscopy, consistent with a rapid transfer from the nucleolus to the cytoplasm (Gleizes *et al*, 2001).

Rps15p was first identified in the mid-1980s as a protein overexpressed in Rat insulinoma cells, and is found in the literature under the name 'RIG protein' (Takasawa *et al*, 1986). It is composed of 142 amino acids in *S. cerevisiae* and is highly conserved in eukaryotes. In accordance with a role of Rps15p in a late nuclear step of the pre-40S pathway, pulse-chase experiments in mammalian cells have found that Rps15p (also called S21) associates with the precursors of the small subunits in the nucleus at a late stage (reviewed by Hadjiolov, 1985). This result can also be correlated with the *in vitro* 30S subunit assembly scheme in *Escherichia coli*, where the Rps15p homologue S19 is considered a secondary binder. Along the same line, Rps15p was not found in yeast 90S particles analysed by mass spectrometry.

Rps18p, which putatively forms a dimer with Rps15p in eukaryotic pre-40S particles, is shown here to be required for pre-rRNA cleavage at site A2. At the time this article was being prepared, a similar phenotype was reported in a strain bearing a deletion of RPS18A, one of the two genes encoding Rps18p (Tabb-Massey *et al*, 2003). Indeed, Rps18p has been previously shown to co-purify with Enp1p, consistent with this protein being part of 90S particles (Grandi *et al*, 2002). Interestingly, the 21S-containing particles in Rps18p-depleted cells do not accumulate in the nucleoplasm, suggesting that they are retained in the nucleolus or rapidly degraded. These observations put Rps18p just upstream of Rps15p in the pre-ribosomal processing scheme and indicate that Rps18p is functional in Rps15p-depleted cells.

Recent proteomic analyses have revealed that pre-40S particles, purified with Enp1p-TAP, contain only a small number of nonribosomal proteins compared to 90S pre-ribosomes (Schäfer *et al*, 2003). Our data show that depletion of Rps15p blocks the pre-40S particles at a yet uncharacterized intermediate stage, with most specific pre-40S factors properly assembled. However, Tsr1p and Rio2p, two proteins among the last to bind to the pre-40S particles, appear to be partially or totally misassembled in the absence of Rps15p. Rio2p remains cytoplasmic in Rps15p-depleted cells despite nuclear retention of pre-40S particles, which clearly indicates that it is not recruited in these pre-ribosomes. Since this protein contains a putative NES (discussed by Schäfer *et al*, 2003), it is possible that Rio2p, in the absence of Rps15p, is actively transported out of the nucleus by Crm1p, because it is not incorporated into the pre-ribosomes. In addition, analysis of the pre-40S particle composition suggests here that Tsr1p is only loosely associated to these particles upon depletion of Rps15p. Loss of function of either of these two proteins has previously been shown to result in a specific defect in 20S pre-rRNA processing (Gelperin *et al*, 2001; Vanrobays *et al*, 2003). Here, we show by FISH that this 20S pre-rRNA accumulates in the cytoplasm, whereas its nuclear export is only marginally affected. Turning off the expression of both genes leads to the same result. Thus, these two proteins are required for 20S pre-rRNA processing in the cytoplasm, as already shown for Rio2p elsewhere (Vanrobays *et al*, 2003).

Figure 8 illustrates the positions along the 40S subunit biogenesis pathway that we may assign to Rps15p and to the other proteins studied here, in terms of assembly and function. In wild-type cells, Tsr1p is located in part in the

nucleolus, suggesting that it associates with newly formed pre-40S particles in this compartment. In contrast, we barely detect Rio2p in the nucleus under normal conditions, and find it to be excluded from the nucleus after depletion of Rps15p. We propose that Rio2p associates with pre-40S particles in the cytoplasm, similar to Rio1p, a homologous protein also required for 20S pre-rRNA processing to 18S rRNA (Vanrobays *et al*, 2001).

In conclusion, incorporation of Rps15p appears to be a prerequisite for assembling nuclear export-competent precursors to the small ribosomal subunits. To our knowledge, this is the first ribosomal protein found to be involved in this transport process. Using *in vivo* labelling followed by nuclear-cytoplasmic fractionation, we show here in an unprecedented way how rapid inhibition of Crm1p hampers nuclear export of the small subunit, without major alteration of pre-rRNA processing, which emphasizes the potential role of this exportin in this transport process. Rps15p may contain a nuclear export signal that would serve as a binding site for Crm1p, but to date we have been unable to find such a functional sequence in Rps15p. Rps15p may instead recruit the nuclear export machinery through interaction with an adaptor protein, such as Rpl10p and Nmd3p in the large subunit precursors. When compared with its eubacterial homologue S19, Rps15p has a long N-terminal extension, as can be found in many conserved ribosomal proteins. This N-terminal domain might be linked to eukaryotic specific functions, including binding of transport factors. Alternatively, Rps15p may be involved in a conformation change of the 20S pre-rRNA preceding the association of Rio2p and of other factors. In the crystal structure of the *Thermus thermophilus* small subunit, S19 has a large contact area with the 16S rRNA and thus might help in RNA folding and stability (Brodersen *et al*, 2002). A conformation change could also be required for association of the nuclear export machinery to the pre-ribosomes, and/or for translocation through the nuclear pore complex. These hypotheses are currently being explored.

Materials and methods

Strains

The strains used in this work and their origins are indexed in Table I. To construct yeast strains expressing RPS genes under control of the conditional GAL promoter, RPS ORFs were PCR-amplified from *S. cerevisiae* genomic DNA and cloned into a CEN URA3 plasmid containing a *GAL1/10-CYC1* promoter (pFL38-GAL). This plasmid was constructed by subcloning the *GAL1/10-CYC1* cassette from pYeDP60 (*Nco*I/*Hin*DIII) into pFL38 (*Nco*I/*Eco*RI). Diploid cells, obtained by crossing Euroscarf strains Y14844 (*rps0A*::*KAN-MX4/RPS0B*) and Y02659 *(RPS0A/rps0B*::*KAN-MX4)*, Y04284 (*rps18A*::*KAN-MX4/RPS18B)* and Y10547 (*RPS18A/rps18B*::*KAN-MX4*), as well as strain Y21731 (*RPS15/rps15*::*KAN-MX4*), were transformed with pFL38-GAL containing the ORFs *RPS0B*, *RPS18B*,

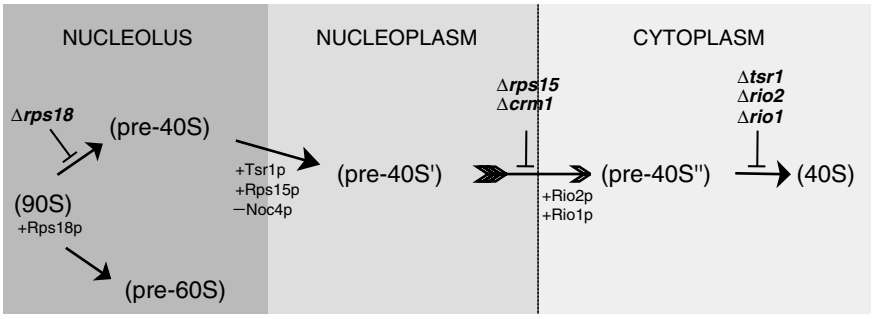

Figure 8 Involvement of Rps15p, Rps18p, and the late pre-ribosomal factors Tsr1p and Rio2p in the small subunit biogenesis pathway. Once released from the nucleolus, the pre-40S particles become competent for nuclear export (pre-40S′), a process that requires at least Rps15p and Crm1p. After translocation to the cytoplasm, association of the Rio proteins precedes the conversion to mature 40S subunits. Tsr1p may be incorporated earlier, in the nucleus (see Discussion).

Table I Yeast strains used in this work

Strains	Genotypes	Origin
BY4742	*Matα*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*	Euroscarf
MNY7	*MAT***a**; *his3*; *trp1*; *ura3*; *CRM1*::*KanMX4*; pRS315(CRM1; LEU2)	Neville and Rosbash (1999)
MNY8	*MAT***a**; *his3*; *trp1*; *ura3*; *CRM1*::*KanMX4*^{r}; pRS315(*crm1*^{T539C}; LEU2)	Neville and Rosbash (1999)
GAL-RPS0	*his3Δ1*; *leu2Δ0*; *ura3Δ0*; *YGR214w*::*KanMX4*; *YLR048w*::*KanMX4*; pFL38(P_{GAL}::*RPS0B*; URA3)	This work; derived from Euroscarf Y14844 and Y02659
GAL-RPS15	*his3Δ1*; *leu2Δ0*; *ura3Δ0*; *YOL040c*::*KanMX4*; pFL38(P_{GAL}::*RPS15*; URA3)	This work; derived from Euroscarf Y21731
GAL-RPS18	*his3Δ1*; *leu2Δ0*; *ura3Δ0*; *YDR450w*::*KanMX4*; *YML026c*::*KanMX4*; pFL38(P_{GAL}::*RPS18B*; URA3)	This work; derived from Euroscarf Y04284 and Y10547
rio2-1	*his3Δ1*; *leu2Δ0*; *ura3Δ0*; *YNL207w*::*KanMX4*; pRS315(*rio2-1*; LEU2)	Schäfer *et al*, 2003; derived from Euroscarf Y22005
SL4078	*Mat***a**; *his3Δ200*; *leu2*; *ura3-52*; GAL2; *HIS3MX6:GAL1:3HA-TSR1*	Gelperin *et al*, 2001
PEGY401	*Mat***a**; *his3*; *leu2*; *ura3*; *HIS3Mx6:GAL1:3HA-TSR1*; *YNL207w*::*KanMX4*; p(P_{GAL}::*RIO2-ProtA*; URA3)	This work; derived from SL4078 and YO471 (Vanrobays *et al*, 2003)

and *RPS15*, respectively. Ura+ diploids were recovered and transferred to sporulation medium. Spores of interest were selected according to their resistance to G418 and sensitivity to 5-FOA and glucose.

TAP-tagged strains were constructed by integration of a TAP-URA3 module into the genome, using a PCR-based strategy according to Rigaut *et al* (1999).

Fluorescence in situ hybridization and immunofluorescence microscopy

Pre-ribosomal RNAs were localized by FISH as described by Gleizes *et al* (2001) with the following oligonucleotidic probes: TT*GCAC AGAAATCTCT*CACCGTTTGGAAT*AGCAAGAAAGAAACT*TACAA GCT*T (ITS1 probe), AT*AGGCCAGCAATTTCAAGTT*AACTCCAA AGAGTATCACT*C (ITS2 probe), where T* represents amino-modified deoxythymidine conjugated to Cy3.

For *in situ* localization of TAP-tagged proteins, cells were fixed in culture medium with 4% paraformaldehyde for 30 min at room temperature (RT). Fixed cells were then rinsed with 20% sorbitol in 0.1 M potassium phosphate, pH 7.5, and centrifuged for 5 min at 3000 *g* at RT. Spheroplasting was performed on pellets using a buffer containing 2 mM vanadium ribonucleoside complex (VRC), 0.2 mM PMSF, 29 mM β-mercaptoethanol, and 0.05 mg/ml zymolyase 100T (Seikagaku Corporation) for 30 min at 30°C. After centrifugation, the pellet was rinsed with 20% sorbitol in 0.1 M potassium phosphate, pH 7.5, and the spheroplasts were left to adhere to coverslips for 30 min at 4°C. The spheroplasts were first rinsed with 0.1% BSA in 150 mM NaCl, 50 mM $NaPO_4$, pH 7.4 (PBS/BSA), and permeabilized by incubating twice for 5 min with PBS/BSA, 0.1% Igepal CA-630. They were then incubated for 2 h at RT with rabbit anti-protein A antibodies (Sigma) in PBS/BSA (1:50 000 dilution). Fluorescent detection was achieved with Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L) antibodies (Molecular Probes) and DNA was counterstained with DAPI. Images were captured with a CoolSnap color CCD camera (Photometrics) mounted on a DMRB microscope (Leica) and processed with Adobe Photoshop.

Ultrastructural in situ hybridization

The procedures for embedding of cells for electron microscopy and *in situ* hybridization with the ITS1 riboprobe on ultrathin sections have been previously reported (Gleizes *et al*, 2001). The ultrastructural observations were performed at the EM facility of the Institut d'Exploration Fonctionnelle des Génomes (IFR 109-CNRS) (Toulouse, France).

Analysis of total rRNA

Pre-rRNA analysis by Northern blotting was performed according to standard techniques as described (Milkereit *et al*, 2003), except that probes were hybridized at 50°C, and membranes were washed twice in 0.1% SSC, 0.1% SDS at RT. The following probes were used for analysis of pre-rRNA processing: probe 5′-A0 (35S-1): 5′-GGCA GATCTGACGATCACC-3′; probe A0–A1: 5′-GATCGTTCTCCCTTACC CAC-3′; probe 18S: 5′-CATGGCTTAATCTTTGAGAC-3′; probe D–A2: 5′-TTAAGCGCAGGCCCGGCTGG-3′; probe A2–A3: 5′-GATTGCTCG AATGCCCAAAG-3′; probe E-C2: 5′-GGCCAGCAATTTCAAGTTA-3′; probe 25S: 5′-CCATCTCCGGATAAACC-3′.

Cell fractionation and metabolic labelling of newly synthesized RNA

Nuclear and cytoplasmic fractions were prepared according to the method of Rozijn and Tonino (1964) with some modifications. Yeast cells grown to an OD of 0.5 were washed at RT in 0.5 volume of 0.5% (v/v) β-mercaptoethanol in water and resuspended in 0.1 volume of SB buffer (1.2 M sorbitol, 10 mM EDTA, pH 8, 10 mM potassium phosphate, pH 7.5, 1 mg/ml zymolyase 100 T). After 20 min at 25°C with mild agitation, cells were centrifuged for 5 min at 2500 rpm and carefully resuspended in 0.1 volume of recovery buffer (2% glucose, 1% peptone, 0.6% malt extract, 0.01% yeast extract, 12% mannitol, 17.8 mM magnesium acetate). After a 45 min incubation at 25°C, with or without the addition of 100 nM leptomycin B (generous gift from Dr Barbara Wolff, Novartis), 10 μCi of 5′,6′[^{3}H]uracil (Amersham) was added to a number of cells equivalent to 10 ml of the initial culture. Incorporation of radiolabelled uracil was stopped after a 15-min incubation at 25°C by cooling the vial containing the cells in ice-cold water. All the following steps were performed at 4°C. The cells were centrifuged for 3 min at 2500 rpm, resuspended in 0.5 ml of Buffer A (8% polyvinylpyrolidone, average MW 40 000, Research Organics), 1 mM magnesium chloride, 20 mM potassium phosphate, pH 6.5 and 0.03% (w/v) Triton X-100 and directly broken by 18 strokes in a Dounce Tissue Grinder (25–75 μm clearance, 1 ml volume, Wheaton). After addition of 0.5 ml of Buffer A containing 0.6 M sucrose, the suspension was charged onto 4 ml of Buffer A/0.45 M sucrose cushion and centrifuged for 10 min at 5000 rpm (4100 *g*) in a swinging rotor. In all, 500 μl of the turbid supernatant was recovered as the cytoplasmic fraction; the pellet, corresponding to the nuclear fraction, was resuspended in 150 μl of Buffer A containing 0.45 M sucrose. Purification of RNA from the fractions was achieved by hot phenol extraction (Kohrer and Domdey, 1991). RNAs were then resolved by denaturing agarose gel electrophoresis and transferred to a Hybond-N+ membrane (Amersham). After treatment with En^{3}Hance spray (Perkin-Elmer), the membrane was exposed to an X-OMAT AR film at −80°C.

Co-immunoprecipitation and TAP purifications

Affinity purification of TAP-tagged Noc4p, Enp1p, Rio2p, Tsr1p, and analysis of co-precipitating RNAs were performed as described (Dez *et al*, 2002). TAP purification was performed with a modification of the original procedure (Rigaut *et al*, 1999). One volume of lysis buffer containing 0.1 M Tris–HCl, pH 7.4, 0.1 M NaCl, 8% glycerol, and complete protease inhibitors (Roche Diagnostics) was mixed with one volume of wet cell pellet, lysed with a French press, and the extract clarified by centrifugation at 20 000 *g* for 45 min. The clarified supernatant was adjusted to neutral pH and Igepal CA-630 (Sigma) was added to a final concentration of 0.1%. After addition of 200 μl IgG-Sepharose beads (Amersham Biosciences), the extract was incubated for 1.5 h on a rotating wheel at 4°C. The beads were washed with 10 ml of a buffer containing 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.1% NP-40, and 0.5 mM DTT, and incubated for 2 h at 16°C with 100 U of recombinant TEV protease (Invitrogen) in a total volume of 200 μl. The eluate was further incubated with 100 μl of calmodulin beads (Stratagene) for 1 h at 16°C after adjusting the concentration of $CaCl_2$ to 2 mM. The final eluate was obtained after washing the calmodulin beads with 5 ml buffer containing 2 mM $CaCl_2$, using 400 μl of buffer that contained 20 mM Tris–HCl, 50 mM NaCl, and 5 mM EGTA. The purified proteins were TCA precipitated and separated on a 5–20% denaturing polyacrylamide gel. Colloidal Coomassie-stained bands were recovered from the gel. Proteins were identified by peptide mass fingerprinting with a Voyager DE-STR matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer. The Investigator Progest system (Genomic Solutions) was used for digestion with modified porcine trypsin (Promega), peptide purification, and preparation of the MALDI plates. A minimum of four matching peptides (at 50-ppm error) were considered necessary for positive protein identifications.

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