DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR
INSTITUTO DE BIOLOGÍA MOLECULAR Y CELULAR DEL CÁNCER
UNIVERSIDAD DE SALAMANCA

Role of Rrp12 in the Formation of Ribosomal Subunits

TESIS DOCTORAL

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Salamanca, 2015

CENTRO DE INVESTIGACIÓN DEL CÁNCER

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Fdo. Mercedes Dosil Castro

Ai miei nonni e a mio marito

ABSTRACT

During the synthesis of small ribosomal subunits in eukaryotes, the pre-40S particles formed in the nucleolus are rapidly transported to the cytoplasm. The mechanisms involved in the nuclear export of these particles and its coordination with other steps of the 40S synthesis pathway are mostly unknown. This thesis focused on the study of Rrp12, an evolutionary-conserved protein previously found to be present in nuclear pre-40S particles. The initial finding of the study was that the conditional depletion of Rrp12 in yeast impairs the production of 40S, but not 60S, ribosomal subunits. A detailed analysis of the depletion phenotype, using a combination of genetic, biochemical, cell-biology and proteomic approaches, unveiled that Rrp12 is specifically required for the exit of pre-40S particles to the cytoplasm. In addition, it was found that Rrp12, together with the Crm1/Xpo1 exportin, participates in processes that occur in early pre-ribosomes in the nucleolus, including the processing of the pre-rRNA and the elimination of processing byproducts. Thus, the two pre-40S export factors Rrp12 and Crm1/Xpo1 participate in maturation steps that take place in the nucleolus, upstream of nuclear export. Altogether, the findings of this work indicate that, in the 40S subunit synthesis pathway, the completion of early pre-40S particle assembly, the initiation of byproduct degradation and the priming for nuclear export occur in an integrated manner in nucleolar pre-ribosomes.

TABLE OF CONTENTS.....1

INTRODUCTION

1. Ribosomes. General features

Ribosomes are highly-conserved macromolecular assemblies responsible for protein synthesis in all kingdoms of life. They are formed by two ribonucleoprotein complexes of unequal size, the 40S and 60S subunits. The small subunit (40S) is in charge of the decoding function, whereas the large subunit (60S) catalyses the formation of peptide bonds. Despite the universal conservation of ribosomes, their composition varies considerably between prokaryotes and eukaryotes (**Table 1**). In eukaryotes, the small subunit is formed by one RNA (18S rRNA) and 33 proteins, and the large subunit consists of three RNAs [5S, 5.8S and 28S (human)/25S (yeast) rRNAs] and 46/47 proteins. The two subunits of eukaryotes, when assembled onto the mRNA, form the 80S ribosome. The functional sites within the ribosome, required for protein synthesis, are well-established. These include the tRNA binding sites (A, P and E), the decoding center, the mRNA entry and exit sites, the peptidyltransferase center, the polypeptide exit tunnel and the translation factor binding sites.

Table 1. Sedimentation coefficients and composition of bacterial and eukaryotic ribosomes

	Bacteria *(T.thermophilus or E. coli)*	Lower eukaryotes *(S. cerevisiae)*	Higher eukaryotes *(H. sapiens)*
Large ribosomal subunit	33 proteins 23S rRNA - 2904 bases 5S rRNA - 121 bases Sedimentation coefficient: 50S	46 proteins 5.8S rRNA - 158 bases 25S rRNA - 3396 bases 5S rRNA - 121 bases Sedimentation coefficient: 60S	47 proteins 5.8S rRNA - 156 bases 28S rRNA - 5034 bases 5S rRNA - 121 bases Sedimentation coefficient: 60S
Small ribosomal subunit	21 proteins 16S rRNA - 1542 bases Sedimentation coefficient: 30S	33 proteins 18S rRNA - 1800 bases Sedimentation coefficient: 40S	33 proteins 18S rRNA - 1870 bases Sedimentation coefficient: 40S

The resolution of the first crystal structures of eukaryotic ribosomes, in yeast and *Tetrahymena*, revealed how the rRNAs are arranged into the core of small and large subunits, and how the ribosomal proteins are placed on the surface, sometimes protruding into the rRNA core (**Figure 1**) [1,2]. The folding of the rRNAs into tertiary structures, and their association with ribosomal proteins generates several characteristic features of the subunits: the body, the shoulder, the platform, the head, the beak and the left and right feet of the small subunit, and the central protuberance, the L1-stalk and the P-stalk of the large subunit (**Figure 1**). In recent years, different groups have solved cryo-electron microscopy and crystal structures of bacterial, protist, plant, yeast and mammalian ribosomes, either by themselves or in complex with translation factors [3-5]. This is adding great detail to our understanding of the process of protein synthesis.

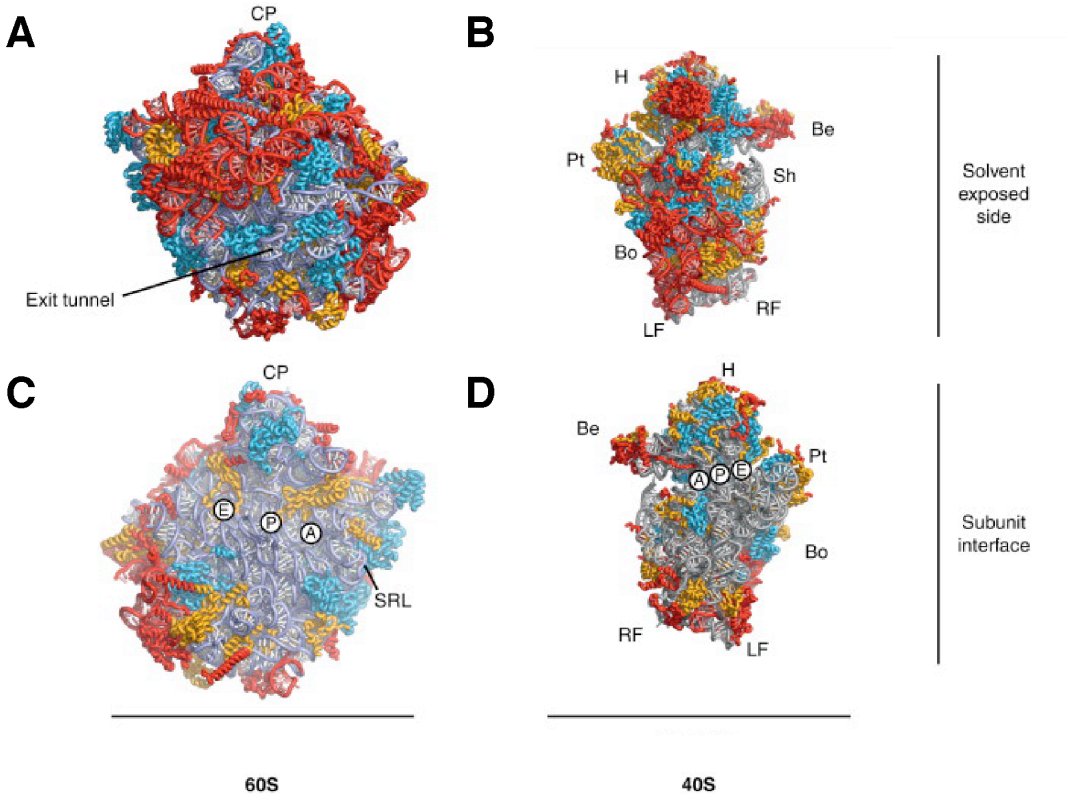


Figure 1. Eukaryotic ribosomal subunits. The figure shows the solvent-exposed sides and interfaces of *Tetrahymena thermophila* large (**A** and **C**) and small (**B** and **D**) subunits. The universally conserved proteins are coloured in light blue, the proteins present in archaea and eukarya are coloured in gold, and the proteins and RNA elements exclusively present in eukarya are coloured in red. The tRNA binding sites (A, P and E) and the hallmarks of the small subunit, such as the head (H), beak (Be), platform (Pt), shoulder (Sh), body (Bo), left foot (LF) and right foot (RF) are indicated. Taken from reference [2].

2. Ribosome synthesis

2.1 Overview of ribosome synthesis in eukaryotes

The formation of ribosomes is a major cellular task in all living organisms. In eukaryotes, it requires the assembly of ≈ 80 ribosomal proteins with four ribosomal RNAs (rRNAs) to yield the two subunits of the ribosome. Due to its complexity, the synthesis of ribosomal subunits has been mostly studied in the yeast *Saccharomyces cervisiae* through a combination of genetic, biochemical and, more recently, systematic proteomic approaches [6-8].

Three of the four rRNAs (18S, 5.8S and 25S/28S) are produced by extensive processing of a pre-rRNA transcript (**Figure 2**), that is synthesized by RNA polymerase I in the nucleolus, a non-membrane bound subcompartment of the cell nucleus. The fourth rRNA (5S) instead is synthesized independently by RNA polymerase III. Processing of the primary pre-rRNA encompasses numerous cleavage reactions that remove external transcribed spacers (5'-ETS and 3'-ETS) and internal transcribed spacers (ITS-1 and ITS-2) through the sequential generation of a series of intermediate pre-rRNA species (**Figure 2**) [8-11].

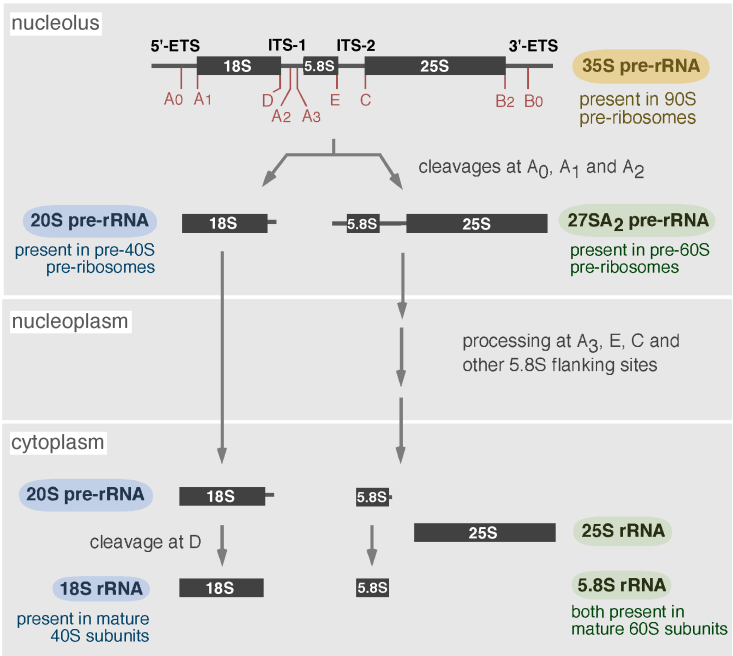
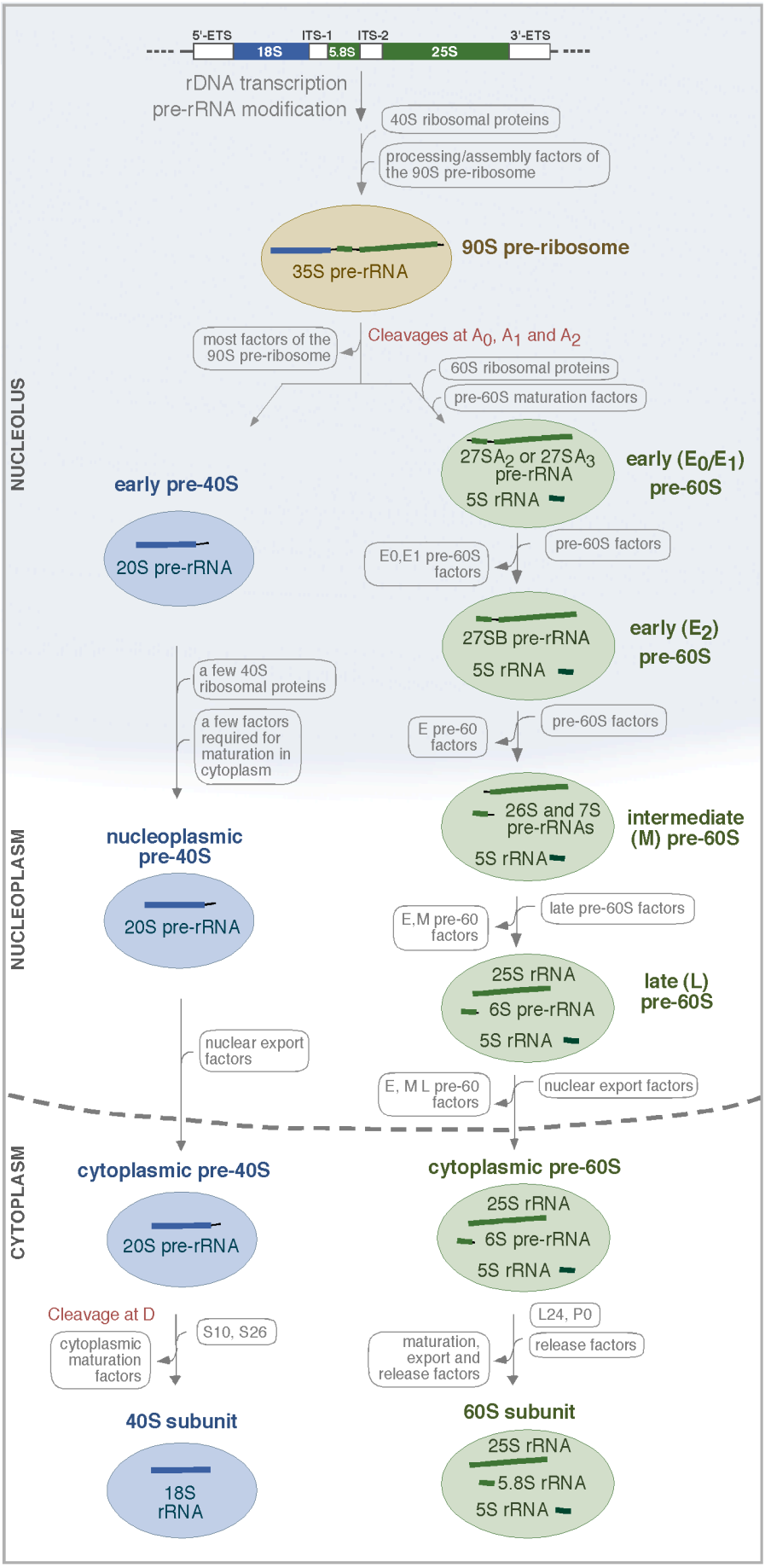


Figure 2. A simplified view of pre-rRNA processing in *Saccharomyces cerevisiae*. The 35S pre-rRNA is the initial precursor and contains the 18S, 5.8S and 25S rRNA sequences. This pre-rRNA is transcribed in the nucleolus and assembles onto 90S pre-ribosomes. It undergoes several cleavages that remove the 5' and 3' external transcribed spacers (5'-ETS and 3'-ETS), and the internal transcribed spacers 1 and 2 (ITS-1 and ITS-2). Cleavage at site A_2 generates the 20S and the $27SA_2$ pre-rRNAs, separating the 40S and 60S maturation pathways. Additional processing steps take place in the nucleoplasm and cytoplasm, leading to the formation of the mature 18S, 25S and 5.8S rRNAs.

The events of pre-rRNA processing do not occur in an isolated manner, but are concomitant to other events of ribosome synthesis, such as the covalent modification of pre-rRNAs and the recruitment of ribosomal proteins. Those activities take place in the context of pre-ribosomes, also known as pre-ribosomal particles or pre-ribosomal complexes, which are the precursors of ribosomal subunits that successively mature in the nucleolus, nucleoplasm and cytoplasm (**Figure 3**). Pre-ribosomes not only include pre-rRNAs and ribosomal proteins, but also contain numerous ribosome biogenesis factors needed for pre-RNA processing and protein assembly. In yeast, there are more than 200 ribosome synthesis factors [6-8]. They comprise different small nucleolar RNAs (snoRNAs) and a large variety of proteins, such as RNA helicases, ATPases, GTPases, kinases, phosphatases, RNA-binding proteins, scaffolding proteins, and a few proteins that are highly homologous to ribosomal proteins. The analysis of the phenotypes observed upon the depletion of each one of these factors, as well as the determination of the pre-rRNAs to which they are associated, have allowed to assign them a function at a particular step of ribosome biogenesis [6-8].

The first pre-ribosomal complex is the 90S particle. It contains the initial pre-rRNA transcript and a large number of factors that mediate the first cleavages on the pre-rRNA, upon which two pre-ribosomal particles are produced: the early pre-40S particle, precursor of the small subunit, and the early pre-60S particle, precursor of the large subunit (**Figures 2 and 3**). After their formation in the nucleolus, the pre-40S and the pre-60S particles follow independent routes that involve successive changes in composition and structure to finally yield the mature subunits (**Figure 3**).

Figure 3. Overview of the 40S and 60S ribosome subunit maturation pathways. The first steps of the pathway are the synthesis of the initial 35S pre-rRNA, its modification and its assembly, together with ribosomal and processing factors, onto the 90S pre-ribosome. In this first pre-ribosomal complex, the primary pre-rRNA is cleaved at sites A_0, A_1 and A_2. Cleavage at site A_2 releases pre-40S and pre-60S particles, whose subsequent maturation and processing proceeds independently. In both pathways the series of intermediate pre-ribosomes are drawn. These include the so-called early, nucleoplasmic, and cytoplasmic pre-40S particles, and the early (E_0/E_1 and E_2), middle (M), late (L) and cytoplasmic pre-60S particles.


5'-ETS
18S
ITS-1
5.8S
ITS-2
25S
3'-ETS
rDNA transcription
pre-rRNA modification
40S ribosomal proteins
processing/assembly factors of the 90S pre-ribosome
35S pre-rRNA
90S pre-ribosome
most factors of the 90S pre-ribosome
Cleavages at A0, A1 and A2
60S ribosomal proteins
pre-60S maturation factors
NUCLEOLUS
27SA2 or 27SA3 pre-rRNA
5S rRNA
early (E0/E1) pre-60S
early pre-40S
20S pre-rRNA
E0,E1 pre-60S factors
pre-60S factors
27SB pre-rRNA
5S rRNA
early (E2) pre-60S
a few 40S ribosomal proteins
a few factors required for maturation in cytoplasm
E pre-60 factors
pre-60S factors
26S and 7S pre-rRNAs
5S rRNA
intermediate (M) pre-60S
NUCLEOPLASM
nucleoplasmic pre-40S
20S pre-rRNA
E,M pre-60 factors
late pre-60S factors
25S rRNA
6S pre-rRNA
5S rRNA
late (L) pre-60S
nuclear export factors
E, M L pre-60 factors
nuclear export factors
CYTOPLASM
cytoplasmic pre-40S
20S pre-rRNA
cytoplasmic pre-60S
25S rRNA
6S pre-rRNA
5S rRNA
Cleavage at D
cytoplasmic maturation factors
S10, S26
L24, P0
maturation, export and release factors
release factors
40S subunit
18S rRNA
60S subunit
25S rRNA
5.8S rRNA
5S rRNA

With more than 2000 ribosomes produced each minute in dividing cells, ribosome synthesis is one of the most metabolically-expensive cellular processes [12]. The whole pathway involves ≈70 different snoRNAs, more than 200 processing/assembly factors and all three RNA polymerases. In exponentially-growing cells, the amount of rRNA constitutes approximately 80% of the total cellular RNA content, and the mRNAs of ribosomal proteins represent about 50% of all cellular transcripts [12]. Because of the large metabolic costs entailed, the ribosome synthesis pathway is under strict regulation. Thus, the expression of rRNAs, ribosomal proteins and ribosome biogenesis factor genes is finely tuned to the cellular energy status and, therefore, to the capacity of the cell to grow [13].

Given that the focus of this thesis is the study of a ribosome biogenesis factor in the yeast *Saccharomyces cerevisiae*, in this Introduction we will describe in some detail the steps of the ribosome synthesis pathway in yeast.

2.2 rDNA transcription

In *Saccharomyces cerevisiae* the ribosomal DNA (rDNA) comprises a large region of chromosome XII that contains a tandem array of 100-200 repeat units. Each one of these repeats contains two genes: the 5S rRNA gene, that is transcribed by RNA polymerase III, and the 35S rRNA gene (containing the sequences of the 18S, 5.8S and 25S rRNAs), that is transcribed in the opposite direction by RNA polymerase I (**Figure 4**).

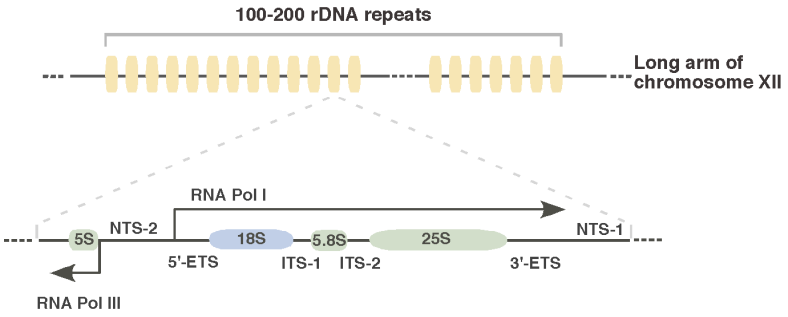


Figure 4. Organization of the rDNA locus in *Saccharomyces cerevisiae* The rDNA repeats (100-200) are located in the long arm of chromosome XII. A single repeat contains the 5S rRNA gene, transcribed by RNA polymerase III (RNA pol III, arrow pointing left), and the 35S pre-rRNA gene, transcribed by RNA polymerase I (RNA pol I, arrow pointing right). NTS, non-transcribed spacer; ETS, external transcribed spacer; ITS, internal transcribed spacer.

The recruitment of RNA polymerase I to the promoter of the 35S rRNA gene requires several specific transcription factors, and the DNA-binding protein TBP (TATA-binding protein), common to all three polymerases [14]. RNA polymerase I specific transcription factors include the UAS-binding upstream activity factor (composed of Rnr5, Rnr9, Rnr10 and histones H3 and H4), the core factor (consisting of Rnr6, Rnr7 and Rnr11) and the transcription factor Rnr3 [14].

2.3 Pre-rRNA processing

In the initial 35S pre-rRNA all the sequences that do not become part of mature rRNAs (5'-ETS, ITS-1, ITS-2, and 3'-ETS) need to be processed away (**Figure 5**) [8,9,11]. The first two cleavages, at sites A_0 and A_1, remove the 5'-ETS and generate the 33S and 32S pre-rRNA species, respectively (**Figure 5**). The following cleavage step, at site A_2 in ITS-1, produces the 20S and the $27SA_2$ pre-rRNAs. As mentioned above, this event is crucial because it separates the processing and maturation pathways of the small and large subunits. The 20S pre-rRNA will undergo just an additional cleavage at site D, which will take place in the context of cytoplasmic pre-40S particles (**Figures 3** and **5**). By contrast, the processing of the $27SA_2$ is much more complex, and includes several steps that take place in the nucleolus, nucleoplasm and cytoplam to render the mature 25S and 5.8S rRNAs (**Figures 3** and **5**). The majority of the $27SA_2$ pre-rRNA (about 85-90%) is shortened to the $27SA_3$ intermediate by cleavage at site A_3 and by exonucleolytic digestion from the site B_0 to the site B_2 (to remove the 3'-ETS) (**Figure 5**). Then, after the exonucleolytic elimination of the remaining ITS-1 sequences, the resulting 27SB pre-rRNA undergoes endonucleolytic cleavage at site C_2 to form the 7S and 26S pre-rRNAs. These two pre-rRNA precursors undergo several steps of exonucleolytic trimming that, at the end, originate the 25S and 5.8S rRNAs. A small proportion of the $27SA_2$ pre-rRNA (about 10-15%) is not cleaved at site A_3 and, instead, is directly cleaved at site B_{1L}. This alternative pathway (not shown in **Figure 5** for simplicity) generates a small pool of 5.8S rRNA, referred to as $5.8S_L$, that is slightly bigger in size than the 5.8S produced by the major pathway ($5.8S_s$).

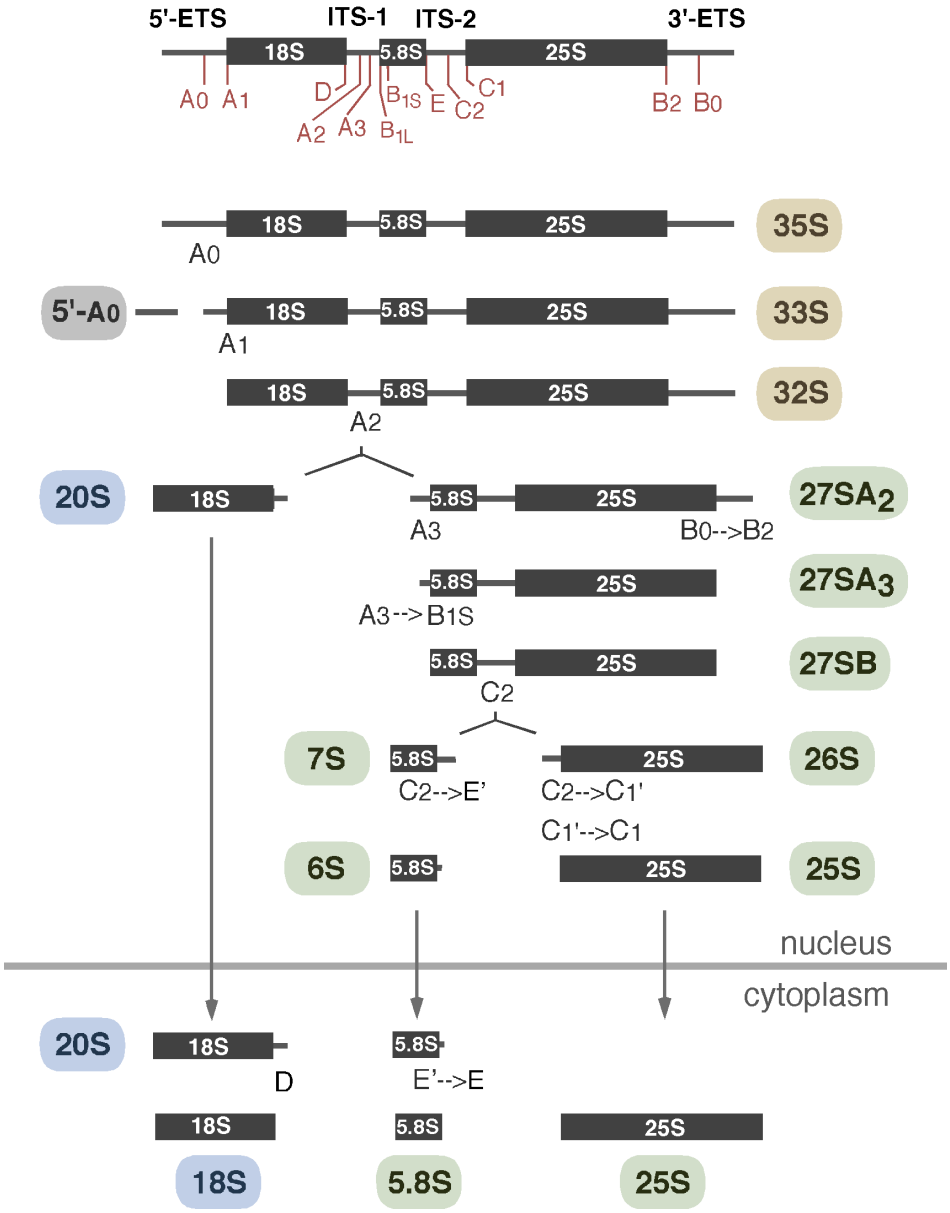


Figure 5. Steps of pre-rRNA processing in *Saccharomyces cerevisiae*. The figure shows the scheme of the primary transcript with the main processing sites (in red). The 18S, 5.8S and 25S mature rRNAs are generated from this primary transcript by a series of endonucleolytic and exonucleolytic processing steps that generate different pre-rRNA intermediate precursors. Cleavage at site B_0 occurs just upon transcription (not shown). The names of the first pre-rRNA species formed are highlighted in brown. All 18S pre-rRNA precursors and 5.8S/25S precursors are highlighted in blue and green, respectively. For simplicity, the minor pathway that forms $27S_{BL}$ pre-rRNA is not shown (see text for details).

As a result of pre-rRNA processing, several RNA fragments are excised and need to be degraded. The A_0-A_1, A_2-A_3 and D-A_2 spacers are degraded by the 5'-3' exonucleases Xrn1 and Rat1 [15], whereas the 5'-ETS is degraded by the exosome, a multi-protein complex that functions as a 3'-5' exonuclease [16,17]. The exosome is also responsible for the 3' processing of the 7S pre-rRNA [16,17]. The action of this complex is facilitated by several cofactors [18]. One of them is Mtr4, a helicase that is thought to promote the unwinding of stem-loop structures in the substrates, making them accessible to the exonuclease subunit of the exosome complex.

2.4 Modifications of the rRNA

The pre-rRNAs are extensively modified at several sites, largely by methylation of the 2'-hydroxyl group of riboses (2'-O-methylation) or conversion of uridine residues to pseudouridines (pseudouridylation) [8]. Those modifications (67 methylated sites and 44 pseudouridylated sites) are mostly present in functional regions of the ribosome and, even though they are not essential for cell viability, they appear to be required for the optimal translation efficiency of ribosomes [8]. The rRNA modifications can occur co-transcriptionally or post-transcriptionally, and are carried out by small nucleolar ribonucleoproteins (snoRNPs). These complexes contain a single snoRNA, which guides the snoRNP by base-pairing to the site of modification, and several proteins, which include the enzyme that catalyzes the modification reaction [8]. The two major classes of snoRNPs are: the box C/D snoRNPs that catalyze the ribose 2'-O-methylation, and the H/ACA snoRNPs, that direct the pseudouridylation. In addition to ribose methylation and pseudouridylation, there is a third type of modification that is not so extensive, and that is base methylation [8]. This modification occurs at only 10 sites of the rRNAs, and it is carried out by methyltransferases. Currently, just a few of these enzymes have been characterized: Dim1, Nep1, Bud23 and Rrp8 [19-22]. The first three proteins methylate the 20S pre-rRNA. Rrp8, instead, modifies the mature 25S rRNA. The significance of base methylation is poorly understood, but there is increasing evidence that the modification itself is not essential. In fact, Dim1, Nep1 are Bud23

are essential enzymes or very important for cell growth, but these functions do not require their base methylation activities [21,23,24].

2.5 Formation of the 90S pre-ribosome

As mentioned above, the 90S pre-ribosome is the first pre-ribosomal complex formed and is required for the initial processing steps of the 35S pre-rRNA, the cleavages at sites A_0, A_1 and A_2 that yield the 20S and $27SA_2$ pre-rRNAs (see **Figure 3** in page 11). It contains the primary pre-rRNA, the U3 snoRNP and ≈ 70 non-ribosomal proteins that play structural, regulatory and cleavage roles [6-8]. The vast majority of the non-ribosomal proteins of the 90S pre-ribosome do not stay as part of the 40S and 60S pre-ribosomes, and are released soon after the cleavage at site A_2. It is important to note that the 90S pre-ribosomal particle is essential for the formation of 40S subunits, because the precursor of the 18S rRNA (the 20S pre-rRNA) only can be generated by the cleavages at sites A_0, A_1 and A_2. By contrast, the 90S pre-ribosome is not required for the production of 60S subunits because, in the absence of the A_0, A_1 and A_2 cleavages, the 35S pre-rRNA can be directly cleaved at site A_3 and produce the $27SA_3$ pre-rRNA. Thus, the depletion of any essential 90S pre-ribosome factor causes a loss of 40S subunits without affecting the production of 60S subunits. The specific functions of the 90S components are mostly unknown, and the role of only a few of them have been studied in some detail. In this regard, it has been described that the GTPase Bms1 is important for recruiting the putative endonuclease Rcl1 to the 90S pre-ribosome [25]. This protein has been proposed as responsible for cleavage at site A_2 [26]. Another candidate to mediate the cleavage at site A_2 is Utp24, a protein structurally related to endonucleases [27].

The 90S pre-ribosome is assembled in a modular and hierarchical manner from different subsets of proteins organized as autonomous subunits (**Figure 6**) [28-30]. Three of the 90S building blocks are the so-called UTP (U three binding proteins) complexes: UTP-A, UTP-B and UTP-C. Other subunits are the U3 snoRNP, the trimeric complex Mmp10/Imp3/Imp4 and the dimer Bms1/Rcl1. The current model for 90S assembly is that UTP-A is the first complex that binds the pre-rRNA. Then, the rest of the modules are incorporated following two independent (but not

mutually exclusive) pathways (**Figure 6**). One of them involves the initial incorporation of Rrp5, which facilitates the subsequent binding of the UTP-C module. The other one entails the co-recruitment of UTP-B and U3 snoRNP, which form a stable intermediate that promotes the incorporation of the Mmp10/Imp3/Imp4 and Bms1/Rcl1 modules. It is thought that all these assembly steps can occur co-transcriptionally, as the nascent pre-rRNA is being synthesized by the RNA polymerase I, or post-transcriptionally on the 35S pre-rRNA [6,8,11]. Some authors refer to the 90S pre-ribosome that is co-transcriptionally assembled as the SSU-processome.

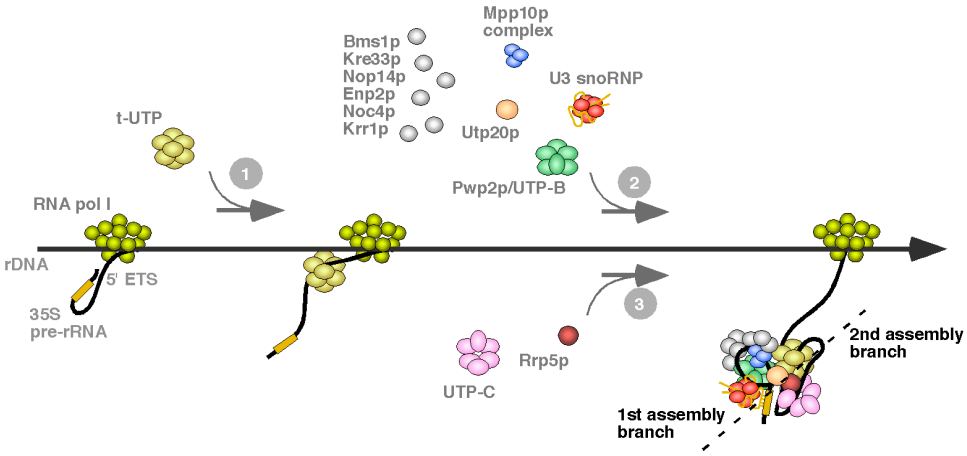


Figure 6. Current model for the stepwise assembly of the 90S pre-ribosomal particle. The 90S pre-ribosome is formed by independent building blocks that follow hierarchical rules of assembly onto the 35S pre-rRNA. The binding of the UTP-A complex to the pre-rRNA precursor (step 1) is required for the subsequent assembly of other 90S pre-ribosome subunits (Pwp2/UTP-B, U3 snoRNP, Mpp10 complex, UTP-C) and isolated factors (steps 2 and 3). Taken from reference [29].

2.6 Maturation of pre-40S complexes

2.6.1 Maturation of pre-40S complexes in the nucleus

Due to the rapid kinetics of transit through the nucleoplasm, it is assumed that the major events of pre-40S particle assembly take place concurrently with the 35S pre-RNA cleavage steps in the nucleolus. Despite this, the nucleolar pre-40S particles have to undergo some transformations before leaving the nucleus (see **Figure 3** in page 11). These include the recruitment of factors that will participate in

cytoplasmic maturation processes, and some conformational changes and interactions with transport proteins, that will promote the transit through nuclear pore complexes. How these nuclear events take place, and how much they affect the assembly of the 40S subunit, is still poorly understood, mostly due to the lack of strategies to purify nucleoplasmic pre-40S complexes or to identify well-defined separable maturation steps within the nucleus.

The composition of pre-40S particles along its maturation pathway is relatively well-known. Cytoplasmic pre-40S complexes contain seven stably-bound ribosome biogenesis factors: Enp1, Dim1, Pno1/Dim2, Tsr1, Ltv1, Nob1 and Rio2 (**Figure 7**) [31]. These factors are incorporated at different steps (**Figure 8**). Three of them, Enp1, Dim1 and Pno1, are already recruited to the 90S pre-ribosome. In fact, these three factors are bona-fide components of the 90S pre-ribosome, as they are required for the cleavages at sites A_0, A_1 or A_2. Enp1 is essential for the three cleavages (A_0, A_1 and A_2) , and Dim1 and Pno1 are required only for the cleavages at sites A_1 and A_2 [32-35]. The other four components of pre-40S particles, Tsr1, Nob1, Rio2 and Ltv1, are recruited after 90S particle disassembly and are essential for the final maturation steps that take place in the cytoplasm [31,36-40]. Tsr1 appears to be incorporated in pre-40S particles in the nucleolus, Ltv1 and Nob1 are recruited in the nucleoplasm, whereas for Rio2 the step of incorporation is not clear (**Figure 8**).

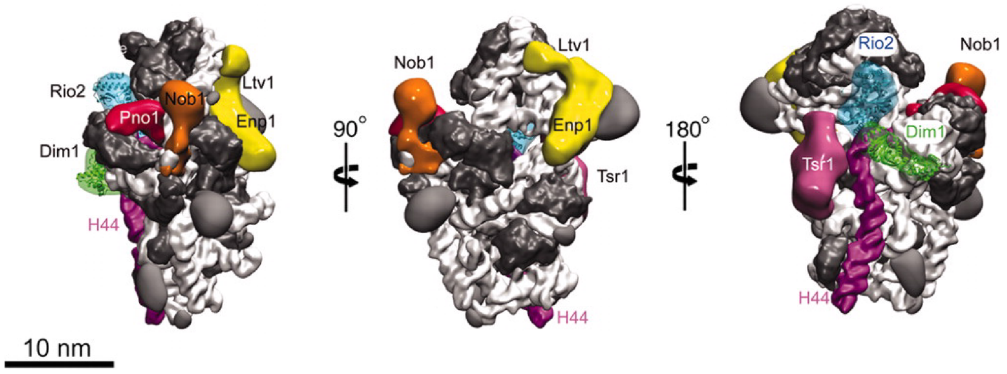


Figure 7. Cytoplasmic pre-40S particles. Cryo-electron microscopy structure of cytoplasmic pre-40S particles showing the positions predicted for the seven stably-bound ribosome biogenesis factors. Nob1 and Pno1 overlap the binding site for eIF3 at the platform. Enp1 and Ltv1, that form a complex with the ribosomal protein Rps3, are in the beak and block the opening of the mRNA channel. Rio2, Tsr1 and Dim1 localize on the subunit interface and overlap the binding sites of translation initiation factors eIF1 and eIF1A. Taken from reference [31].

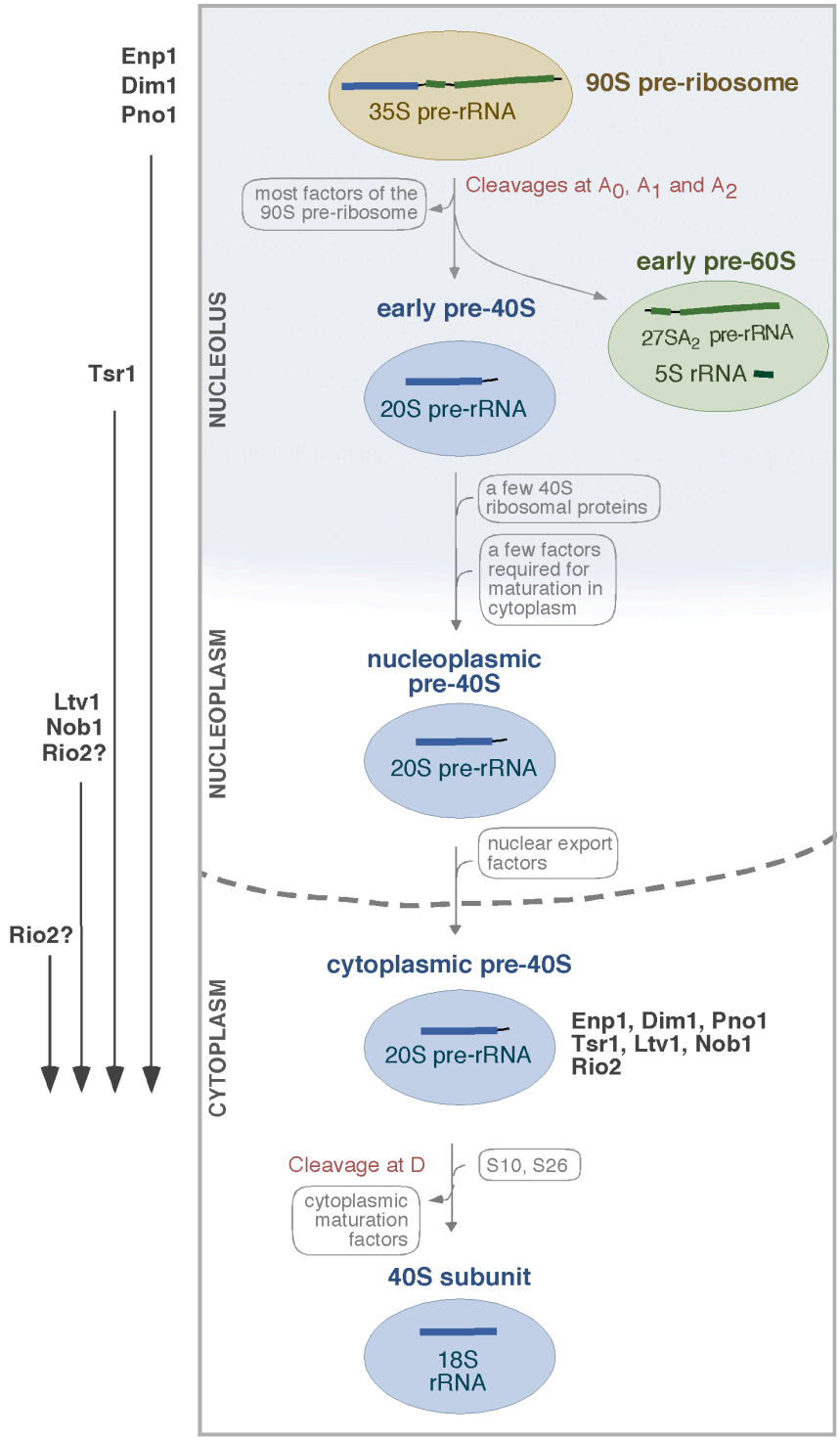


Figure 8. Maturation pathway of pre-40S particles. Scheme of the pre-40S maturation pathway indicating the order of incorporation of the seven major maturation factors present in cytoplasmic pre-40S particles. Enp1, Dim1 and Pno1 are recruited to 90S particles. Tsr1 is recruited to early pre-40S particles in the nucleolus. Ltv1 and Nob1 join pre-40S particles in the nucleoplasm. The step of incorporation of Rio2 remains ill-defined.

In addition to the seven major factors that follow the pre-40S particles to the cytoplasm, there are a few proteins that interact with them in the nucleus and not in the cytoplasm. These include Rrp12, a stably-bound component of nuclear pre-40S complexes, and Bud23, Slx9 and the kinase Hrr25, three factors that interact with those complexes in a transient or weak manner [36,41,42]. The molecular functions of Rrp12, Bud23 and Slx9, both in 40S subunit structure organization and nuclear export, are not well-known. Only the role of Hrr25 has been studied at the molecular level (see below).

2.6.2 Nuclear export of pre-40S complexes

The dimensions of ribosomal subunit precursors and of the nuclear pore channel are comparable (≈25-30 nm), making it possible that one pre-ribosome passes through a nuclear pore at any given time [43]. The free diffusion of these big hydrophilic ribonucleoproteins is likely to be prevented by the hydrophobic Phe-Gly (FG)-repeats of the nucleoporins that fill the lumen of the nuclear pore complexes. For this reason, their translocation requires exportins able to interact with the FG-repeats allowing their transit. Two factors that are absolutely required for the transport of pre-40S particles are the general exportin Crm1/Xpo1 and the small GTPase Ran/Gsp1 [44-46]. Crm1 belongs to the karyopherin-β-like family of export receptors. Such receptors bind proteins carrying short aminoacidic sequences, known as nuclear export signals (NES), in the presence of Ran-GTP, promoting their export out of the nucleus (**Figure 9**) [47]. An adaptor that mediates the binding of Crm1 to 40S pre-ribosomal particles has not been identified, although Ltv1 (in yeast) and Pno1 and Rio2 (in humans) have been proposed to function as redundant adaptors [6,45,46].

In addition to Crm1, another receptor implicated in the export of pre-40S particles in yeast is the mRNA transport complex Mex67-Mtr2 [42]. This receptor functions in a Ran-independent manner, interacts directly with nucleoporins, and uses a common structural domain to associate to mRNAs, 40S and 60S pre-ribosomes. The mechanisms that specify the use of Mex67-Mtr2 by mRNAs or pre-ribosomes are unknown.

A third protein that has been implicated in the export of pre-40S subunits is Rrp12 [48]. This protein binds to pre-40S complexes in the nucleus and has the capacity to bind Ran and FG-repeat nucleoporins, two properties that suggest a direct role in nuclear export. However, a demonstration of its ability to promote export has not yet been provided (see below). Other proteins, such as Rps15, Slx9 and Bud23, have also been proposed to contribute to the export of 40S pre-ribosomal particles [21,42,49]. These proteins, when depleted or mutated, cause an accumulation of pre-40S complexes, and not pre-60S complexes, in the nucleus. However, there is no evidence for their direct relation with the export machinery and, like in the case of Rrp12, it is unclear whether they specifically influence nuclear export or some upstream event involved in the formation of export-competent pre-40S particles.

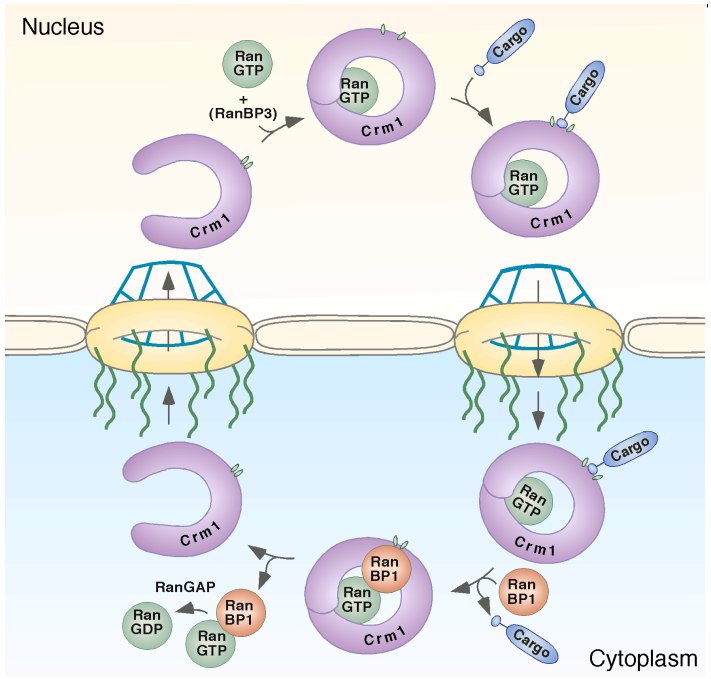


Figure 9. Schematic overview of a Crm1 export cycle. In its free form, Crm1 displays a conformation that prevents the interaction with NES-containing cargoes. The association to the GTP-bound form of Ran (RanGTP) increases the affinity of Crm1 for its cargoes. Several Ran binding proteins (RanBPs) are needed for proper and efficient export complex formation. RanBP3, for example, is able to keep RanGTP bound to Crm1 until the cargo binds to the NES cleft. The ternary complex translocates through the nuclear pore complex (NPC) and reaches the cytoplasm. Here, the interaction with the Ran binding protein RanBP1 produces several structural rearrangements that lead to the release of the cargo. Subsequently, the RanGTP-RanBP1 complex dissociates from Crm1, the GTP is hydrolyzed to GDP by RanGAP1, and the free form of Crm1 is recycled back to the nucleus.

In addition to the interaction with transport factors, there is another event specifically required for the nuclear exit of pre-40S complexes, and that is a structural rearrangement of the particle. In mature 40S subunits, the protrusion of helix 33 of the 18S rRNA, bound by the ribosomal proteins Rps3, Rps10 and Rps12, generates a rigid structure known as the "beak" (see **Figure 1** in page 8). It is thought that such structure, if formed prematurely inside the nucleus, should prevent the export of the pre-40S pre-ribosomes. Based on this, it has been proposed that the region where the beak will be formed has to be flexible to be able to pass through the nuclear pore complexes. A mechanism for the regulation of the beak flexibility in pre-40S pre-ribosomal particles has been described [50]. A trimeric complex formed by the proteins Rps3, Enp1 and Ltv1 is weakly associated with the pre-40S particles in the nucleus due to the phosphorylation of Rps3 and Ltv1 by the kinase Hrr25. Upon export, Rps3 is dephosphorylated and bound stably to the 40S particle, leading to the formation of the rigid beak [50]. This Hrr25-mediated conformational change is the only known functionally-relevant transformation of pre-40S pre-ribosomes in the nucleus before their export.

2.6.3 Final maturation of pre-40S complexes in the cytoplasm

The events and challenges faced by the pre-40S particles during their final maturation in the cytoplasm are the following: (1) the cleavage of the 20S pre-rRNA at site D to form the mature 18S rRNA; (2) the assembly of the final ribosomal proteins (Rps26 and Rps10); (3) the prevention of a premature association with the translational machinery; and (4) the confirmation of correct assembly of all functional domains.

The newly-exported 40S pre-ribosomes find in the cytoplasm an environment where mRNAs, tRNAs and translation factors are very abundant and, therefore, premature translation initiation represents a risk that must be avoided. In fact, immature ribosomes could be prone to translational errors and stalled ribosomes would be rapidly degraded together with their bound mRNAs. It is thought that a major function of the seven ribosome biogenesis factors (Enp1, Dim1, Pno1, Tsr1, Nob1, Ltv1 and Rio2) bound to pre-40S particles is to prevent

premature initiation of translation [31]. They are all positioned in strategical places of the pre-40S complexes that impede their association with translation factors and the opening of the mRNA channel (see **Figure 7** page 18).

Despite the fact that they cannot initiate translation, it has been shown that pre-40S complexes interact with 60S subunits in the cytoplasm to undergo a conformational change (head to body rotation) that is mediated by the GTPase Fun12/eIF5b [51-53]. This conformational change appears to be important for the final pre-rRNA processing step because it brings the active site of the endonuclease Nob1 close to the site D of the 20S pre-rRNA, stimulating its cleavage (**Figure 10**) [51,52]. It is hypothesized that the maturation step inside these 80S-like complexes could also serve as a proofreading step to ensure that the ribosome subunits will be translational competent [51-53]. It is thought that during this process several features of the pre-40S pre-ribosomes are checked, including the capacity to bind the 60S subunits and the ability to recruit and activate several translation factors.

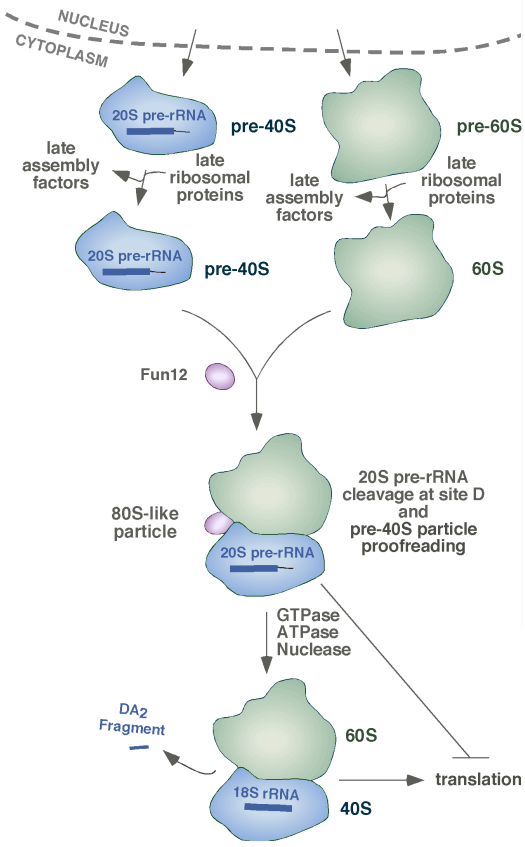


Figure 10. Model for the last maturation steps of pre-40S particles in the cytoplasm. In their last steps of maturation, the pre-40S and pre-60S particles that reach the cytoplasm associate with the final ribosomal proteins (RPs) and release a few assembly factors (AFs) that participated in previous maturation events. Then, as the translation initiation factor Fun12/ eIF5B binds to the pre-40S complex, the joining of the 60S subunit is stimulated, and a 80S-like particle is formed. Such complex is not competent for translation, but allows to test if the subunits will be translational competent. If not, they should be targeted for degradation. The formation of the 80S-like particle triggers the hydrolysis of GTP by Fun12 which leads to a conformational change inside the pre-40S particle that promotes the cleavage of the 20S pre-rRNA at site D. Final maturation of 60S subunits includes 6S pre-rRNA processing to mature 5.8S rRNA (not shown). Note that the precise order of the events shown in this cartoon has not yet been defined.

2.7 Maturation of pre-60S complexes

2.7.1 Maturation of pre-60S complexes in the nucleus

Maturation of 60S pre-ribosomes includes different pre-rRNA processing steps that take place within six successive pre-ribosomal intermediates (see **Figure 3** in page 11) [7,8]. The first three are generated in the nucleolus and are known as the early E_0, E_1 and E_2 particles. Then, in the nucleoplasm, two other pre-ribosomal intermediates are formed: the middle (M) and late (L) particles. The final intermediate is the cytoplasmic pre-60S particle.

The E_0 particles, the first formed, contain the $27SA_2$ pre-rRNA, the 5S rRNA, different snoRNPs and eight putative helicases. These particles were identified after mass spectrometry analysis of complexes associated to the Npa1 protein [54]. The E_1 particles are very similar in composition to the E_0 ones, and have been identified through purification of complexes associated to Ssf1 and Nsa3 [55,56]. They assemble onto the $27SA_2$ pre-rRNA to promote, in most cases, its cleavage at site A_3 by the MRP RNase and the processing to site B_{1S} (see **Figure 5** in page 14). The E_2 particles, identified through the purification of complexes associated to the Nop7 protein [56,57], contain mostly the 27SB pre-rRNA and the 5S rRNA. They remain assembled until the cleavage at site C_2 takes place and the 26S and 7S pre-rRNAs are formed.

The M particles are formed at the nucleolus-nucleoplasm transition. They have been defined as the complexes that co-purify with the factors Nug1, Rix1 and Sda1 [56]. These are a heterogeneous population of particles that include complexes containing the mature 5S rRNA, the 7S pre-rRNAs and the 26S or a 26S partially-processed species, the 25S' pre-rRNA (see **Figure 5** in page 14). The events that presumably occur in this particle are the cleavage at site C_2, and the initial exonucleolytic digestion of 26S pre-rRNA from C_2 to $C_{1'}$ to generate the 25S' and the 7S pre-rRNAs.

The L particles (L) are defined as the complexes obtained upon purification of the Nog2/Nug2 and Arx1 proteins [56]. The exonucleolytic digestion of the 25S' pre-rRNA (from site $C_{1'}$ to site C_1) and of the 7S pre-rRNA (from C_2 to site E'), occur within these pre-ribosomal intermediates. Therefore, in addition to the mature 5S rRNA, the L particles contain the 6S pre-rRNAs and the mature 25S rRNA. They also

include a few ribosome biogenesis factors, such as Nmd3 and Mtr2, which participate in export to the cytoplasm. In fact, it seems that the L particles are the intermediates that have already released most of the processing machinery and are ready to be exported.

For most pre-60S maturation factors, the specific functions are unknown. Only a few have been characterized in some detail. Among these, it is a group of twelve proteins, named "A3 factors", that are required for the removal of the ITS-1 sequence from the $27SA_3$ pre-rRNA: Ebp2, Brx1, Pwp1, Nop12, Nop7, Ytm1, Erb1, Rpl7, Bop15, Nsa3, Dsr1 and Has1 [8]. They are present in the E_0 and E_1 particles and promote the activity of the Rat1 exonuclease, which degrades the fragment A_3-B_{1S} in the $27SA_3$ pre-rRNA. Another group of factors that have been functionally characterized are the so-called "B factors", which are involved in the C_2 cleavage [8]. These include four helicases (Spb4, Dsr1, Has1 and Dbp10), five putative RNA-binding proteins (Nip7, Rpf2, Nsa2, Rpl24 and Tif6), three GTPases (Nog1, Nog2 and Nug1), one possible scaffold protein (Mak11) and one potential methyltransferase (Nop2).

2.7.2 Nuclear export of pre-60S complexes

The export of pre-60S particles is much better understood than that of pre-40S particles [6,44-46]. In addition to the long-known involvement of the general exportin Crm1, it has been reported that the exit of pre-60S complexes requires the function of at least two proteins and one complex: Nmd3, Arx1 and Mex67-Mtr2 (**Figure 11**). Nmd3 is the NES adaptor for Crm1 that, in the presence of Ran-GTP, facilitates the transport of pre-60S complexes through the nuclear pore complex [58]. Arx1 is a non-essential component of pre-60S particles that binds to nucleoporins and aids in the translocation of pre-60S complexes [59,60]. The Mex67-Mtr2 heterodimer works as an export receptor [61] that, as mentioned above, it is also involved in the nuclear export of mRNAs and pre-40S particles. It directly binds to nucleoporins and to the 5S rRNA. In addition to these proteins that have been clearly implicated in the export process itself, there are others, like

Rrp12, Bud20 and Ecm1 that have been proposed to act as pre-60S export factors [48,62,63]. However, their direct involvement in export remains to be demonstrated.

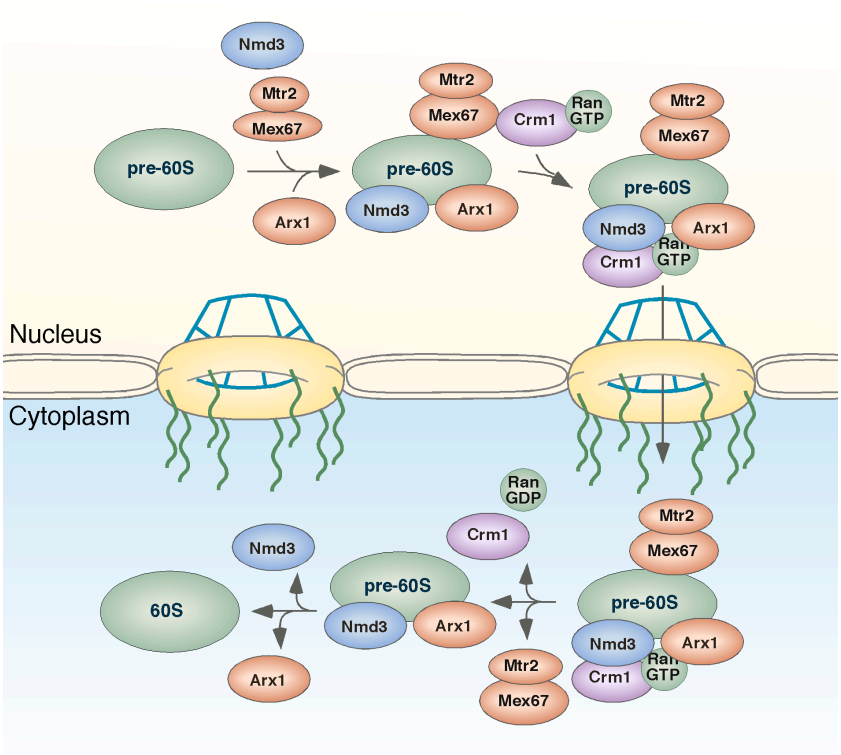


Figure 11. Nuclear export of pre-60S particles. The export of pre-60S particles is mediated by several known export receptors: Crm1, Arx1 and Mex67-Mtr2. The exportin Crm1 binds in the presence of RanGTP to the nuclear export signal containing adaptor Nmd3. Mex67-Mtr2 and Arx1 export receptors can bind directly to the pre-60S particles. After export, all these factors are released.

2.7.3 Final maturation of pre-60S complexes in the cytoplasm

Cytoplasmic pre-60S particles are defined by the presence of the Kre35/Lsg1 protein [56]. Within these pre-ribosomes occur the last steps of maturation: the final processing of the pre-rRNA, the dissociation of ribosome biogenesis factors and the assembly of the remaining ribosomal proteins (L10, L24, L29, L40, L42, P0, P1 and P2).

The pre-60S complexes transported from the nucleus to the cytoplasm contain the 5S rRNA, the fully mature 25S rRNA and the 6S pre-rRNA (5.8S rRNA 3' extended by 5-8 nucleotides). It is in the cytoplasm when the exonucleases Rex1 and Rex2, together with the endonuclease Ngl2, remove the last nucleotides to produce the mature 5.8S rRNA [8].

In addition to the formation of the 5.8S rRNA, the cytoplasmic pre-60S complexes have to get rid of some maturation factors that are still associated to them. These include several export factors (Nmd3, Arx1, Ecm1 and Mex67-Mtr2 dimer) and a few assembly proteins (Rpl24, Tif6, Nog1 and Alb1). The release of export and assembly factors is catalyzed by GTPases (Kre35, Efl1), ATPases (Drg1, Hsp70), and their cofactors (Sdo1, Rei1 and Jjj1). Concomitant to the release of the export and assembly factors, there is a recruitment of ribosomal proteins L24 and P0, which replace their paralogues Rlp24 and Mtr4, respectively [8].

3. The protein Rrp12

Rrp12 is an evolutionary-conserved protein of 138 KDa that predominantly localizes in the nucleolus. It is essential for cell viability, and the first clue about its function came from high-throughput proteomic studies in yeast cells, which found Rrp12 associated to different pre-ribosomal complexes [36,48,64-66]. The involvement of Rrp12 in ribosome synthesis was studied in detail by the laboratory of David Tollervey at the University of Edinburgh. This group described in the year 2004 that Rrp12 is essential for the nuclear export of both pre-40S and pre-60S particles in yeast [48]. Based on several pieces of evidence, it was proposed that Rrp12 is an export factor for both pre-40S and pre-60S particles. This was an important finding in the field because it identified a novel essential export factor for the two ribosomal subunits. It is important to note that most ribosome biogenesis factors participate in the formation of either small subunits or large subunits, but not both, consistent with the fact that they follow routes of maturation that are largely separated.

In a second unrelated study, performed by Mercedes Dosil at the University of Salamanca, Rrp12 was found to be required for proper S phase progression and

for the DNA damage response in yeast [65]. These roles of Rrp12 are not shared by other ribosome biogenesis factors, and appear to be unrelated to its function in ribosome synthesis. Thus, it was described that Rrp12 facilitates the nuclear import of some proteins, such as the ribonucleotide reductase subunits Rnr2 and Rnr4, that are required for normal DNA synthesis and for the DNA damage response [65].

Since the work of this thesis focuses on the role of Rrp12 in ribosome synthesis, we will detail the data relative to this function of the protein.

3.1 Role of Rrp12 in ribosome biogenesis

As mentioned above, Rrp12 was described as a nuclear export factor for pre-40S and pre-60S complexes [48]. This conclusion was based on the following pieces of evidence obtained from studies in yeast:

-Rrp12 is associated to pre-40S and pre-60S particles. Proteomic analysis of different pre-ribosomal complexes revealed that Rrp12 is present in 90S, pre-40S and pre-60S pre-ribosomal particles [36,48,64-66]. Furthermore, Northern blot analysis of RNAs that coimmunoprecipitate with Rrp12 indicated that this protein interacts with pre-rRNA precursors present in 90S (35S pre-rRNA), pre-40S (20S pre-rRNA) and pre-60S complexes (the $27SA_2$, 27SB and 7S pre-rRNAs, and the 25S rRNA) [48].

-The depletion of Rrp12 causes an accumulation of pre-40S and pre-60S particles in the nucleus. The conditional elimination of Rrp12 in yeast cells leads to a a loss of the 18S rRNA that is caused by a block in the maturation of the 20S pre-rRNA [48]. This precursor is accumulated in the nucleus [48], consistent with a function of Rrp12 in pre-40S nuclear maturation and/or export. In regard to the 60S synthesis pathway, it was observed that the mature 25S and 5.8S rRNAs are produced in the absence of Rrp12, but there is an abnormal accumulation of 5.8S pre-rRNA precursors (7S, 5.8S + 30 and 6S pre-rRNAs) [48]. Most importantly, using a reporter of pre-60S subunits (the ribosomal protein Rpl11 fused to GFP) it was found that the depletion of Rrp12 causes an accumulation of pre-60S complexes in the nucleus

[48]. These data are consistent with a function of Rrp12 in pre-60S nuclear maturation and/or export.

-Rrp12 is a nucleolar-cytoplasmic shuttling protein. Rrp12 is largely located in the nucleus and is mostly enriched in the nucleolus. Using heterokaryon assays, it was found that Rrp12 is able to shuttle between the nucleus and the cytoplasm, a property expected for a nuclear export factor [48].

-Rrp12 is a HEAT-repeat protein similar to export factors. Sequence similarity searches and secondary structure predictions revealed that Rrp12 is a HEAT-repeat containing protein [48]. The majority of the protein (with the exception of the last 196 aminoacids) contains repeated motifs termed HEAT repeats (Huntingtin, elongation factor 3, protein phosphatase 2A and yeast kinase TOR1) that are related to Armadillo-like repeats (**Figure 12A** and **12B**). HEAT domains consist of arrays of HEAT repeats, which are ≈50 aminoacidic residues that form two antiparallel α-helices and two turns arranged around a common axis (**Figure 12C**) [67]. The HEAT repeats contain a set of conserved hydrophobic residues that mediate protein-protein interactions. One large family of HEAT-repeat proteins are β-karyopherins, which are transport factors that interact directly through their HEAT repeats with FG-repeats of nucleoporins, mediating the passage of associated cargos through the lumen of nuclear pore complexes [68]. One example of HEAT-repeat karyopherin is the exportin Crm1 (**Figure 12D**). The similarity of Rrp12 to β-karyopherins is consistent with a direct role in nuclear export.

-Rrp12 is able to interact directly with the GTPase Ran, FG-repeat nucleoporins and RNA. By performing *in vitro* protein-protein and protein-RNA binding assays it was demonstrated that Rrp12 has the ability to bind directly to both Ran-GDP and Ran-GTP in the absence of other proteins [48]. It was also observed that Rrp12 shows strong *in vitro* binding to the FG-repeat regions of nucleoporins Nup100 and Nup116 [48]. In regard to the RNA-binding capacity, the protein was found to bind *in vitro* to homopolymeric nucleotides, poly(A) and poly(U), and to a transcript corresponding to a pre-rRNA region extending from ITS-1 to ITS-2 [48]. The ability to bind Ran, FG-repeats and RNA is consistent with a direct role in pre-ribosome export.

The above-mentioned findings were taken to propose that Rrp12 associates to both pre-40S and pre-60S particles in the nucleus to promote their export by establishing interactions with FG-repeats nucleoporins in the presence of Ran-GTP. However, the lack of information about the maturation status of the pre-ribosomes accumulated upon Rrp12 depletion does not allow to rule out the possibility of Rrp12 being involved in pre-ribosome assembly or maturation in the nucleus.

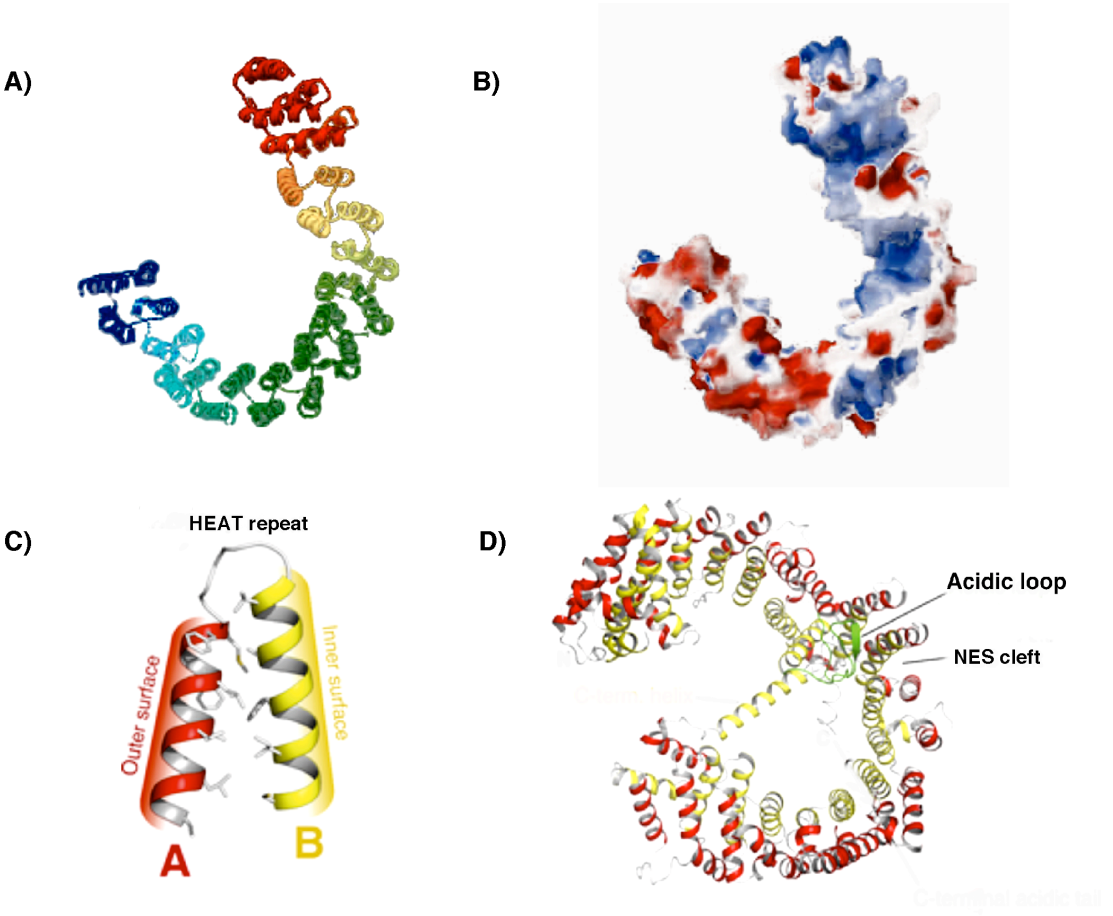


Figure 12. HEAT-repeat architecture and overall structure of Rrp12 and Crm1. (**A**) Predicted structure of Rrp12. The model comprises residues 452-1018 of the protein. Blue color corresponds to the amino terminus. (**B**) Electrostatic surface representation of the Rrp12p model. The inner concave surface of the molecule has extensive regions of positive charge and is proposed to interact with RNA. (**C**) Structural organization of a HEAT repeat. HEAT repeats are structural motifs formed by two anti-parallel helices connected by a short linker. Commonly, the A-helix (red) is located at the outer, convex surface and the B-helix (yellow) lines the inner, concave area. HEAT repeats are characterized by pronounced intra-HEAT-repeat hydrophobic interactions. (**D**) Structure of Crm1, a HEAT repeat protein that is member of the β-karyopherin family. Modified from references [47,48].

OBJECTIVES

Despite the significant progress in the understanding of the compositional changes that take place between 90S and pre-40S pre-ribosomes, there are still many questions about the nucleolar assembly and nuclear maturation of 40S subunits that remain unanswered. For example, it is still unclear how the early pre-40S particles are assembled within the 90S pre-ribosome and how similar they are, at the structural level, to the pre-40S particles that reach the cytoplasm. It is also unknown how and when pre-40S particles become competent for export, and how the export process itself takes place. The Ran GTPase and the Crm1 exportin are both essential for pre-40S particles to exit the nucleus, but the factors or mechanisms that mediate their interaction with those particles are not known. Tackling these questions has been difficult so far due to the large number of components involved, the transient nature of nucleoplasmic transit and nuclear exit, and the lack of success in dissecting these activities in separable or mechanistically simple steps.

One approach that might shed light onto the nuclear events of 40S subunit synthesis is to try to unveil the function of Rrp12, the only major non-ribosomal factor that is associated to nuclear, but not to cytoplasmic, pre-40S particles. Rrp12 was previously found to be essential for the export of pre-40S and pre-60S particles out of the nucleus, but it is presently unclear whether such function is due to an implication in the assembly of pre-ribosomal complexes, their maturation in the nucleus, the actual transport event, or compound roles in some of the above processes.

The objective of this thesis has been the elucidation of the role of Rrp12 in pre-ribosome assembly, maturation and nuclear export in *Saccharomyces cerevisiae***.**

RESULTS

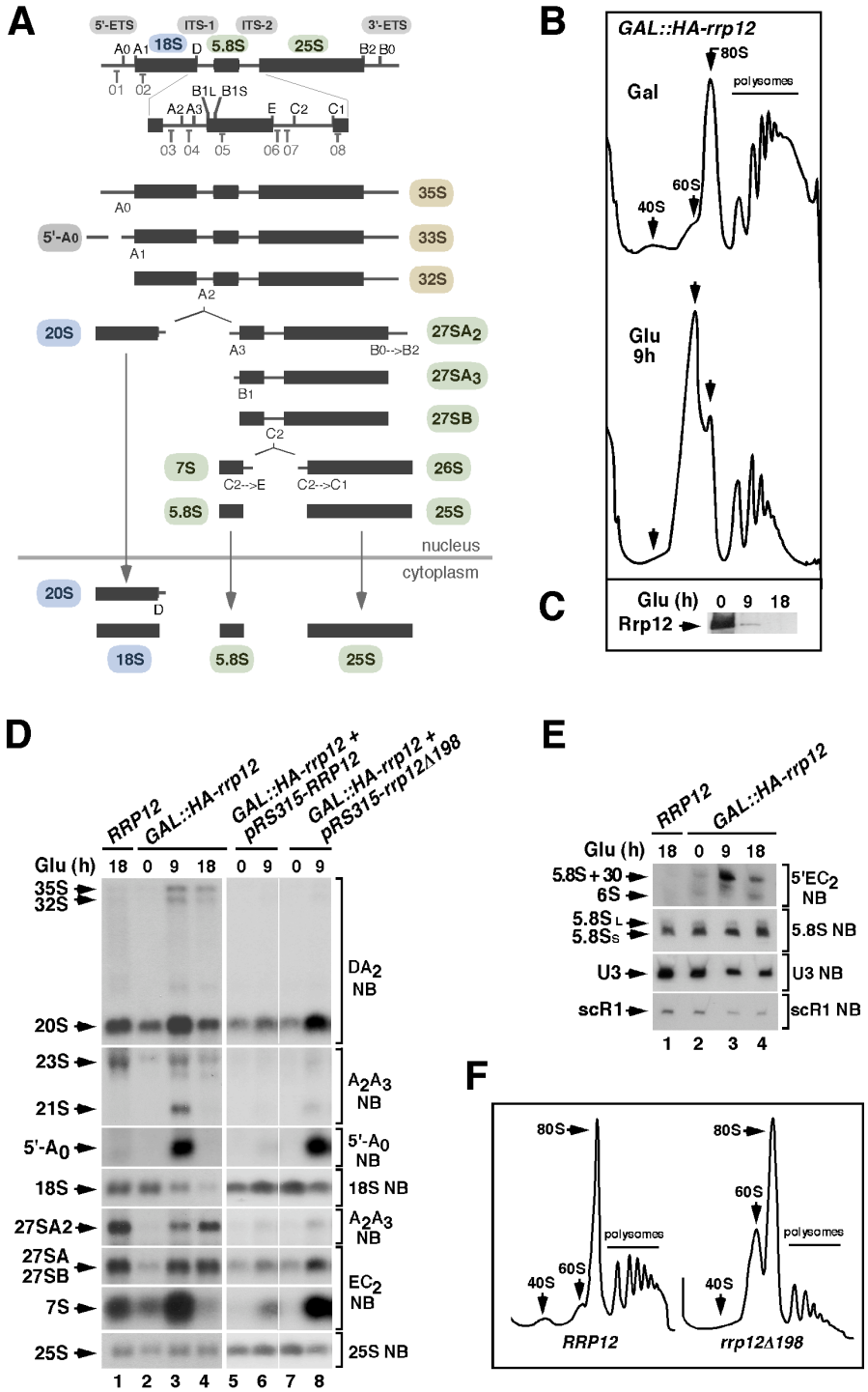
1. Rrp12 is primarily required for the synthesis of 40S ribosomal subunits

A previous report described that Rrp12 was required for export of both 40S and 60S pre-ribosomes from the nucleus to the cytoplasm [48]. However, we observed using a yeast strain with the *RRP12* gene under a galactose-inducible promoter (*GAL::HA-rrp12*) that this protein was specifically involved in the biosynthesis of 40S subunits. Evidence in favor of such conclusion included:

(**i**) Polysome profile analyses showing that the loss of Rrp12 was associated with reductions in the content of free 40S subunits and polysomes, but not of free 60S subunits (**Figure 13B** and **Figure 13C**). In fact, the relative abundance of the large subunits was clearly increased in the absence of Rrp12 (**Figure 13B** and **Figure 13C**).

(**ii**) Northern blot analyses demonstrating a decrease in the steady-state amount of the 18S rRNA (present in 40S subunits) but not in those of the 5.8S and 25S rRNAs (present in 60S subunits) in Rrp12-depleted cells (**Figure 13D**, left panels; and **Figure 13E**). Such alterations were found to be associated with an increase in the abundance of the 20S pre-rRNA, the immediate upstream precursor for the 18S rRNA (**Figure 13D**; see scheme in **Figure 13A**), indicating that the cleavage at site D is inhibited. Consistent with previously published data [48], we also observed some accumulation of the 35S and 32S pre-rRNAs, a reduction in the content of the $27SA_2$ pre-rRNA, and the generation of the aberrant 21S pre-rRNA (a species produced from direct cleavage of the 32S pre-rRNA at site A_3) (**Figure 13D**; see scheme in **Figure 13A**). These results indicate that, in addition to the major defect in the cleavage at site D, the loss of Rrp12 causes partial defects in the early cleavages at sites A_0 and A_1 and, to a larger extent, at site A_2. We also detected a delay in the processing events of 5.8S rRNA precursors manifested by the presence of both the 7S pre-rRNA and aberrant 3'-extended forms of the 5.8S rRNA (5.8S+30) in Rrp12-depleted cells (**Figure 13D** and **Figure 13E**). Curiously, we found that the absence of Rrp12 led to an increase in the abundance of the 5'-A_0 fragment (**Figure 13D**), a byproduct produced when the rRNA precursor is cleaved at site A_0 (**Figure 13A**). Similar defects, although milder in intensity, were observed in a constitutive manner when pre-rRNA analyses were performed in a yeast strain (*rrp12-Δ198*) expressing a hypomorphic version of Rrp12 (**Figure 13D**, right panels; **Figure 13F**).

Taken together, these data indicate that Rrp12 is absolutely required for the generation of the 18S rRNA from 20S pre-rRNA and, in addition, important for both the rapid elimination of the 5'-A_0 fragment and the normal processing of both 32S and 5.8S pre-rRNA precursors. Despite this latter function, Rrp12 does not seem to have any major influence on the overall production of 60S ribosomal subunits.

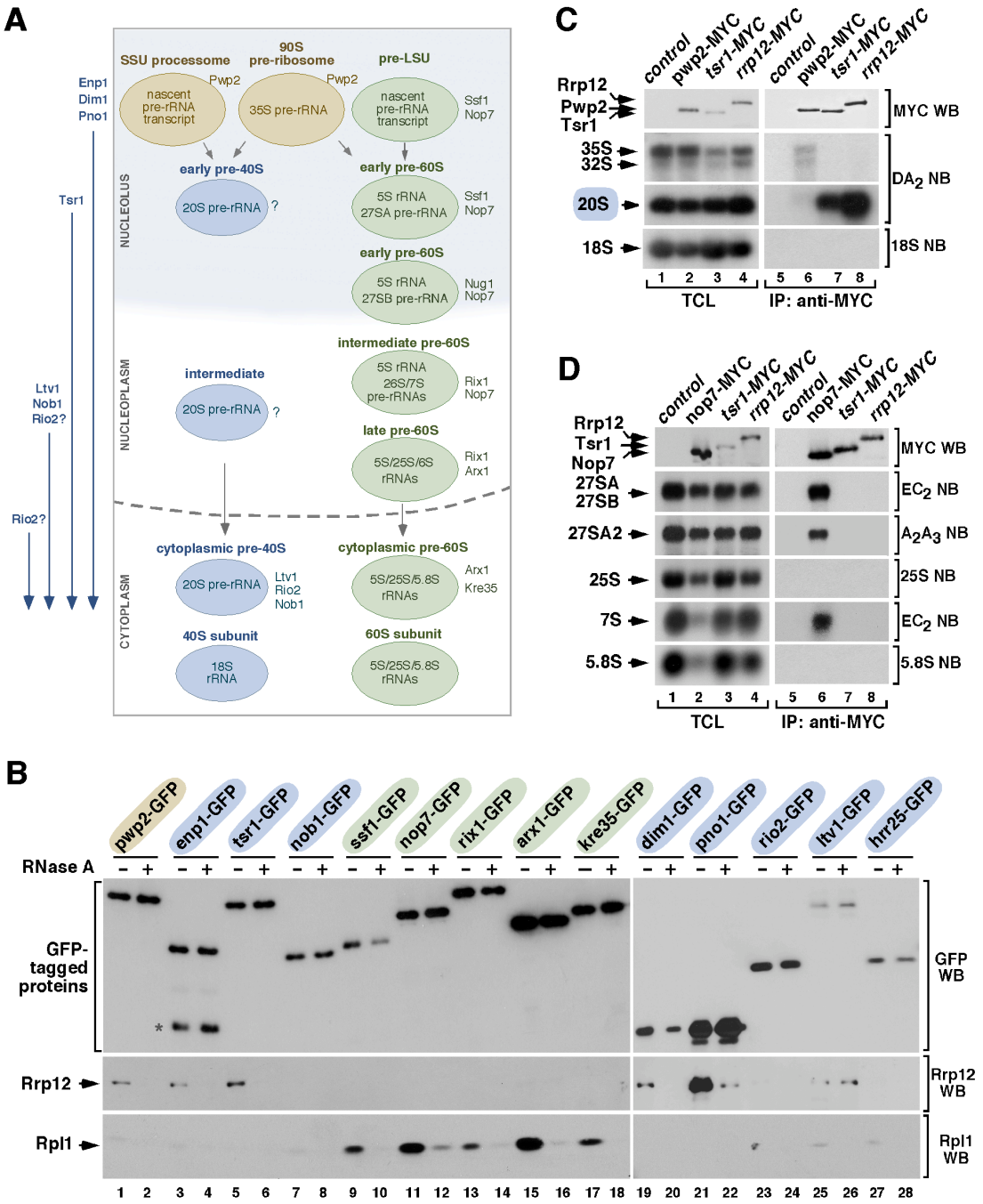


2. Rrp12 is present in both 90S and pre-40S particles

Our group and others have previously shown that Rrp12 copurifies with components of 90S and pre-40S particles [36,48,64,65]. However, there is no detailed information about its relative content in different subsets of pre-40S complexes. Using coimmunoprecipitation experiments, we observed that endogenous Rrp12 interacted with green fluorescent protein (GFP)- tagged versions of factors present in nucleolar 90S (Pwp2, Enp1, Dim1, Pno1; **Figure 14A** and **Figure 14B**, lanes 1 to 4 and lanes 19 to 22) and nucleoplasmic pre-40S (Enp1, Dim1, Pno1, Tsr1; **Figure 14A** and **Figure 14B**, lanes 3 to 6 and lanes 19 to 22) particles. These interactions took place within the context of ribonucleproteic complexes, because they were eliminated by RNase treatment (**Figure 14B**, lanes 1 to 6 and lanes 19 to 22). By contrast, we did not detect any association of Rrp12 in these experiments with either Nob1 or Rio2, two proteins mostly present in cytoplasmic pre-40S particles (**Figure 14A** and **Figure 14B**, lanes 7,8 and lanes 23,24). Rrp12 did show an interaction with Ltv1, a protein that, like Nob1 and Rio2, is mainly detected in cytoplasmic pre-40S complexes (**Figure 14A** and **Figure 14B**, lanes 25,26). This interaction is the only one that cannot be disrupted by RNase treatment (**Figure 14B**, lanes 25,26), indicating that it survives pre-40S particle disassembly or,

◀ **Figure 13. Defects in Rrp12 function block the synthesis of 40S subunits but not of 60S subunits.** (**A**) Structure of the 35S pre-rRNA and major intermediates of the rRNA processing pathway. The names of the initial pre-rRNA species are highlighted in brown. Those for the 18S pre-rRNA precursor, 5.8S/25S precursors, and 5'-A_0 processing byproduct are highlighted in blue, green and grey, respectively. For simplicity, an alternative pathway to form $27SB_L$ pre-rRNA is not shown. Binding sites for oligonucleotide probes (01 to 08) used in Northern blot experiments are indicated in the upper diagram. Those included probe 03 for the DA_2 region, probe 04 for the A_2–A_3 region, probe 01 for the 5'-A_0 region, probe 02 for the 18S region, probe 07 for the E-C_2 region, probe 08 for the 25S region, probe 06 for the 5'EC_2 region and probe 05 for the 5.8S region. (**B**) Sucrose-gradient sedimentation analysis of ribosomal fractions (40S, 60S, 80S and polysomes) of cell lysates from *GAL::HA-rrp12* cells that have been grown in galactose (Gal)-containing media or shifted to a glucose (Glu)-containing media for 9 hours. Depletion of Rrp12 protein was analyzed by Western blot (**C**). (**D** and **E**) Northern blot (NB) analysis of total RNAs extracted from *RRP12*, *GAL::HA-rrp12*, and *GAL::HA-rrp12* cells containing plasmids encoding either wild type Rrp12 or the hypomorphic Rrp12 (deletion Δ1-198) mutant. Cells were grown at 30°C in galactose-containing media and shifted to glucose-containing media for the indicated times. The specific region of the 35S pre-rRNA recognized by the Northern blot probe is indicated on the right. This will be similarly indicated in the rest of analyses presented in this work. The thin white lines between lanes 6 and 7 indicate the presence of in-between lanes in the same blot that have been removed. The experiment shown in E also includes, as a loading control, the RNA of the signal recognition particle scR1. (**F**) Sucrose-gradient sedimentation analysis of ribosomal fractions (40S, 60S, 80S and polysomes) of cell lysates from *GAL::HA-rrp12* cells containing plasmids encoding either wild type Rrp12 or the hypomorphic Rrp12 (Δ1-198) mutant. Cells were grown continuously in glucose-containing media.

alternatively, that takes place outside those particles. In agreement with the results presented in **Figure 13**, we could not detect interactions of Rrp12 with proteins present in early (Ssf1, Nop7; **Figure 14A** and **Figure 14B**, lanes 9 to 12), intermediate nuclear (Rix1; **Figure 14A** and **Figure 14B**, lanes 13,14), late nuclear (Arx1; **Figure 14A** and **Figure 14B**, lanes 15,16) or cytoplasmic (Kre35; **Figure 14A** and **Figure 14B**, lanes 17,18) pre-60S complexes.



These results suggest that Rrp12 is predominantly associated to both nucleolar and nuclear pre-40S pre-ribosomes while it is weakly associated, or not bound at all, to the cytoplasmic ones. Further analyses of Rrp12-MYC immunoprecipitates by Northern blot confirmed the predominant presence of this protein in the 40S synthesis pathway and, in addition, evidenced that its interactions with nucleolar and nucleoplasmic particles exhibit differential features. Thus, we observed that the association of Rrp12 to pre-40S particles had to be rather strong, as inferred by the stable coimmunoprecipiation of the 20S pre-rRNA with Rrp12-MYC (**Figure 14C**). Indeed, the amount of this pre-RNA in those complexes is even higher than that seen in the case of immunoprecipitations performed with Tsr1, a factor that stably associates with both nucleolar- and cytoplasmic-located pre-40S particles (**Figure 14A** and **Figure 14C**). By contrast, we could not detect any significant amount of 35S and 32S pre-RNAs in the Rrp12-MYC immunoprecipitates, suggesting that the association with the 90S particle is either labile or restricted to a minor pool of Rrp12-containing complexes (**Figure 14C**). As control, we found that these two pre-RNAs do coimmunoprecipitate with Pwp2 (**Figure 14C**), an integral component of the 90S pre-ribosome (**Figure 14A**). Consistent with the lack of Rrp12 in the purifications of pre-60S complexes (see above **Figure 14B**), we could not observe any interaction of Rrp12-MYC with the 27S or 7S pre-rRNAs. As expected, these two

◀ **Figure 14. Rrp12 is present in both 90S pre-ribosomes and pre-40S particles.** (**A**) Scheme of the maturation of pre-ribosomes. The names of specific factors frequently used for purifying each pre-ribosome are indicated on the right. In rapidly growing cells, 60% of primary transcripts are cleaved at A_0–A_1–A_2 co-transcriptionally within the small subunit (SSU) processome and, after this, the precursor of the large subunit (pre-LSU) is assembled onto the nascent pre-rRNA. When not cleaved co-transcriptionally, the full-length 35S pre-rRNA is assembled into the 90S pre-ribosome, a particle very similar to the SSU-processome. The order of incorporation of the seven major maturation factors present in cytoplasmic pre-40S particles is shown on the left. Enp1, Dim1 and Pno1 are recruited to 90S/SSU particles. Tsr1 is recruited to early pre-40S particles in the nucleoplasm. Ltv1 and Nob1 join pre-40S particles in the nucleus. The step of incorporation of Rio2 remains ill-defined. (**B**) Western blot analysis showing coimmunoprecipitation of Rrp12 (second panels from top) and of the control protein Rpl1 (bottom panels) with the indicated 90S pre-ribosome and nuclear pre-40S factors (top) in the presence (+) or absence (-) of RNase A in cell lysates. Factors present in 90S, pre-40S and pre-60S particles are shaded in brown, blue and green, respectively. The amount of GFP-Trap purified bait is shown in the first panels from top. The asterisk indicates a protein species in the Enp1-GFP purification lane that probably corresponds to a partial degradation product. (**C** and **D**) Northern blot analysis showing coimmunoprecipitation of pre-rRNA species (second to bottom panels on the right) with the indicated MYC-tagged proteins in normal cells. As control, parallel Northern blots were performed on total RNAs prepared from the same total cell lysate samples used for the immunoprecipitations (second to bottom panels on the left). Western blot experiments were performed to analyze the amount of MYC-tagged protein present in the total cell lysates (top panel on the left) and immunoprecipitations (top panel on the right). TCL, total cell lysates. IP, immunoprecipitation.

pre-rRNAs do coimmunoprecipitate with the early pre-60S particle component Nop7-MYC (**Figure 14D**, see scheme in **Figure 14A**). These results indicate that Rrp12 does not stably associate with pre-60S particles.

3. Rrp12 is not required for pre-40S particle assembly

We next focused on the cause of the block in the maturation of 20S pre-rRNA to 18S rRNA found in Rrp12-depleted cells. Given the restricted presence of Rrp12 to nucleolar 90S and nucleoplasmic pre-40S complexes, this phenotype could be due to defects in the assembly of the pre-40S particle inside the nucleus. However, this does not seem to be the case because the depletion of Rrp12 does not affect the stability of both early and late nuclear pre-40S components (Enp1, Dim1, Tsr1, Rio2, Nob1; **Figures 15A** to **C**; top panels). Likewise, it does not block the interaction of those proteins with the 20S pre-rRNA (**Figures 15A** to **C**; bottom panels). However, the depletion of Rrp12, although not affecting the steady state levels of Ltv1 in cell lysates prepared by TCA precipitation (**Figure 15D**, compare lanes 7 and 9), does cause a destabilization of that protein under the conditions used for the pre-rRNA coimmunoprecipitation analyses (**Figure 15C**; top panel, compare lanes 4 and 6). Such behavior may reflect a functional relationship of Rrp12 and Ltv1 *in vivo*, because we observed using sucrose gradient fractionation experiments that the loss of Rrp12 leads to a substantial decrease in the amount of Ltv1 that is stably incorporated onto 40S complexes (**Figure 16A**). This effect is specific, because the depletion of Rrp12 does not affect the incorporation of both Enp1 and Rio2 onto those complexes (**Figure 16B** and **Figure 16C**).

Mass spectrometry experiments further confirmed that the absence of Rrp12 does not have a major effect in the composition of pre-40S complexes. Indeed, we found that both the pattern and strength of the associations exhibited by four pre-40S factors (Enp1, Tsr1, Nob1 and Rio2) with the rest of major pre-40S particle components are quite similar to those observed in wild-type cells (**Figure 17** compare columns 1 to 4 with columns 5 to 8). The only exception observed is the loss of the interaction of both Enp1 and Tsr1 with Ltv1 (**Figure 17**, compare columns 1 and 2 with columns 5 and 6), a defect probably derived from the impaired recruitment of Ltv1 to the pre-40S particle seen in above experiments.

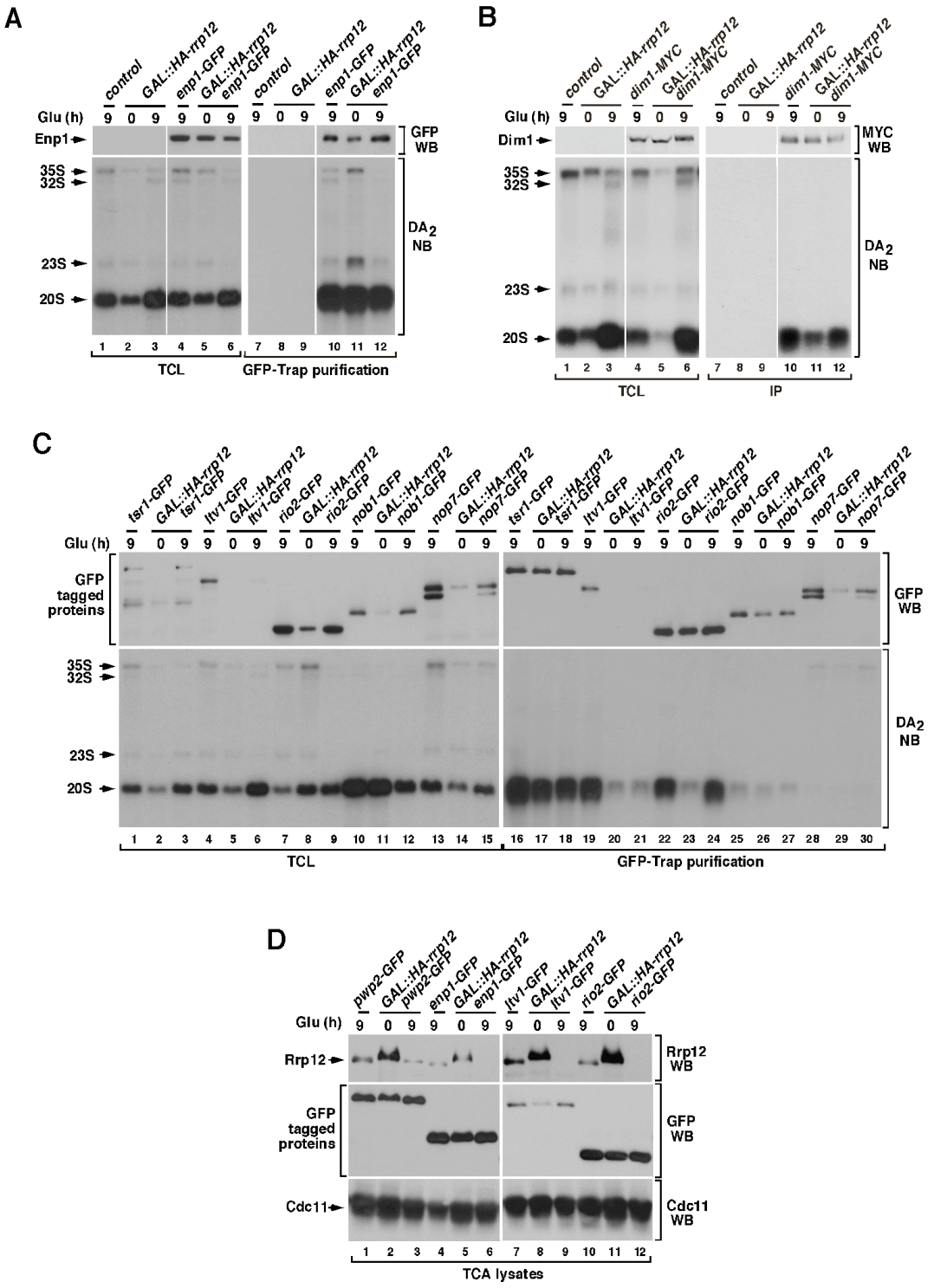


Figure 15. Rrp12 is not involved in pre-40S particle assembly. (**A** to **C**) Bottom panels, Northern blot analysis showing coimmunoprecipitation of the 20S pre-rRNA with Enp1-GFP (**A**), Dim1-MYC (**B**), Tsr1-GFP (**C**), Ltv1-GFP (**C**), Rio2-GFP (**C**), Nob1-GFP (**C**) and Nop7-GFP (**C**) before (0) and upon depletion of Rrp12 for 9 hours. Top panels, Western blot analysis showing the amount of immunoprecipitated proteins in these experiments. Mobility of pre-RNA species is indicated on the left of each bottom panel. Antibodies used in the immunoblots and Northern blot probes are shown on the right of the top and bottom panels, respectively. The thin white lines between lanes 3 and 4, and 9 and 10, shown in **A** and **B**, indicate the presence of in-between lanes in the same blot that have been removed. (**D**) Western blot analyses of trichloroacetic acid (TCA) precipitated cell lysates showing the amount of Rrp12 (top panels) and the indicated GFP-tagged proteins (middle panels) under the indicated growth conditions. The amount of Cdc11 was used as loading control (bottom panel).

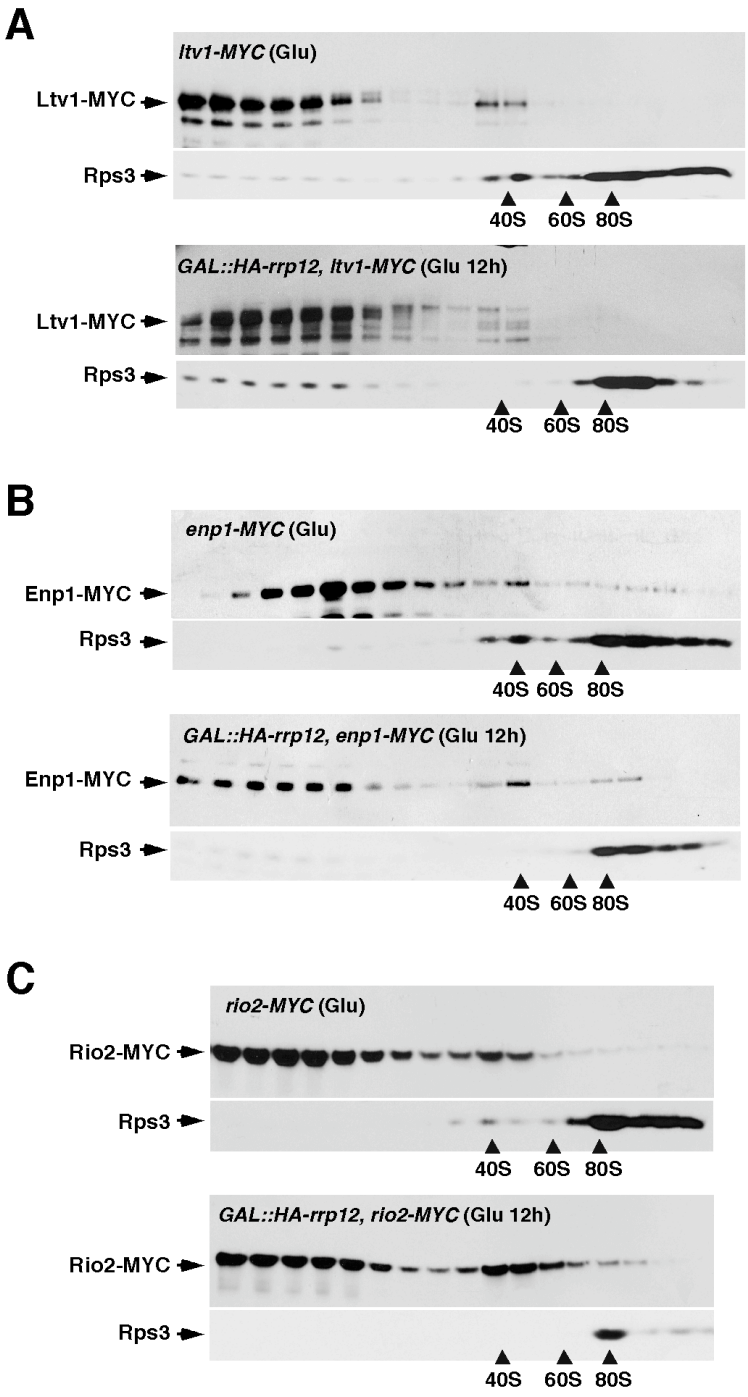


Figure 16. Recruitment of maturation factors to pre-40S particles in the absence of Rrp12 (A–C) Sucrose gradient analysis showing the sedimentation behavior of Ltv1-MYC **(A)**, Enp1-MYC **(B)** and Rio2-MYC **(C)** in the presence (top two panels) and absence of Rrp12 (bottom two panels). Each set of gradient fractions was analyzed by Western blot with anti-MYC, and anti-Rps3. The positions of the gradient where 40S, 60S and 80S complexes sedimented are indicated by arrows.

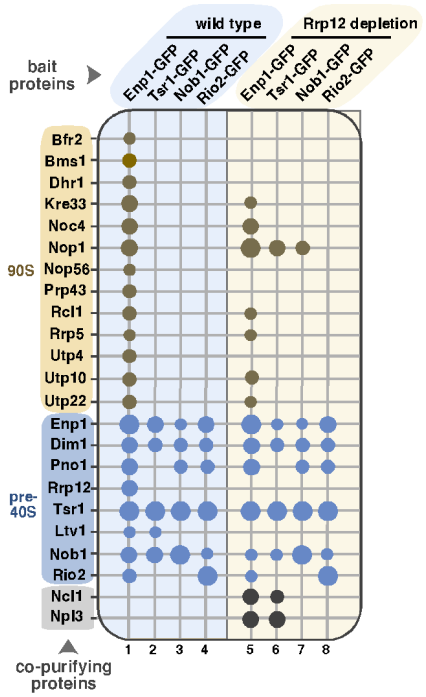


Figure 17. Proteomic analysis of pre-40S complexes formed upon Rrp12 depletion. Pre-ribosomal factors (listed on the left) copurifying with the indicated GFP-tagged proteins (top) in the presence (columns 1 to 4) or absence (columns 5 to 8) of Rrp12. Copurification of a factor with the bait is indicated with a dot. For Rrp12 depletion, *GAL::HA-rrp12* cells were shifted from galactose-containing media to glucose-containing media for 12 hours. The pre-ribosomal particles that contain the prey proteins are indicated on the left. Size of dots represents the relative amount of coimmunoprecipitated protein in each case (see Materials and Methods).

Interestingly, we observed that the loss of Rrp12 promotes the formation of new interactions of both Enp1 and Tsr1 with the tRNA methyltransferase Ncl1 and the abundant hnRNP protein Npl3 (**Figure 17**, compare columns 1 and 2 with columns 5 and 6). Likewise, Tsr1 and Nob1 interact with the 90S particle component Nop1 (**Figure 17**, compare columns 2 and 3 with columns 6 and 7). These results indicate that Rrp12 is not required for the formation of the core structure of the pre-40S particle, although it may contribute to the release of specific nucleolar factors such as Nop1. In addition, they show that Rrp12 appears to be dispensable for the recruitment of some factors with hitherto unknown roles in the synthesis of 40S subunits (i.e., Ncl1, Npl3). Also consistent with a correct particle assembly in the absence of Rrp12, we found using Western blot analyses that Prp43 and Mex67 [42,69-71], two factors that are not usually detected in this type of proteomics analysis due to their weak interaction with pre-40S particles, remain particle-associated in the absence of Rrp12 (**Figure 18A** and **Figure 18B**). Interestingly, the absence of Rrp12 does promote a reduction in the association of Enp1 with some, but not all, of its usual partners within the 90S particle (**Figure 17**, compare columns 1 and 5). These data indicate that the lack of Rrp12 may affect either the composition or maturation dynamics of 90S pre-ribosomes.

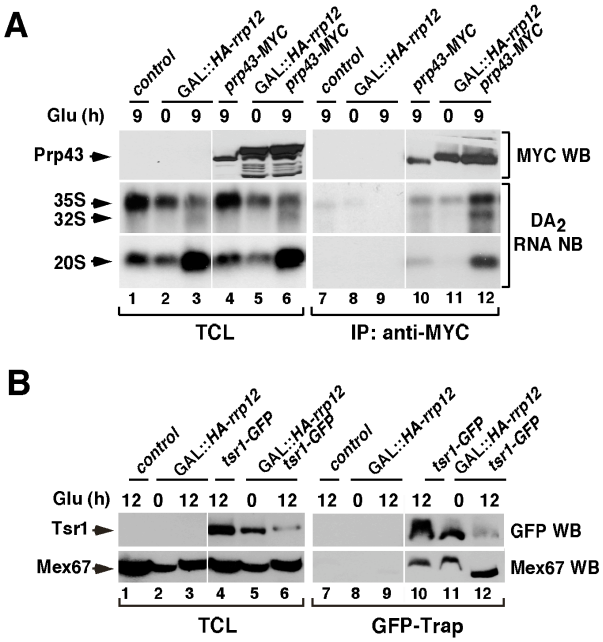
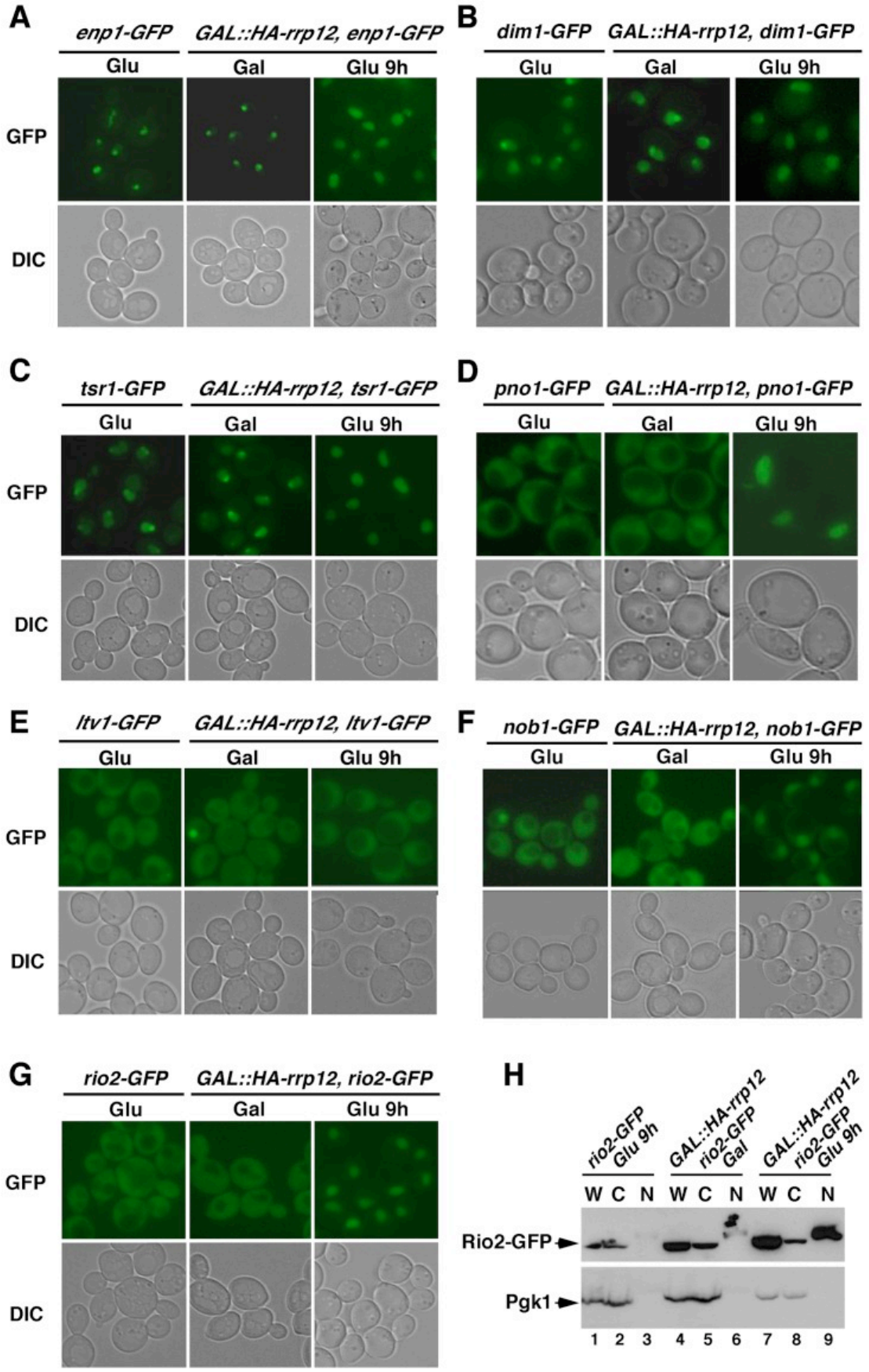


Figure 18. Rrp12 is not required for the association of Prp43 and Mex67 with pre-40S particles. (**A**) Northern blot analysis showing coimmunoprecipitation of the indicated pre-RNA species with Prp43-MYC in the presence and absence of Rrp12. Total RNAs (middle and bottom panels, lanes 1 to 6) and RNAs present in Prp43-MYC immunoprecipitates (middle and bottom panels, lanes 7 to 12) obtained from the indicated strains, grown under the indicated conditions, were analyzed with a probe that maps to the pre-rRNA D-A_2 region. Western blot experiments were performed to analyze the amount of Prp43-MYC present in the total cell lysates (top panels, lanes 1 to 6) and immunoprecipitations (top panels, lanes 7 to 12). (**B**) Western blot analysis showing copurification of Mex67 with Tsr1-GFP in the presence and absence of Rrp12. Total cell lysates (lanes 1 to 6) and GFP-Trap purified complexes (lanes 7 to 12) obtained from the indicated yeast strains, grown under the indicated conditions, were analyzed with anti-MYC and anti-Mex67 antibodies. The thin white lines between lanes 3 and 4, and lanes 9 and 10, shown in A and B, indicate the presence of in-between lanes in the same blot that have been removed.

4. Rrp12 is required for nuclear export of pre-40S particles

The above findings indicated that the lack of Rrp12 blocks the 40S synthesis pathway at a step downstream the assembly of pre-40S particles. To investigate if this block occurred in the nucleolus, nucleoplasm or cytoplasm, we analyzed the subcellular localization of GFP-tagged versions of pre-40S particle (Enp1, Dim1, Pno1, Tsr1, Ltv1, Nob1, Rio2) and mature 40S subunit (Rps2) components in control and Rrp12-depleted cells (**Figure 19** and **Figure 20A**).

Figure 19. Rrp12 is required for the export of pre-40S particles out of the nucleus. (**A** to **G**) Top panels, epifluorescence microscopy analysis of the subcellular distribution of GFP-tagged Enp1 (**A**), Dim1 (**B**), Tsr1 (**C**), Pno1 (**D**), Ltv1 (**E**), Nob1 (**F**) and Rio2 (**G**) in the indicated yeast strains and culture conditions (top). Bottom panels, differential interference contrast (DIC) images of the above preparations. (**H**) Western blot analysis showing the distribution of Rio2-GFP (top panel) and Pgk1 (bottom panel) in whole cell lysates (W), cytosolic (C) and nuclear (N) fractions obtained from either control *rio2-GFP* cells (lanes 1 to 3) or *GAL::HA-rrp12/rio2-GFP* cells growing in galactose-containing medium (lanes 4 to 6) or upon a shift to glucose-containing medium for 9 hours (lanes 7 to 9).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GAL::HA-rrp12, enp1-GFP
Glu
Gal
Glu 9h
GFP
DIC
B
dim1-GFP
GAL::HA-rrp12, dim1-GFP
Glu
Gal
Glu 9h
C
tsr1-GFP
GAL::HA-rrp12, tsr1-GFP
Glu
Gal
Glu 9h
GFP
DIC
D
pno1-GFP
GAL::HA-rrp12, pno1-GFP
Glu
Gal
Glu 9h
E
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Glu 9h
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nob1-GFP
GAL::HA-rrp12, nob1-GFP
Glu
Gal
Glu 9h
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rio2-GFP
GAL::HA-rrp12, rio2-GFP
Glu
Gal
Glu 9h
GFP
DIC
H
rio2-GFP Glu 9h
GAL::HA-rrp12 rio2-GFP Gal
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W C N W C N W C N
Rio2-GFP
Pgk1
1 2 3 4 5 6 7 8 9

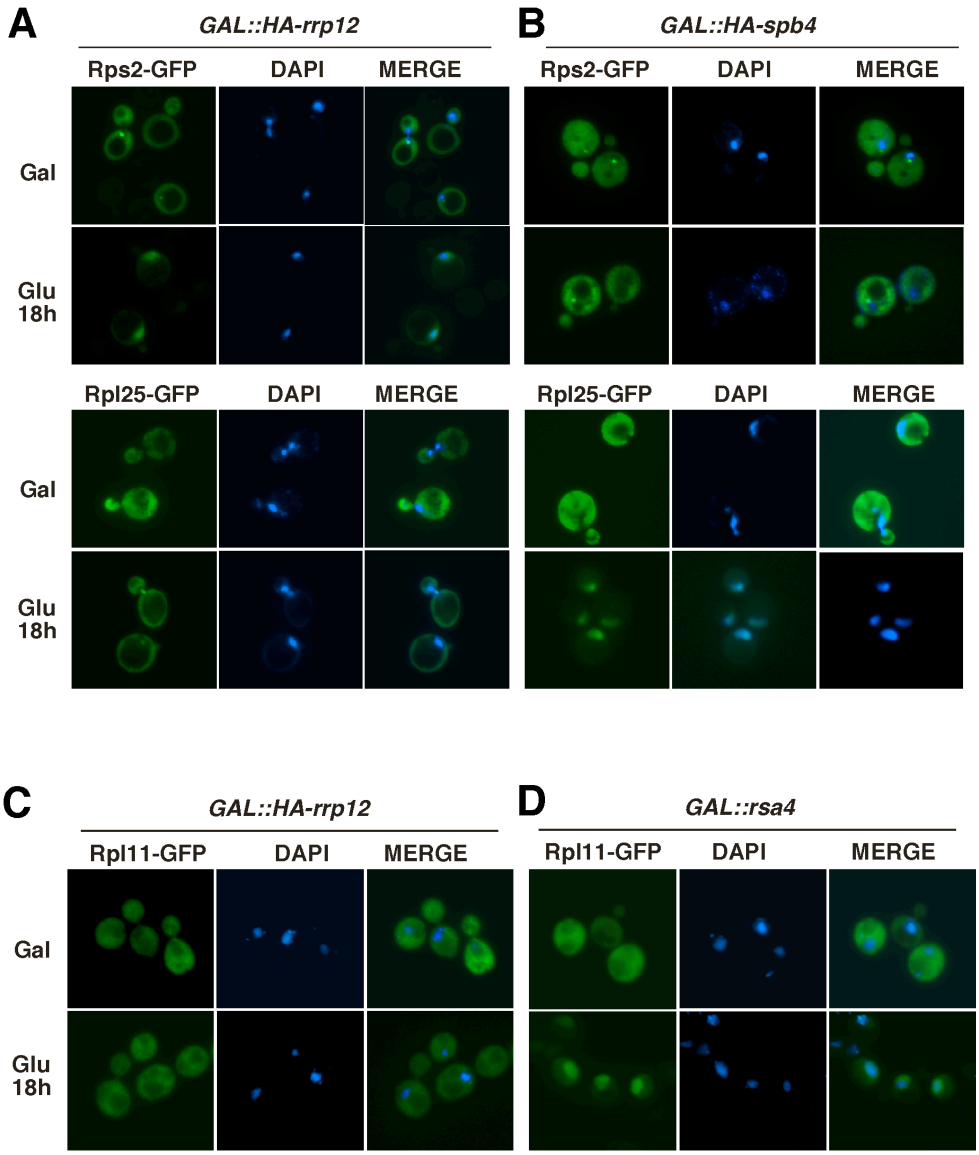


Figure 20. The loss of Rrp12 causes accumulation of pre-40S, but not pre-60S, complexes in the nucleus. Epifluorescence microscopy analysis of *GAL::HA-rrp12* cells (**A, C**), control *GAL::HA-spb4* cells (**B**), and control *GAL::HA-rsa4* cells (**D**) expressing 40S (Rps2-GFP; top and second panels in **A** and **B**), 60S (Rpl25-GFP, third and bottom panels in A and B; and Rpl11-GFP, top and bottom panels in **C** and **D**) subunit reporters. These cells were grown in galactose-containing medium or shifted to glucose-containing medium for 18 h as indicated. Spb4 and Rsa4 are 60S subunit synthesis factors that, when depleted, cause accumulation of pre-60S particles in the nucleus. The GFP signal, the DAPI-stained nuclei and the GFP-DAPI merge are shown in the left, middle and right panels, respectively.

Consistent with previous reports [32,34,36,38,39], we found that these proteins exhibit nucleolar (Enp1, **Figure 19A**), nucleolar and nucleoplasmic (Dim1, Tsr1; **Figure 19B** and **Figure 19C**, respectively), and nucleoplasmic plus cytoplasmic (Pno1, Ltv1, Nob1, Rio2 and Rps2; **Figures 19D to G**, and **Figure 20A**, respectively) localizations in both wild type cells and control *GAL::HA-rrp12* cells. However, in Rrp12-depleted *GAL::HA-rrp12* cells, we detected that most of those proteins undergo a major relocalization towards the nucleoplasm (**Figures 19A to G**; and **Figure 20A**). The only exception was again Ltv1, since its subcellular distribution is fully Rrp12-independent (**Figure 19E**). The nuclear accumulation of Rio2, but not of the cytosolic Pgk1 control protein, in the absence of Rrp12 was demonstrated using independent subcellular fractionation experiments (**Figure 19H**). This effect is specific for the 40S subunit synthesis pathway, because the loss of Rrp12 does not alter the normal subcellular distribution of Rpl25 and Rpl11 (**Figure 20A and Figure 20C**), two 60S subunit components. These results show that pre-40S particles are blocked in the nucleoplasm when Rrp12 is absent. Collectively, our data indicate that Rrp12 does not participate in the major assembly events of pre-40S particles in the nucleus, and that it is essential for some event that immediately precedes or is concomitant to nuclear export.

5. Rrp12 influences an intermediate maturation step within a 90S transitional particle

In addition to the block in pre-40S particle export, the depletion of Rrp12 causes defects in the cleavage of the pre-rRNA at site A_2 and in the elimination of the 5′-A_0 fragment. The accumulation of this byproduct appears to be a rather specific feature, because it is not observed upon depletion of other factors (**Figure 21A** and **Figure 21B**). We also found that the 5′-A_0 fragment associates to Rrp12 in wild type cells (**Figure 22**), suggesting that Rrp12 might influence directly the elimination of this fragment.

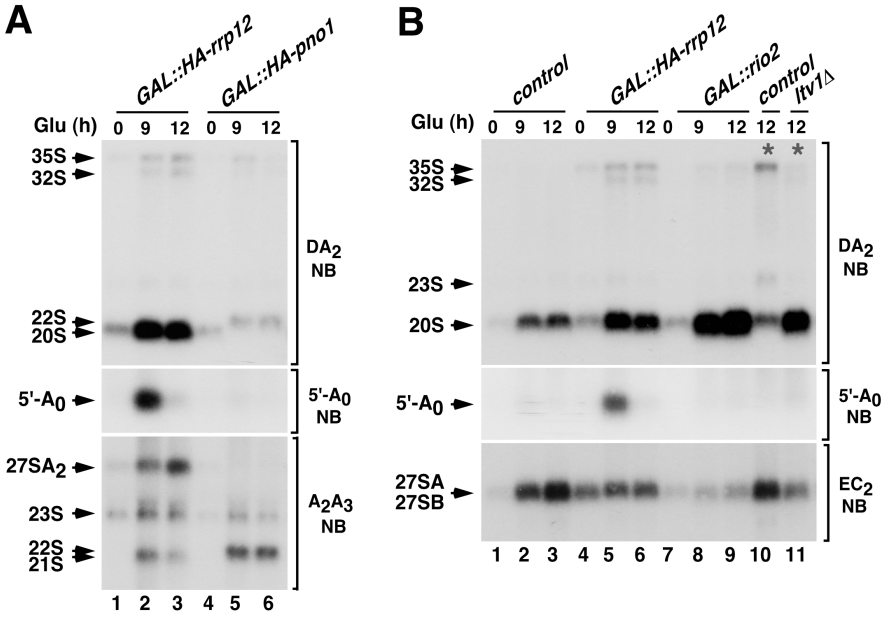


Figure 21. The loss of Pno1, Rio2 or Ltv1 does not cause accumulation of the 5'-A_0 fragment. Northern blot analysis of total RNAs extracted from *GAL::HA-rrp12* and *GAL::HA-pno1* cells (**A**), and from *GAL::HA-rrp12, GAL::rio2* and *ltv1Δ* cells (**B**). Cells were grown at 30°C (except those corresponding to the lanes marked with an asterisk in **B**) in galactose-containing media or shifted to glucose-containing media for the indicated times. The samples marked with an asterisk (lanes 10 and 11 in **B**) were prepared from cultures grown at 25°C, the temperature at which the defects of the *LTV1* deletion are most patent.

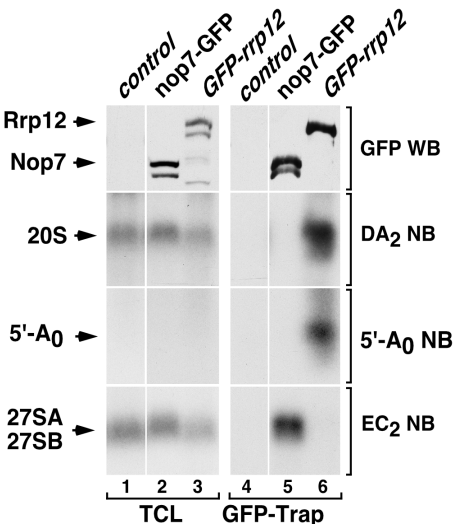


Figure 22. Interaction of the 5'-A_0 fragment and Rrp12 in wild type cells. As control, a parallel Northern blot analysis was performed on total RNAs prepared from the same total cell lysate samples used for the GFP-Trap protein purifications (second to bottom panels on the left). Western blot experiments were performed to analyze the amounts of the GFP-tagged proteins present in the total cell lysates (top panel on the left) and in the purifications (top panel on the right). The strains used in this experiment were W303 (control), JDY851 (*nop7-GFP*) and YPM7-R (*GAL::HA-rrp12* containing a pRS416-GFP-rrp12 plasmid). These strains were maintained continuously in glucose-containing media. The specific region of the 35S pre-rRNA recognized by each Northern blot probe is indicated on the right.

As a first approximation to obtain clues about the role of Rrp12 in this process, we decided to study the sedimentation behavior of the 5′-A_0 fragment on sucrose gradients in the presence and absence of Rrp12. These experiments corroborated the increase in the abundance of the 5′-A_0 fragment already seen by Northern blot analyses in Rrp12-depleted cells (see above, **Figure 13D** in page 38) and, in addition, revealed that this fragment was present in complexes that sediment broadly between the 60S and 90S regions of the gradient (**Figure 23A**; right panels, gradient fractions 12 to 15). A significant proportion of these entities cosedimented with the 32S pre-rRNA and U3 snoRNA (**Figure 23A**; right panels, gradient fractions 14, 15), suggesting that they form part of a 90S transitional particle that has initiated, but not completed, the processing of the 35S pre-rRNA. This interpretation is consistent with the delay in the A_2 cleavage evidenced by the formation of aberrant 21S pre-rRNA (see above **Figure 13D**, in page 38), and the increased coimmunoprecipitation of the 5′-A_0 fragment with the 90S pre-ribosome-specific Pwp2 protein in Rrp12-depleted cells (**Figure 23B**, compare lanes 10 and 12). The interaction of Pwp2 with the 5′-A_0 fragment appears to take place in the context of a 90S pre-ribosome-like particle, as inferred from the presence of Pwp2 in 80–90S complexes in Rrp12-depleted cells (**Figure 23C**). In agreement with an abnormal accumulation of a 90S transitional particle, we observed by microscopy experiments that Pwp2 shifted from an exclusively nucleolar localization to a more disperse distribution between the nucleolus and the nucleoplasm upon depletion of Rrp12 (**Figure 23D**). These results indicate that the loss of Rrp12 delays some event during pre-40S particle assembly in the nucleolus, leading to both the accumulation and delocalization of 90S transitional particles in the nucleoplasm.

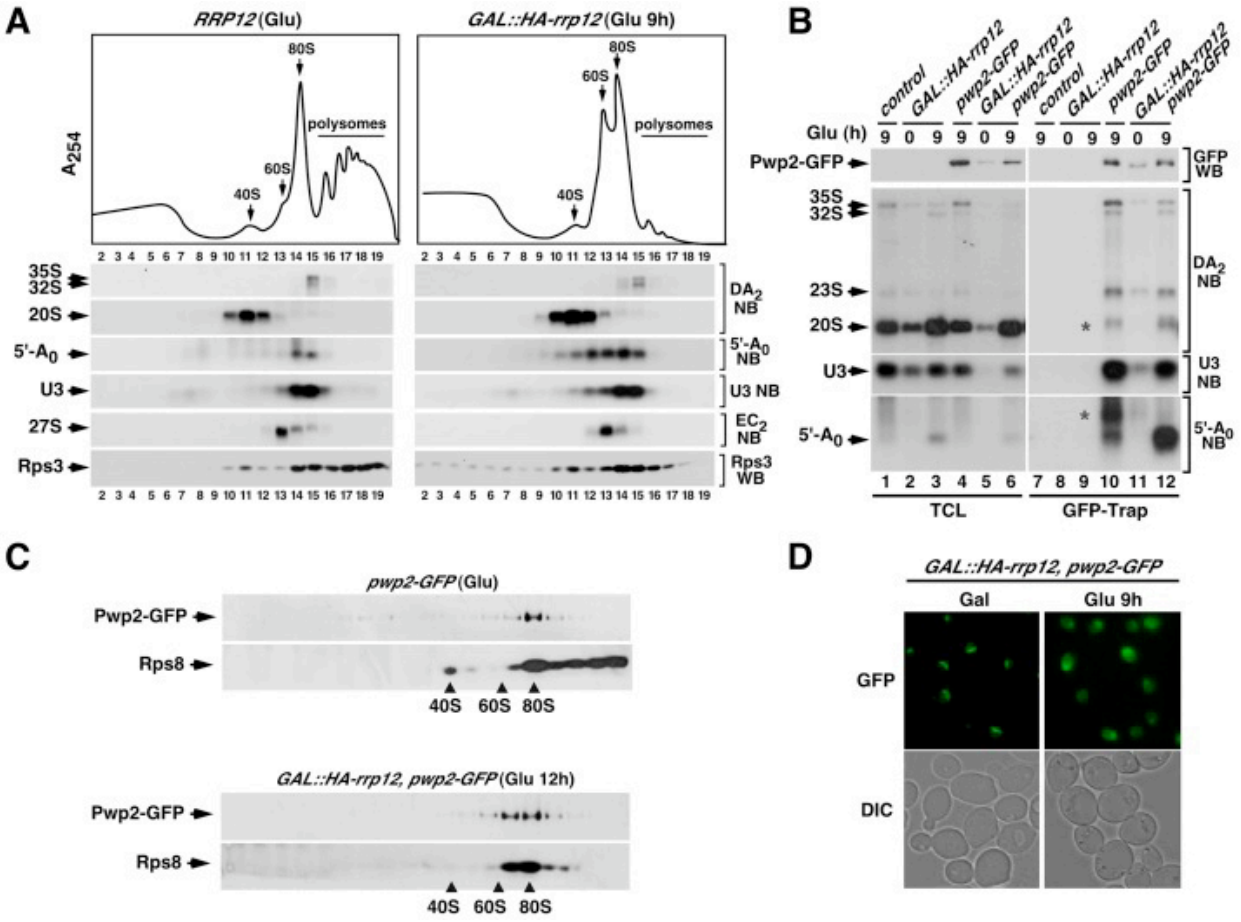
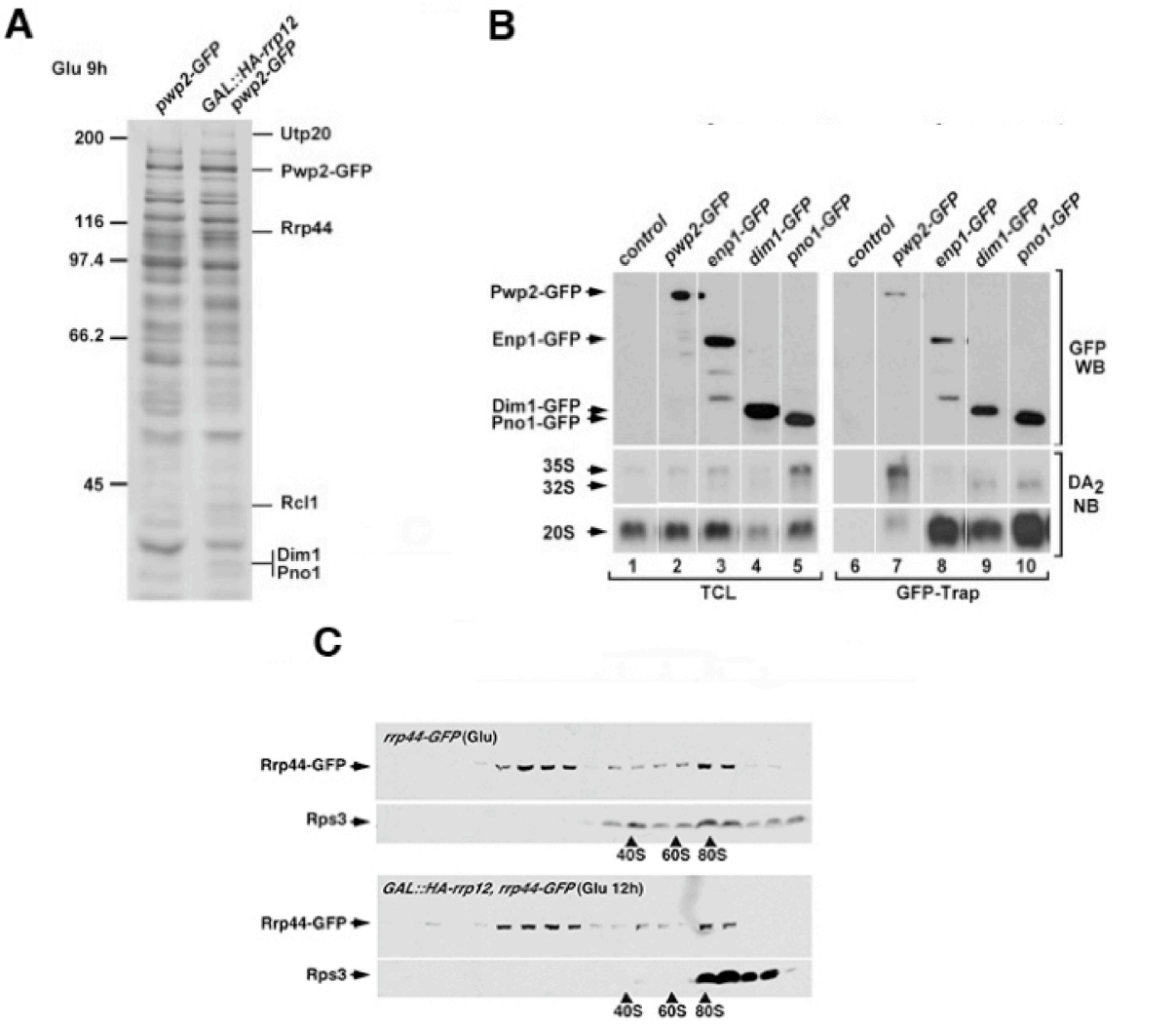


Figure 23. Loss of Rrp12 causes accumulation of 5'-A0-containing 90S pre-ribosomes. (**A**) Top panel, sucrose-gradient sedimentation analysis of ribosomal fractions (40S, 60S, 80S and polysomes) of cell lysates from the control wild type strain grown in glucose-containing media, and the *GAL::HA-rrp12* strain grown in galactose-containing media and shifted to glucose-containing media for 9 hours. Bottom panels, Northern (second to sixth panels from top) and Western (bottom panel) blot analyses of indicated components of pre-ribosomal particles in fractions obtained in the gradients. Numbers of fractions are shown at the bottom. Blotting probes and antibodies are indicated on the right. (**B**) Northern blot analysis showing copurification (second to fourth panels on the right) of the indicated pre-RNA species, U3 snoRNA and 5'-A_0 fragment with Pwp2-GFP in the indicated yeast strains and culture conditions (top). As control, parallel Northern blots were performed on total RNAs prepared from the same total cell lysate samples used for the immunoprecipitations (second to third panels on the left). Western blot experiments were performed to analyze the amount of Pwp2-GFP present in the total cell lysates (top panel on the left) and GFP-Trap purified complexes (top panel on the right). Asterisks indicate pre-rRNA species that do not correspond to any major processing intermediate, which probably are 35S partial degradation products. (**C**) Sucrose gradient analysis showing the sedimentation behavior of Pwp2-GFP and Rps8 in the presence (top two panels) and absence (bottom two panels) of Rrp12. The positions of the gradient where 40S, 60S and 80S complexes sedimented are indicated by arrows. (**D**) Top panels, epifluorescence microscopy analysis of the subcellular distribution of Pwp2-GFP before (top left panel) and upon depletion (top right panel) of Rrp12. Bottom panels, DIC images of above preparations.

6. The Rrp12-dependent maturation step precedes the A_2 cleavage and the exosome-mediated degradation of the 5′-A_0 fragment

We next characterized by mass spectrometry the complexes formed by Pwp2 in the absence of Rrp12 to investigate possible differences in the composition of 90S pre-ribosomes. Although highly similar to those formed in control cells, we observed the presence of new Pwp2 partners in the absence of Rrp12 (**Figure 24A**). Those included 90S pre-ribosome components involved in the cleavage of the 35S precursor at the A_0–A_1–A_2 (Utp20, Rcl1) and A_1–A_2 (Dim1, Pno1) sites [33-35,72-74]. Interestingly, we observed using RNA coimmunoprecipitation experiments that two of the above partners, Dim1 and Pno1, preferentially bind the 32S rather than the earliest 35S pre-rRNA (**Figure 24B**). This suggests that they become stably assembled onto the 90S pre-ribosome upon cleavage of the 35S precursor at the A_0 and A_1 sites (see above, **Figure 13A**, page 38). We also found among the new partners the nuclease Rrp44 (also known as Dis3), an exosome subunit shown to be involved in the direct physical interaction with the 5′-A_0 fragment [75]. This finding was quite interesting for us, because previous results have shown that this interaction seems to be crucial for poising the 5′-A_0 fragment for productive degradation [75,76]. Thus, we surmised that the Rrp44-Pwp2 interaction detected in Rrp12-depleted cells could indicate that the exosome is normally recruited to 90S pre-ribosomes and that, in the absence of Rrp12, there is an enrichment or stabilization of some of those exosome-containing 90S pre-ribosomes. In agreement with this idea, we found using sucrose gradient sedimentation analyses that Rrp44 is indeed present in 80–90S complexes both in control and Rrp12-depleted cells (**Figure 24C**). These data raised the possibility that the defect in the elimination of 5′-A_0 fragment found in Rrp12-depleted cells could be due to an impairment of exosome function. Consistent with this idea, we found that the elimination of the exosome cofactor Mtr4 (also known as Dob1) elicited the expected accumulation of the 5′-A_0 fragment (**Figure 24D** and **Figure 24E**) [77] and, most importantly, that such accumulation occurs in the context of 80–90S complexes, similarly to what is observed in Rrp12-depleted cells (**Figure 24E**; see above, **Figure 23**).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GAL::HA-rrp12 pwp2-GFP
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97.4
66.2
45
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Rrp44
Rcl1
Dim1
Pno1
B
control
pwp2-GFP
enp1-GFP
dim1-GFP
pno1-GFP
Pwp2-GFP
Enp1-GFP
Dim1-GFP
Pno1-GFP
GFP WB
35S
32S
20S
DA2 NB
1 2 3 4 5
TCL
6 7 8 9 10
GFP-Trap
C
rrp44-GFP (Glu)
Rrp44-GFP
Rps3
40S 60S 80S
GAL::HA-rrp12, rrp44-GFP (Glu 12h)
Rrp44-GFP
Rps3
40S 60S 80S
D
MTR4
GAL::HA-mtr4
Glu (h) 0 9 12 0 9 12
35S
32S
23S
20S
5'-A0
27SA
27SB
7S
DA2 NB
5'-A0 NB
EC2 NB
1 2 3 4 5 6
E
MTR4 (Glu)
GAL::HA-mtr4 (Glu 9h)
A254
80S
polysomes
60S
40S
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
35S
32S
20S
5'-A0
U3
27S
DA2 NB
5'-A0 NB
U3 NB
EC2 NB

Interestingly, Rrp12-depleted cells do not exhibit the sustained high levels of the 5′-A_0 fragment seen in Mtr4-depleted cells (**Figure 13D** in page 38 and **Figure 24D**), indicating that the exosome activity is affected but not fully compromised upon the loss of Rrp12. Consistent with this, we have seen that the loss of this protein does not trigger other terminal defects typically observed in exosome-deficient cells, such as the abnormal accumulation of the 35S pre-rRNA, the total block of 7S pre-rRNA maturation, and the balanced decrease in the contents of both ribosomal subunits (**Figure 24D** and **Figure 24E**) [77,78]. Taken together, our data indicate that the loss of Rrp12 causes a 90S pre-ribosome maturation defect that precedes the A_2 cleavage and the exosome-dependent 5′-A_0 fragment degradation steps. As a result, it promotes either a delay or partial inhibition, but not a block, in the A_2 cleavage of the pre-rRNA and the elimination of the 5′-A_0 fragment.

◀ **Figure 24. Rrp12 is required at a 90S particle-mediated maturation step that precedes exosome action.** (**A**) Protein complexes formed by Pwp2-GFP in control and Rrp12-depleted cells. Bands and proteins identified by mass spectrometry are indicated on the right. Molecular weight markers (in kDa) are indicated on the left. (**B**) Northern blot analysis showing copurification (lanes 6 to 10) of the indicated pre-RNA species with GFP-tagged Pwp2, Enp1, Dim1 and Pno1 in normal cells. As control, parallel Northern blots were performed on total RNAs prepared from the same samples used for the immunoprecipitations (second and third panels on the left). Western blot experiments were performed to analyze the amount of the GFP-tagged protein present in the corresponding total cell lysates (top panel on the left) and GFP-Trap purifications (top panel on the right). (**C**) Sucrose gradient analysis showing the sedimentation behavior of Rrp44-GFP in the presence (top two panels) and absence of Rrp12 (bottom two panels). The gradient fractions were analyzed by Western blot with anti-GFP, and anti-Rps3. The positions of the gradient where 40S, 60S and 80S complexes sedimented are indicated by arrows. (**D**) Northern blot analysis of total RNAs extracted from *MTR4*, and *GAL::HA-mtr4* cells to show the relative contents of pre-rRNA species and 5′-A_0 fragment. Cells were grown at 30°C in galactose-containing media and shifted to glucose-containing media for the indicated times. Northern blot probes are indicated on the right. (**E**) Top panel, sucrose-gradient sedimentation analysis of ribosomal fractions (40S, 60S, 80S and polysomes) of cell lysates from control (*MTR4*) and Mtr4-depleted (*GAL::HA-mtr4* (Glu 9h)) strains. Bottom panels, Northern blot analysis of indicated components of pre-ribosomal particles in gradient fractions obtained in the above experiment. Numbers of fractions are shown at the bottom. Blotting probes and antibodies are indicated on the right.

7. The Crm1 exportin is also involved in the Rrp12-dependent 90S pre-ribosome maturation step

Given the implication of Rrp12 in the export of pre-40S particles (see above, **Figures 15 to 20**), we decided to investigate whether the pre-40S export step was associated to the Rrp12 dependent 90S pre-ribosome maturation step. If that were the case, we expected that the elimination of any other protein involved in pre-40S export would induce the same defects seen in Rrp12-depleted cells. To test this idea, we chose a yeast strain that constitutively expressed a mutant version of the Crm1 ($Crm1^{T539C}$) exportin. This mutant protein, unlike its wild type counterpart, can be specifically inhibited by leptomycin B [79]. Using this strategy, we found that the inhibition of Crm1 recapitulates all the defects observed in Rrp12-depleted cells, including increased abundance of the 35S, 32S and 21S pre-RNA species (**Figure 25A**), abnormal levels of the 5′-A_0 fragment (**Figure 25A** and **Figure 25B**), accumulation of this fragment in 80–90S complexes (**Figure 25B**) and, as expected [80], an increase in the content of the 20S pre-rRNA due to the halt in pre-40S particle nuclear export (**Figure 25A**). These results indicate that the 40S subunit export machinery facilitates a late 90S pre-ribosome maturation event that promotes the rapid cleavage of the pre-rRNA at site A_2 and the efficient degradation of the 5′-A_0 fragment. This function is quite specific for export regulators, because the elimination of factors specifically involved in the cytoplasmic maturation of pre-40S complexes (Rio2 and Ltv1) does not trigger any of the above defects (**Figure 21A** and **Figure 21B**) [38,81,82]. The above results led us to investigate whether Crm1, like Rrp12, was present in 90S pre-ribosomes. We first assessed the potential interaction of Crm1 with two 90S pre-ribosome components, the 35S pre-RNA and Pwp2, using coimmunoprecipitation analyses similar to those that detect Rrp12 in 90S and pre-40S particles (see above **Figure 14** in page 40). This approach however did not reveal associations of Crm1 with any pre-ribosomal component, not even with pre-rRNAs or proteins present in the pre-40S and pre-60S complexes transported by this exportin. We therefore decided to change the experimental conditions of our coimmunoprecipitation assays. In particular, we changed the Triton X-100-containing lysis buffer by a NP-40-containing buffer that was similar to buffers used by others to detect interactors of

Crm1 *in vivo* [83-85]. Notably, when we purified Crm1-GFP using the NP-40 buffer, we could readily observe that it interacts with the 35S pre-rRNA, the 20S pre-rRNA, 27S pre-rRNAs and the 25S rRNA (**Figure 26A**).

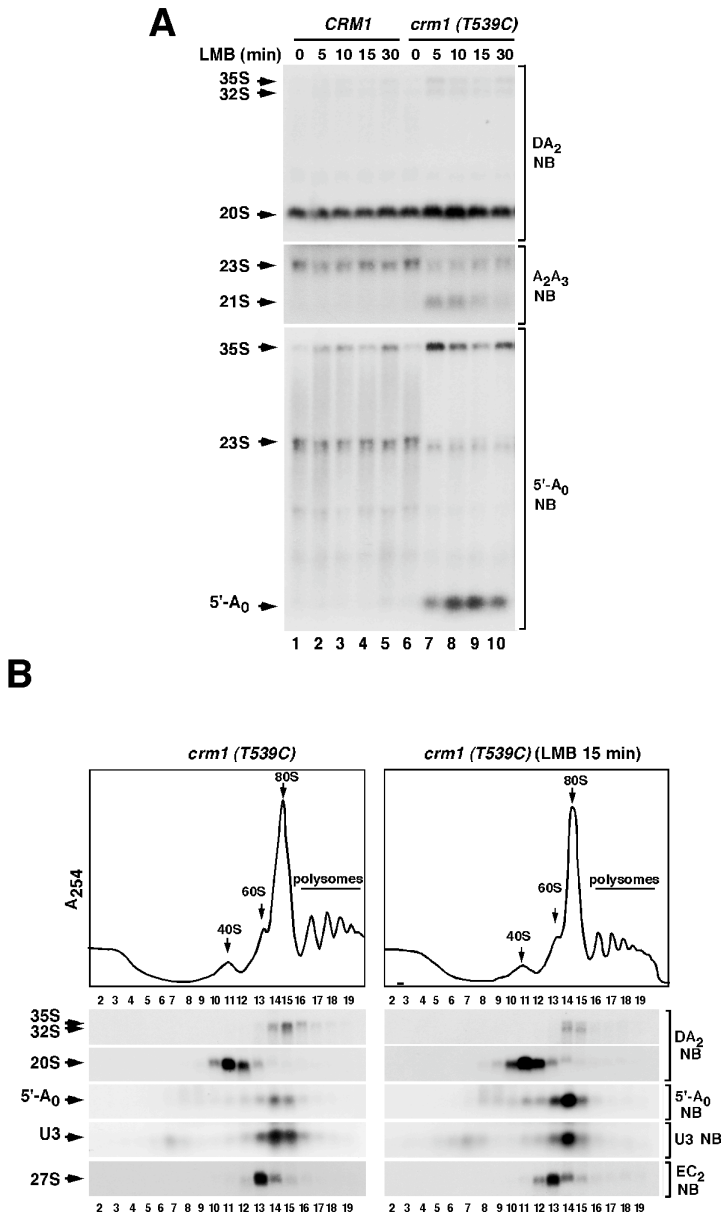

Figure 25. The inhibition of Crm1 recapitulates all the defects observed in Rrp12 depleted cells. (**A**) Northern blot analysis showing the amount of the indicated pre-RNA intermediaries and 5′-A_0 byproduct (left) in control *CRM1* and mutant *crm1 (T539C)* strains treated with leptomycin B (LMB) for the indicated periods of time (top). (**B**) Top panel, sucrose-gradient sedimentation analysis of ribosomal complexes (40S, 60S, 80S and polysomes) of cell lysates from *crm1 (T539C)* cells that were either nontreated (left panels) or treated (right panels) with leptomycin B for 15 min. Bottom panels, Northern blot analysis of the indicated pre-rRNA species in the gradient fractions. Numbers of fractions are shown at the bottom. Blotting probes are indicated on the right.

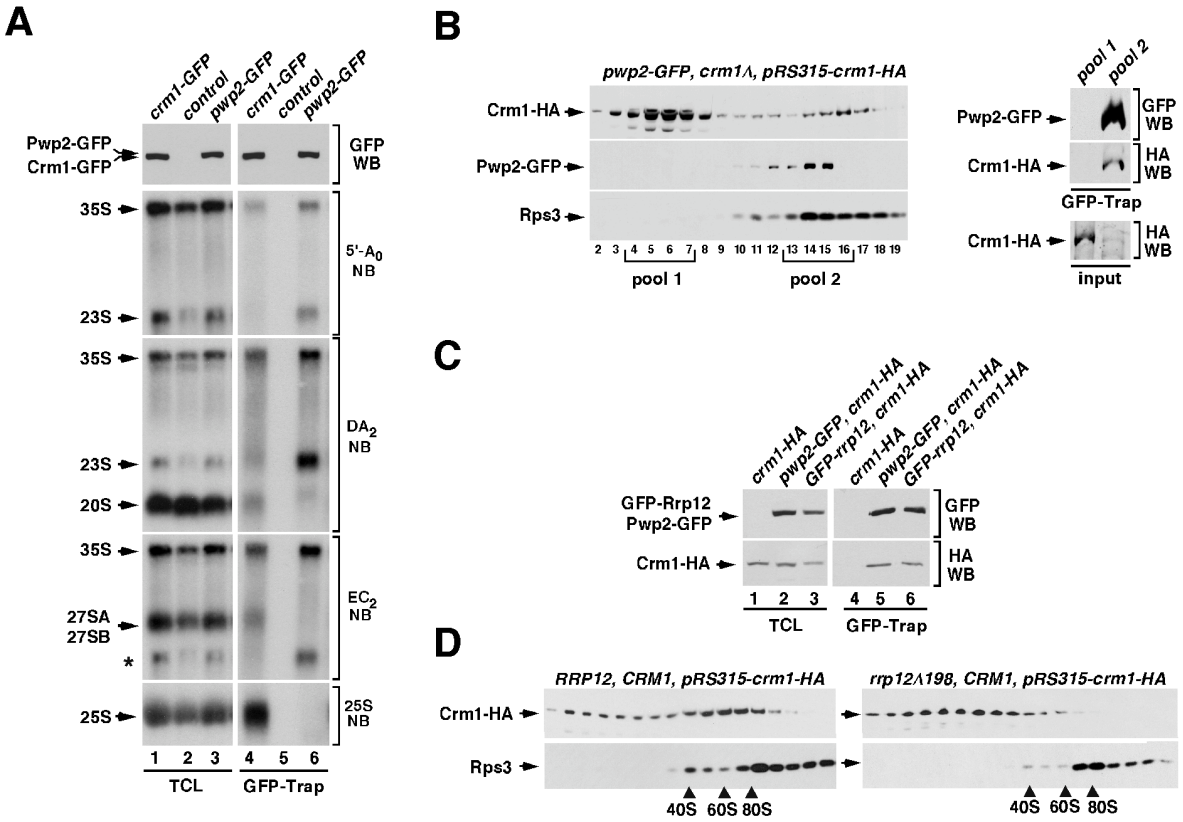

Figure 26. Crm1 is present in 90S pre-ribosomes and its recruiting depends on Rrp12. (**A**) Northern blot analysis showing copurification (second to fifth panels on the right) of the indicated pre-RNA species with GFP-tagged Crm1 (lane 4) and GFP-tagged Pwp2 (lane 6) in normal cells. Control samples were wild-type cells expressing endogenous untagged Crm1 and Rrp12. Parallel Northern blots were performed on total RNAs prepared from the same total cell lysate samples used for the purifications (second to fifth panels on the left). Western blot experiments were performed to analyze the amount of Crm1-GFP and Pwp2-GFP present in the total cell lysates (top panel on the left) and GFP-Trap purified complexes (top panel on the right). The asterisk in the EC_2 blot indicates the signal of the 23S pre-rRNA from previous hybridization with the DA_2 probe. (**B**) Sucrose gradient analysis of Crm1-HA, Pwp2-GFP and Rps3 in *pwp2-GFP*/*crm1Δ* cells containing a pRS315-crm1-HA plasmid. Gradient fractions were analyzed by Western blot with anti-HA, anti-GFP and anti-Rps3 (left three panels). The right panels show copurification of Crm1-HA with GFP-Trap purified complexes from pooled fractions of the 80–90S gradient region (pool 2). A GFP-Trap purification on pooled fractions of the 10–20S gradient region (pool 1) was used as a control. Parallel Western blots analyzed the amount of Crm1-HA in each one of the pool samples used for the GFP-Trap purifications (input) (right bottom panel). (**C**) Western blot analysis showing copurification of Crm1-HA with GFP-tagged Pwp2 (lane 5) and with GFP-tagged Rrp12 (lane 6) in *pwp2-GFP* cells containing a pRS315-crm1-HA plasmid (lane 5), and in *GFP-rrp12* cells containing a pRS315-crm1-HA plasmid (lane 6), respectively. Parallel Western blots were performed to analyze the amounts of the coimmunoprecipitated proteins in total cell lysates (lanes 1 to 3). (**D**) Sucrose gradient analysis showing the sedimentation behavior of Crm1-HA and Rps3 in *RRP12/CRM1* (left panels) and *rrp12-Δ198/CRM1* (right panels) cells containing a pRS315-crm1-HA plasmid. The positions of the gradient where 40S, 60S and 80S complexes sedimented are indicated by arrows.

The associations with these RNAs were specific because in the same Northern blots Pwp2-GFP coprecipitated the 35S and 23S pre-rRNAs, but not the 20S, 27S and 25S RNAs. These results indicate that Crm1 binds to pre-40S and pre-60S particles, as expected from its role in export, and also that it is already recruited to early 90S particles. Consistent with this, we found using sucrose gradient sedimentation analysis that Crm1 is indeed present in large 80–90S complexes that co-sediment with Pwp2 (**Figure 26B**). Furthermore, when 90S pre-ribosomes were purified from sucrose gradients using Pwp2 as bait it was confirmed that they do contain Crm1 (**Figure 26B**, right set of panels). Western blot analysis of Rrp12-containing complexes from total cell lysates evidenced that Crm1 interacts with Rrp12 (**Figure 26C**), a result consistent with the common presence of the two proteins in both 90S and pre-40S pre-ribosomes. In our final set of experiments, we investigated whether the recruitment of Crm1 to 90S pre-ribosomes was Rrp12-dependent. For this purpose we analyzed the sedimentation behavior in sucrose gradients of a HA-tagged version of Crm1 that was coexpressed with the endogenous Crm1 either in wild type or in *rrp12Δ198* cells. We found that in wild type cells the Crm1-HA protein is recruited to large assemblies, including 80–90S-like complexes (**Figure 26D**, left two panels). This sedimentation in large complexes is drastically reduced in *rrp12Δ198* cells (**Figure 26D**, right two panels), suggesting that the incorporation of Crm1 onto large 80–90S pre-ribosomal particles is Rrp12-dependent. Altogether, our data indicate that Rrp12 and Crm1 act on 90S pre-ribosomes in a concerted manner.

DISCUSSION

The results presented here identify Rrp12 as a factor required for a number of intertwined steps of the 40S ribosomal subunit synthesis pathway (**Figure 27**). We have observed that Rrp12, together with Crm1, is first recruited to the pathway to facilitate the processing of the 35S pre-rRNA and the elimination of the 5′-A_0 fragment in the context of a late 90S transitional particle (**Figure 27**). A lack of Rrp12 or Crm1 at this step delays but does not halt the assembly and release of early pre-40S particles. Interestingly, this early function of Rrp12 occurs immediately upstream and temporally close to the export of the pre-40S particles, a process that absolutely requires Rrp12 and Crm1. In addition to revealing a hitherto unknown role for export-related factors in a specific maturation step in the nucleolus, our results shed light onto the dynamics of 90S pre-ribosome factors upon cleavage of the 35S pre-rRNA at site A_2. Indeed, some authors previously suggested that, after the A_2 cleavage, the non-ribosomal components of the 90S particle are released en bloc in association with the 5′-A_0 fragment [36,64]. However, the formation of such disassembly complexes, and when and how was the exosome recruited, remained unclear. We find no evidence for the formation of a post-disassembly complex containing the 5′-A_0 fragment upon which the exosome acts (**Figure 24E,** page 54). Rather, our results indicate that the exosome is present in transitional 90S pre-ribosomes to degrade the 5′-A_0 fragment, either in the last step of pre-40S particle assembly or at the very time of pre-40S particle release (**Figure 27**). The implication of Crm1 in steps of ribosome synthesis, other than nuclear export, is also a new finding in yeast. In human cells, Crm1 has been implicated in the targeting of snoRNP complexes to the nucleolus [83,86]. Whether Rrp12 and Crm1 utilize the same domains for the export-related and maturation-related functions, and whether the two proteins need to interact directly to exert their functions, remains to be determined. We have found that Rrp12 and Crm1 purified from bacteria do not stably interact *in vitro* (unpublished data). However, we cannot exclude the possibility that such interaction could require the participation of other proteins. Indeed, it has been shown before that the interaction of Crm1 with other molecules involves the participation of additional factors, including the Ran GTPase in its GTP-bound state.

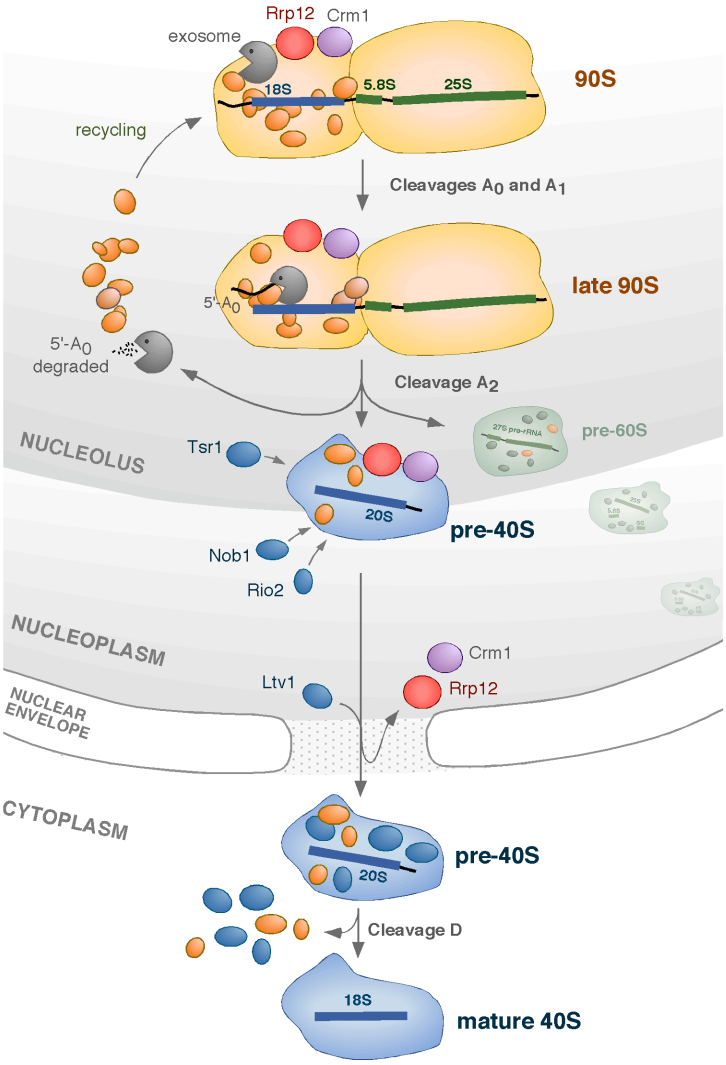

Figure 27. Model for the integration of different processes in the nucleolus during synthesis of 40S subunits. The 90S pre-ribosome contains ≈ 70 factors (represented in orange) that are specifically required for the cleavage of the primary pre-rRNA at sites A_0, A_1 and A_2, and for the assembly of ribosomal proteins (not represented). In addition, the 90S pre-ribosome engages two other sets of proteins that participate in activities that will be initiated at the time of, or immediately after, the A_2 cleavage: the exosome complex, and Rrp12/Crm1. The exosome degrades the 5′-A_0 fragment, allowing the liberation and recycling of bound 90S proteins. Rrp12 and Crm1 act as export factors for the released pre-40S particle. The cleavage of the pre-rRNA at site A_2 is intertwined with the initiation of 5′-A_0 degradation and the priming of the emergent pre-40S particle for nuclear export. During the rapid transit of the pre-40S particle from the nucleolus to the cytoplasm, a few maturation factors (Tsr1, Rio2, Nob1) that will be required in the cytoplasm are incorporated in a manner independent of nuclear export. Another maturation factor, Ltv1, requires Rrp12 for its stable incorporation onto pre-40S particles, but whether or not it is dependent on the export process itself remains to be ascertained. Further details about this model, and the evidence supporting it, is given in the text.

Ran can in fact be involved in these interactions, as suggested by the identification of allele-specific Ran mutants that elicit defects in the degradation of the 5′-A_0 fragment [87]. Based on the present results, we hypothesize that such defects could be associated to the Rrp12- and Crm1-dependent mechanism reported here. An involvement of Ran on the association of Crm1 with pre-ribosomes could also explain the difficulties for detecting Crm1 in purified 90S and pre-40S pre-ribosomes, because these complexes are normally prepared under conditions that favor the conversion of Ran-GTP to Ran-GDP. Here we describe that, using a buffer that contains 0.2% NP-40, it is possible to detect the specific association of Crm1 to both pre-rRNAs and pre-ribosomal components by coimmunoprecipitation analysis. The reason for the efficiency of this buffer is unclear, but it must somehow favor the maintenance of some Ran-GTP levels and/or affect other currently unknown features that improve the stability or solubilization of Crm1-containing complexes.

In our model we propose that Rrp12 is an export factor rather than a nuclear maturation factor (**Figure 27**). Consistent with this, we have observed that the elimination of Rrp12 leads to the accumulation of pre-40S particles that, in addition to being dissociated from the 90S pre-ribosome machinery, are fully-assembled. This is evidenced by the recruitment to those particles of factors that are predominantly cytoplasmic in normal cells (Rio2, Nob1), and that therefore must join the pathway just before nuclear exit. One important inference of our results is that the major assembly events involved in the formation of pre-40S particles are separable and fully independent from the subsequent export step. A direct participation of Rrp12 in the export process is also supported by the previously-described interactions of this protein with some nucleoporins and with Ran [48]. Unexpectedly, we could not find any significant role for Rrp12 in the export of pre-60S subunits, as it had been previously published [48]. In addition to the phenotypic analysis of Rrp12-depleted cells, the prominent role of Rrp12 in the 40S rather than the 60S subunit pathway is supported by the RNA-protein interaction data showing the specific binding of Rrp12 to the 20S but not the 27S and 7S pre-rRNAs. The reason for these different results is not readily apparent. We have found that the loss of Rrp12 elicits the 40S subunit-specific phenotype both in the W303 and in BY4743 strains, indicating no influence of the genetic background. Still, it is worth

noting that the depletion of Rrp12 causes delays in the processing of 5.8S rRNA precursors in the nucleus by a hitherto unknown mechanism. According to our results, such delays do not impact the overall production of 60S subunits, but it could be possible that, under some experimental conditions or in strains with genetic modifications that subtly affect ribosome biogenesis, the defect in 5.8S rRNA production became exacerbated and caused nuclear accumulation of pre-60S particles. It is also plausible that Rrp12 could interact either weakly or very transiently with some pre-60S particle subpools, as it would be expected if its influence on the processing of 5.8S precursors were direct. This possibility would be in agreement with the previously-reported detection of Rrp12 bound to 27SB pre-rRNAs using primer-extension analyses [48]. Despite the possibility of these alternative scenarios, we believe that our data clearly indicate that Rrp12 is not essential for 60S subunit synthesis. Consistent with this idea, it is also worth noting that mammalian Rrp12 has been shown to be required exclusively for 40S subunit synthesis [88,89].

One distinctive feature of the intermediate particle formed in the absence of Rrp12 is the lack of Ltv1, a factor not essential for nuclear export. Previous studies indicate that this protein is recruited in the nucleus [39,49], but some evidence suggests that its interaction with the nuclear pre-ribosomes that are about to be exported might be weak [50]. Thus, a possible explanation for the absence of Ltv1 in the pre-40S particles of Rrp12-depleted cells is that those particles are ready to be exported and have Ltv1 loosely associated. Alternatively, it is possible that Rrp12 could be actively required for the docking of Ltv1 to those particles during the export process. We currently favor the latter possibility, since we have observed that the interaction of these two proteins can occur in a pre-rRNA-independent manner. Based on the present data, we believe that Rrp12 probably promotes the recruitment of Ltv1 onto the pre-40S particle immediately prior to the step of transport (**Figure 27**). Upon this docking step, Rrp12 is carried along with the particle through the nuclear pores to be finally released when the particles reach the cytosol. Consistent with this hypothesis, our co-purification experiments and other proteomic analyses have shown that Rrp12 is not a major component of cytoplasmic pre-40S particles. Alternatively, it is also possible that Rrp12 could

remain associated to cytoplasmic pre-40S particles and only becomes released upon completion of a specific maturation event that takes place right after the nuclear export step. This model would explain previous results indicating that Rrp12 can associate with di-methylated 20S pre-rRNA, a modified form of the 20S pre-rRNA that is generated in the cytoplasm [48]. Further work will be required to dissect the fate and specific roles of Rrp12 in these late maturation stages.

The reason for using the pre-40S export machinery to facilitate late 90S pre-ribosome-mediated processes is unknown. We propose that such mechanism could ensure a timely coordination of the recycling kinetics of 90S pre-ribosome components with pre-40S particle release and rapid export (**Figure 27**). An inter-relation between these three processes is indicated by our data, which shows that the impairment of nuclear export causes defects in the function, disassembly and subcellular localization of the 90S pre-ribosome. Future work will be needed to explain the precise mechanisms by which the export factors influence the activities of the exosome and A_2 cleavage complexes within the 90S pre-ribosome.

CONCLUSIONS

The main conclusions of this thesis are the following:

1. The protein Rrp12 is essential for the formation of 40S ribosomal subunits but is dispensable for the formation of 60S subunits.

2. Rrp12 is a stable constituent of nuclear pre-40S complexes that is absolutely required for their transport to the cytoplasm.

3. Rrp12 is first recruited to the 40S synthesis route in the nucleolus, where it is important for the processing of the primary pre-rRNA and the elimination of the 5'-A_0 fragment in the context of a transitional 90S pre-ribosome.

4. The protein Crm1, an exportin that mediates the transport of pre-40S complexes out of the nucleus, is also required for the same 90S pre-ribosome maturation events that involve Rrp12.

5. Nuclear export factors of the 40S subunit synthesis pathway play roles in maturation steps that take place in the nucleolus, upstream of nuclear export.

6. A new model emerges for the 40S subunit synthesis pathway in which the completion of pre-40S particle assembly, the initiation of byproduct degradation and the priming for nuclear export occur in an integrated manner in late 90S pre-ribosomes.

MATERIALS AND METHODS

1. Yeast strains, genetic methods and plasmids

The *Saccharomyces cerevisiae* strains and plasmids used in this study are listed in **Tables 2** and **3**, respectively. The conditional strain for *RRP12* under the control of the *GAL1* promoter (YPM7) was generated by one-step insertion of a *KAN-MX6-GAL1* cassette upstream of the ATG of the *RRP12* gene [90]. This strain (referred to in the text as *GAL::HA-rrp12*), and the other *GAL1*-driven strains used in this study, JDY144, WDG72, YGM168, YO470 and YGM174 (referred to in the text as *GAL::HA-spb4*, *GAL::rsa4*, *GAL::HA-pno1*, *GAL::rio2-ProtA* and *GAL::HA-mtr4*, respectively) were cultured at 30°C in media containing galactose (YPGal, 0.4% yeast extract, 0.8% peptone, 0.1 mM adenine, 2% galactose) or glucose (YPD, 0.4% yeast extract, 0.8% peptone, 0.1 mM adenine, 2% glucose). For protein depletion, the incubation times in YPD varied from 9 to 18 h, as indicated in figure labelings. The *ltv1Δ* strain was cultured at 25°C, the temperature at which the 40S subunit biogenesis defects of this strain are more exacerbated. For the experiments of inactivation of Crm1 we employed a strain with the *CRM1* gene depleted that carried a plasmid for the expression of the *crm1-T539C-HA* allele (strain MNY8, plasmid pDC-crm1-T539C). As a control for those experiments, we employed the corresponding strain carrying a plasmid for the expression of *crm1-HA* (strain MNY7, plasmid pDC-CRM1). MNY7 and MNY8 cells were treated with 100 ng/ml of leptomycin B (LMB) for 5–15 min. All strains with MYC, hemagglutinin (HA) or green fluorescent protein (GFP) carboxy-terminal tagged alleles, except the *crm1-HA* and *GFP-rrp12* ones, were generated by in-frame one-step integration of PCR cassettes in the corresponding locus of wild type cells. In these strains, the epitope-tagged versions are the only source of the proteins in the cell, and their expression is driven from the endogenous gene promoters. All epitope-tagged alleles were fully functional, as measured by normal growth rates and normal contents of rRNAs, pre-rRNAs and ribosomal subunits. The sedimentation analysis of Crm1-HA shown in **Figure 26B** (page 58) was performed on the YGM193 strain (referred to in the figure as *pwp2-GFP*, *crm1Δ*, pRS315-crm1-HA). The coimmunoprecipitation experiment in **Figure 26C** (page 58) was performed with the YMD6 strain carrying the pDC-CRM1 plasmid (referred to in the figure as *pwp2-GFP*, *crm1-HA*) and with the YPM7 strain carrying the pGM58 and pDC-CRM1 plasmids (referred to in the figure as *GFP-rrp12*,

crm1-HA). The sedimentation analysis of Crm1-HA shown in **Figure 26D** (page 58) was performed on the following strains maintained in glucose-containing media: YPM7 carrying the pBN18 and pDC-CRM1 plasmids (referred in the figure as *RRP12*, *CRM1*, *pRS315-crm1-HA*), and YPM7 carrying the pBN19 and pDC-CRM1 plasmids (referred in the figure as *rrp12Δ198*, *CRM1*, *pRS315-crm1-HA*). Preparation of media, yeast transformation and genetic manipulations were performed according to established procedures.

TABLE 2. Yeast strains used in this study

Name	Genotype	Source
W303a	*MATa, ade2, his3, leu2, trp1, ura3*	Euroscarf
JDY144	*ade2, his3, leu2, ura3, spb4D::TRP [pGAL::HA-spb4 LEU2]*	[91]
JDY207	*MATa, his3, leu2, trp1, ura3, mtr4Δ::HIS3MX [pYCplac33-MTR4 URA3]*	[77]
JDY850	*MATa, ade2, his3, leu2, trp1, ura3, ssf1-GFP(S65T)::natNT2*	[92]
JDY851	*MATa, ade2, his3, leu2, trp1, ura3, nop7-GFP(S65T)::natNT2*	[92]
JDY852	*MATa, ade2, his3, leu2, trp1, ura3, rix1-GFP(S65T)::natNT2*	[92]
JDY853	*MATa, ade2, his3, leu2, trp1, ura3, kre35-GFP(S65T)::natNT2*	[92]
JDY855	*MATa, ade2, his3, leu2, trp1, ura3, arx1-GFP(S65T)::natNT2*	[92]
MNY7	*MATa, ade2, his3, leu2, trp1, ura3, crm1D::KANMX6 [pDC-CRM1 LEU2]*	[79]
MNY8	*MATa, ade2, his3, leu2, trp1, ura3, crm1Δ::KANMX6 [pDC-crm1 (T539C) LEU2]*	[79]
WDG72	*MATa, ade2, leu2, ura, rsa4Δ::URA3 [pGAL::HA-rsa4 TRP1]*	[93]
YBN11	*MATa, ade2, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, rrp44-GFP(S65T)::HIS3MX*	This study
YBN13	*MATa, ade2, leu2, trp1, ura3, rrp44-GFP(S65T)::HIS3MX*	This study
YGM1	*MATa, ade2, his3, leu2, trp1, ura3, tsr1-GFP(S65T)::TRP1*	This study
YGM6	*MATa, ade2, his3, leu2, trp1, ura3, tsr1-MYC::HIS3MX*	This study
YGM92	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, nob1-GFP(S65T)::HIS3MX*	This study
YGM94	*MATa, ade2, his3, leu2, trp1, ura3, nob1-GFP(S65T)::HIS3MX*	This study
YGM96	*MATa, ade2, his3, leu2, trp1, ura3, enp1-GFP(S65T)::HIS3MX*	This study
YGM93	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12*	This study
YGM98	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, pwp2-GFP(S65T):: HIS3MX*	This study
YGM99	*ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, nop7-GFP(S65T)::natNT2*	This study
YGM102	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, enp1-GFP(S65T)::HIS3MX*	This study
YGM104	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, tsr1-GFP(S65T)::TRP1*	This study
YGM119	*MATa, ade2, his3, leu2, ura3, ltv1Δ::TRP1*	This study
YGM143	*MATa, ade2, his3, leu2, trp1, ura3, prp43-MYC::HIS3MX*	This study
YGM145	*MATa, ade2, his3, leu2, trp1, ura3, ltv1-MYC::HIS3MX*	This study

YGM147	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, prp43-MYC::HIS3MX*	This study
YGM148	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, ltv1-MYC::HIS3MX*	This study
YGM149	*MATa, ade2, his3, leu2, trp1, ura3, enp1-MYC::HIS3MX*	This study
YGM151	*MATa, ade2, his3, leu2, trp1, ura3, rio2-MYC::HIS3MX*	This study
YGM151	*MATa, ade2, his3, leu2, trp1, ura3, rio2-MYC::HIS3MX*	This study
YGM154	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, rio2-MYC::HIS3MX*	This study
YGM155	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, enp1-MYC::HIS3MX*	This study
YGM156	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, dim1-MYC::HIS3MX*	This study
YGM168	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-pno1*	This study
YGM174	*MATa, leu2, his3, trp1, ura3, mtr4Δ::HIS3MX [pGAL::HA-mtr4 LEU2]*	This study
YGM193	*MATa, ade2, his3, leu2, trp1, ura3, pwp2-GFP(S65T)::HIS3MX, crm1Δ::KANMX6 [pDC-CRM1 LEU2]*	This study
YLG1	*MATa, ade2, his3, leu2, trp1, ura3, rio2-GFP(S65T)::HIS3MX*	This study
YLG2	*MATa, ade2, his3, leu2, trp1, ura3, ltv1-GFP(S65T)::HIS3MX*	This study
YLG5	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, rio2-GFP(S65T)::HIS3MX*	This study
YLG7	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, dim1-GFP(S65T)::HIS3MX*	This study
YLG9	*MATa, ade2, his3, leu2, trp1, ura3, dim1-GFP(S65T)::HIS3MX*	This study
YLG11	*MATa, ade2, his3, leu2, trp1, ura3, pno1-GFP(S65T)::HIS3MX*	This study
YLG13	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, pno1-GFP(S65T)::HIS3MX*	This study
YMD6	*MATa, ade2, his3, leu2, trp1, ura3, pwp2-GFP(S65T)::KANMX*	This study
YMD44	*MATa, ade2, his3, leu2, trp1, ura3, nop7-MYC::HIS3MX*	[93]
YMD57	*MATa, ade2, his3, leu2, trp1, ura3, pwp2-MYC::HIS3MX*	[93]
YMD230	*MATa, ade2, his3, leu2, trp1, ura3, rrp12-MYC::KANMX*	[65]
YMD392	*MATa, ade2, his3, leu2, trp1, ura3, hrr25-GFP(S65T)::HIS3MX*	This study
YMD393	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12, ltv1-GFP(S65T)::HIS3MX*	This study
YO470	*MATa, ade2, his3, leu2, trp1, ura3, rio2Δ:: KANMX6 [2μ pGAL::rio2-PROTA URA]*	[38]
YPM7	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12*	This study
YPM7-R	*MATa, ade2, his3, leu2, trp1, ura3, KANMX-GAL::HA-rrp12 [pGM57]*	This study

TABLE 3. Plasmids used in this study

Name	Relevant information	Source
pBN18	CEN, HIS3, RRP12	This study
pBN19	CEN, HIS3, rrp12 (198-1228 aa)	This study
pDC-CRM1	CEN, LEU2, CRM1	[77,79]
CEN, LEU2, crm1 (T539C)	CEN, LEU2, crm1 (T539C)	[79]
pGM57	CEN, URA3, GFP-rrp12	This study
pGM58	CEN, HIS3, GFP-rrp12	This study
pLG1	CEN, LEU2, RRP12	This study
pLG2	CEN, LEU2, rrp12 (198-1228 aa)	This study
pRS316-RPS2-GFP	CEN, URA3, rps2-GFP	[94]
pRS316-RPL25-GFP	CEN, URA3, rpl25-GFP	[58]
pRPL11-GFP	CEN, LEU2, rpl11-GFP	Jesús de la Cruz

2. RNA preparation and Northern blot analysis

RNAs from total cellular lysates, gradient fractions and coimmunoprecipitations were prepared by the hot-phenol method [95]. Oligonucleotide labeling, RNA separation, Northern blotting and hybridization were performed as described previously [93]. The sequences of the oligonucleotides used as probes are shown in **Table 4**.

TABLE 4. Probes used in Northern blot analysis

Probe (region recognized)	Sequence (5'-3')
Probe 01 (5'-A_0)	TCAGGTCTCTGCTGC
Probe 02 (18S)	AGCCATTCGCAGTTTCACTG
Probe 03 (D-A_2)	TTAAGCGCAGGCCCGGCT
Probe 04 (A_2-A_3)	TGTTACCTCTGGGCC
Probe 05 (5.8S)	GCGTTCTTCATCGATGC
Probe 06 (5'E-C_2)	TGAGAAGGAAATGACGCT
Probe 07 (E-C_2)	GGCCAGCAATTTCAAGTTA
Probe 08 (25S)	TACTAAGGCAATCCCGGTTGG
U3	GGATTGCGGACCAAGCTAA
scR1	ATCCCGGCCGCCTCCATCAC

3. Protein purification and analysis

Preparation of total celular lysates for immunoblot, Western blot analysis, purification of GFP-tagged proteins and mass spectrometry analysis were performed as described previously [28], except for the Pwp2-GFP/Crm1-HA and GFP-Rrp12/Crm1-HA coimmunoprecipitation analysis in **Figure 26C** (page 58). In this case, instead of lysing

cells in IP buffer (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 150 mM potassium acetate, 1 mM dithithreitol, 0.2% Triton X-100, supplemented with Complete [Roche]), cells were lysed in IP-NP40 buffer (15 mM Na_2HPO_4, 10 mM NaH_2PO_4, pH 7.2, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM $NaVO_4$, 0.5% NP-40 Alternative [Calbiochem], supplemented with Complete). Before purification of Pwp2-GFP and Rrp12-GFP with GFP-TRAP (Chromotek), the pre-cleared lysates were diluted to 0.2% NP-40. The anti-Rrp12 antibody used for Western blot in **Figure 14B** (page 40) is a rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of yeast Rrp12 (this study). Other antibodies used in Western blot analysis were: anti-MYC (Roche), anti-GFP (Clontech), anti-HA (Covance), anti-Nop1 (Pierce), anti-Mex67 (kind gift of C. Dargemont, Institut Jacques Monod), anti-Rps3 (kind gift of M. Seedorf, University of Heidelberg), anti-Rps8 (kind gift of G. Dieci, University of Parma), anti-Rpl1 (kind gift of F. Lacroute, Centre de Génétique Moléculaire, Gif-sur-Yvette), anti-Pgk1 (Abcam), and anti-Cdc11 (Santa Cruz). For the represention of the results of the proteomic analysis shown in **Figure 17**(page 45), the four different dot sizes are indicative of the amount of the copurifying protein relative to the amount of bait: >80%, 60–80%, 40–60%, and <40%.

4. Polysome preparation and sucrose gradient analysis

Cell cultures (200 ml) were grown to an optical density at 600 (OD_{600}) between 0.8 and 0.1 and, before harvesting, cycloheximide was added to a final concentration of 0.1 mg/ml. After an incubation on ice for 5 min, cells were collected and lysed in 700 µl of HK buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol (DTT) and 0.1 mg/ml cycloheximide) using a Fastprep apparatus. Cell lysates were pre-cleared by high-speed centrifugation, and extracts equivalent to 5–20 absorption units at 260 nm (A_{260}) were loaded on 7–50% sucrose gradients (10 ml), which had been prepared in HK buffer without cycloheximide. Ultracentrifugation, subsequent fraction collection and polysome profile recording were performed as previously described [93]. For Western blot analysis, 40 µl samples of each fraction were mixed directly with 10 µl of SDS-PAGE loading buffer (SPLB) and loaded onto SDS

polyacrylamide gels. For Northern blot analysis, total RNA was prepared by the hot-phenol procedure from 100 µl samples of each fraction and separated on 1.2% agarose-formadehyde gels. For the analysis of purified complexes shown in **Figure 26B** (page 58), two sets (pools 1 and 2) of four combined fractions were concentrated 6-fold by spinning on Microcon-10 (Millipore) filters. The recovery of proteins after the concentration step was ~10 fold more efficient for pool 1 than for pool 2, probably due to the higher sucrose concentration in pool 2. Before performing the GFP-Trap purification, each concentrated pool was taken to 1 ml with NP-40 buffer (0,2% final concentration).

5. Protein-RNA coimmunoprecipitation experiments

Cell cultures were grown to OD_{600} between 0.8 and 1.0, and polysome extracts were prepared as described above. Extract equivalents to 15 A_{260} units were taken to 250 µl with HK buffer and mixed with 0.5 ml of IP buffer containing Complete and 600 U/ml of RNasin (Promega). In the Crm1-RNA coimmunoprecipitations shown in **Figure 26A** (page 58), instead of using IP buffer it was used IP-NP40 (0.2% final concentration) buffer. For evaluation of protein content in total cell lysates, a 30 µl aliquot of the pre-cleared lysate was mixed with 30 µl of SPLB and kept frozen until analysis by Western blot. The rest of the extract was incubated with 2 µg of anti-MYC 9E10 (Roche) antibody or with 25 µl of GFP-TRAP beads at 4°C for 2 h. When using anti-MYC antibody, immunoprecipitates were immobilized with GammaBind sepharose beads (GE Healthcare). Immunoprecipitates were washed four times at 4°C with IP or IP-NP40 buffer. For protein analyses, one fifth of the immunoprecipitated material was resuspended in SPLB and analyzed, in paralel with the samples of total protein, by Western blot. For RNA analyses, the rest of the immunoprecipitated material was resuspended in 400 µl of 50 mM sodium acetate, 10 mM EDTA (pH 5.2), and processed for RNA extraction by the hot phenol method. After ethanol precipitation, the whole amount of recovered RNA was resuspended in formaldehyde loading buffer, separated on 1.2% agarose-formadehyde gels and analyzed by Northern blot. In parallel, in the same Northern blot experiments, it was evaluated the pre-rRNA content in cell lysates before immunoprecipitation, using 5 µg of total RNA prepared by the hot phenol

method directly from extract equivalents to 10 A_{260} units of the corresponding polysome preparations.

6. Fluorescence microscopy

Cells were visualized using a Zeiss Axioplan 2 microscope equiped with a 63× objective, a Hammamutsu ORCA-ER digital camera and Openlab (Improvision) cell imaging analysis software. The Rpl25-EGFP and Rps2-GFP reporter assays to monitor pre-40 and pre-60S nuclear accumulation were performed as previously described [58].

7. Subcellular fractionation

Cells were grown to OD_{600} between 0.8 and 0.1, harvested and spheroplasts prepared by incubation in S buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1.2 M sorbitol, 1 mM dithiothreitol, 5 mg/ml Zymolyase T-100 (Seikagaku) at 30°C for 15 min. After two washes with the same buffer, the spheroplasts were lysed using a manual homogenizer in Ficoll buffer (10 mM Tris-HCl, pH 7.5, 20 mM KCl, 5 mM $MgCl_2$, 3 mM dithiothreitol, 1 mM EDTA, 1 mM PMSF, 180 mg/ml Ficoll-400, supplemented with Complete). Pre-cleared lysates were ultracentrifuged in a TLA 100.3 rotor at 23.000 rpm for 15 min, and the supernatant cytosolic fraction collected. The nuclei pellet was resuspended in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 30 mM $MgCl_2$, 0.25% NP-40 supplemented with Complete. Aliquots of the precleared whole lysate (W), cytosolic fraction (C) and nuclei (N) were mixed with SPLB and loaded onto a SDS polyacrilamide gel for Western blot analysis.

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FINANCIAL SUPPORT

This reaserch has been supported by:

- A graduate student contract from the University of Salamanca co-financed by the Santander Bank.
- Grants from both the Spanish Ministry of Economy and Competitiveness (BFU2011-23668, RD06/0020/0001 and RD12/0036/0002), the Samuel Solórzano Barruso Foundation (FS/17-2013) and the Castilla y León Autonomous Government (CSI039A12-1).

ACKNOWLEDGEMENTS

Esta tesis doctoral es el resultado de cinco años de trabajo en el que, directa o indirectamente, participaron muchas personas, opinando, corrigiendo, teniendo paciencia conmigo, dándome ánimos, acompañándome tanto en los momentos más duros como en los de felicidad. Cinco años de una vida podrían no ser muchos, pero yo nunca os olvidaré a todos vosotros ni estos días en Salamanca.

En primer lugar, quiero agradecer a la Dra. Dosil, directora de esta tesis doctoral, ya que sin su orientación y apoyo todo esto no hubiera sido posible. Su experiencia y educación han sido fuente de motivación y curiosidad para mí durante estos años.

Un agradecimiento particular al Dr. Bustelo por sus criticas científicas, sus sugerencias y sobre todo por su pasión hacia la investigación, que siempre he admirado y ha sido para mi un ejemplo.

No puedo no dar las gracias a Blanca, mi compañera de labo y amiga, con la cual he compartido incontables horas de trabajo. Gracias por tu sonrisa que nunca ha faltado día tras día.

Gracias a los compañeros del laboratorio 2, presentes y pasados, con los que he compartido trabajo y muchos buenos ratos, lo cual no tiene precio. En particular, grazie Salvatore perchè per me ci sei sempre stato. Grazie Carmen per la tua amicizia, per le nostre lunghe chiacchierate, e per i bellissimi momenti passati insieme. Gracias Javi por tu amistad, no puedo imaginar como habría sido este camino sin ti. Gracias Maite por tus clases de español, por nuestras risas y por tu apoyo. Gracias Maribel por tu sensibilidad. Gracias Miriam por tu sencillez. Gracias Fran por tu inteligente ironía. Gracias Sonia por llevarme a un mundo mágico con tus colores de princesa. Gracias Antonio por nuestras charlas y tus cuentos de viajes aventureros.

Grazie Marcella perchè con il tuo buonumore e il tuo sorriso hai reso bella ogni singola giornata di lavoro. Grazie perchè riesci a toccare il cuore delle persone con una semplicità estrema, la tua amicizia non ha prezzo.

Gracias Rosa y Nieves, no solo por vuestra preciosa ayuda con los experimentos de proteomica, si no por vuestro apoyo y vuestra amistad.

Gracias Sonia, por aguantarme a la hora de hacer pedidos, y por participar directa o indirectamente en momentos importantes de mi vida. Gracias por tus consejos.

Grazie Josè, Miriam, Francesca per la vostra amicizia sincera.

Un ringraziamento speciale va alla mia famiglia, in particolare a mia zia Anna. Zia, senza di te non mi troverei qui oggi. Grazie per avermi dato coraggio quando continuare gli studi sembrava impossibile. Grazie per essermi sempre accanto.

Grazie Mattia perchè anche se fossimo ai due poli della Terra non potrei fare a meno di sentirti vicino. Grazie per esserci, grazie per essere come sei.

Non posso poi non ringraziare te Masa, per aver sopportato il mio periodo pre-tesi e pre-pubblicazione. Per tutte le volte che hai dovuto sorbire i miei lunghi monologhi su Rrp12, per tutti i giorni in cui che mi sono trattenuta al lab fino a tardi, per il tuo appoggio e amore incondizionato.

I miei ultimi ringraziamenti sono per i miei nonni. Grazie per avermi insegnato con l'esempio ciò che nessun libro potrà mai insegnare: l'umiltà, il sacrificio e la forza dell'ottimismo. Grazie per il vostro amore che sempre mi ha accompagnato in questi anni, ne custodiró gelosamente il ricordo finchè avrò vita.

PUBLICATIONS

The work of this thesis has been recently published in the research article:

Moriggi G., Nieto B., Dosil M. Rrp12 and the exportin Crm1 participate in late assembly events in the nucleolus during 40S ribosomal subunit biogenesis. *PLoS Genet.* 10 (12): e1004836, 2014

APPENDIX

RESUMEN EN CASTELLANO

TÍTULO DE LA TESIS

Papel de Rrp12 en la formación de las subunidades ribosómicas

ÍNDICE DE LA TESIS TRADUCIDO AL CASTELLANO

INTRODUCCIÓN

La formación de los ribosomas en células eucarióticas implica la producción y subsiguiente ensamblaje de los cuatro rRNAs y ≈ 80 proteínas que constituyen las subunidades ribosómicas pequeñas (40S) y grandes (60S). En la levadura *Saccharomyces cerevisiae* tres de los cuatro rRNAs (18S, 5,8S y 25S) se transcriben conjuntamente a partir de un pre-rRNA policistrónico común (pre-rRNA 35S) (Fernandez-Pevida et al., 2014; Woolford and Baserga, 2013). Una vez sintetizado, ese pre-rRNA interacciona con un conjunto de proteínas ribosómicas, con la ribonucleoproteína nucleolar U3 (U3 snoRNP) y con ≈ 70 factores de ensamblaje y procesamiento, formando la llamada partícula pre-ribosómica 90S o pre-ribosoma 90S [8]. Seguidamente, el pre-rRNA 35S empieza a ser procesado y sufre tres cortes endonucleolíticos (en los sitios A_0, A_1 and A_2), dando lugar a dos especies de pre-rRNA distintas, el 20S y el $27SA_2$. Una vez finalizado ese primer procesamiento del pre-rRNA, el pre-ribosoma 90S se desensambla y se forman las partículas pre-40S y pre-60S, que contienen los pre-rRNAs 20S y $27SA_2$, respectivamente. Este proceso provoca la liberación de la gran mayoría de los factores de ensamblaje y procesamiento presentes en el pre-ribosoma 90S y la degradación rápida de los fragmentos de RNA que formaban parte del pre-rRNA primario pero que, tras los cortes en A_0, A_1 and A_2, quedan excluidos de los pre-rRNAs 20S y el $27SA_2$.

Las partículas iniciales pre-60S contienen más de 40 factores asociados, y sufren múltiples pasos de maduración dentro del núcleo que conllevan cambios drásticos en su composición. Por el contrario, las partículas iniciales pre-40S tienen una composición relativamente simple y son exportadas rápidamente al citoplasma, lo cual se explica porque, a diferencia del $27SA_2$, el pre-rRNA 20S no sufre ningún paso de procesamiento dentro del núcleo. Este pre-rRNA se procesa y da lugar al rRNA 18S en el citoplasma. Es también en el citoplasma donde tiene lugar un proceso de control de calidad mediante interacción con subunidades 60S que permite la maduración final de las subunidades 40S.

Aunque las partículas pre-40S iniciales, una vez liberadas en el nucleolo, son transportadas rápidamente al citoplasma, se sabe que sufren algunas transformaciones durante su tránsito por el nucleoplasma. Así, se ha visto que algunos

factores que serán necesarios para la maduración en el citoplasma son reclutados en el nucleoplasma (Henras et al., 2008; Woolford and Baserga, 2013).. También se sabe que las partículas pre-40S nucleares tienen que unirse a factores de transporte, como la GTPasa Ran y la exportina Crm1, para poder atravesar los poros de la envuelta nuclear. Lo que no se sabe es cómo ocurren estos eventos (Moy and Silver, 2002; Zemp and Kutay, 2007). Por ejemplo, en relación con el transporte, no está claro cómo y cuándo las partículas pre-40S adquieren su competencia para ser exportadas, y tampoco se conoce cómo interaccionan con Ran y con Crm1.

Una estrategia que podría contribuir a entender mejor la maduración y exportación nuclear de subunidades 40S es intentar dilucidar la función de Rrp12, un factor que se asocia a los pre-ribosomas pre-40S nucleares, pero no a los citoplasmáticos (Oeffinger et al., 2004). El objetivo general de esta tesis ha sido el estudio de la función de Rrp12 en el ensamblaje, maduración y exportación nuclear de pre-ribosomas en la levadura *Saccharomyces cerevisiae*.

RESUMEN DE LOS RESULTADOS

Con anterioridad a la realización de esta tesis, se había descrito que Rrp12 es una proteína necesaria para la exportación nuclear tanto de partículas pre-40S como pre-60S y, por lo tanto, que es una proteína esencial para la formación de las dos subunidades ribosómicas (Oeffinger et al., 2004).

En los primeros experimentos de la tesis se obtuvieron una serie de resultados inesperados que contradecían los datos publicados, ya que indicaban que la proteína Rrp12 es esencial para la producción de las subunidades 40S pero no de las 60S. Se llegó a esta conclusión tras realizar una caracterización detallada tanto de cepas de levadura en las que se eliminó Rrp12 de forma condicional, como de cepas que contenían una versión mutada de Rrp12 que era parcialmente funcional. Para esta caracterización se realizaron análisis de perfiles de polisomas, que permitieron estimar el contenido de subunidades 40S y 60S en células deficientes en Rrp12, y experimentos de Northern blot, que permitieron evaluar los niveles de todas las especies de pre-rRNAs y de rRNAs maduros en esas mismas células. Los resultados obtenidos demostraron de forma concluyente que Rrp12 es absolutamente necesaria para que se procese el pre-rRNA 20S en rRNA 18S y, por tanto, para la producción de las subunidades 40S, pero que no tiene ningún papel relevante ni en la producción de los rRNAs y proteínas, ni en el ensamblaje, de las subunidades 60S. El estudio de la asociación de Rrp12 con diferentes pre-ribosomas, mediante experimentos de co-inmunoprecipitación proteína-proteína y proteína-RNA, demostró que Rrp12 forma parte de pre-ribosomas 90S y de partículas pre-40S nucleares, pero no de partículas pre-40S citoplásmáticas ni de pre-ribosomas 60S. Todos los datos obtenidos, en su conjunto, indicaron que Rrp12 es un factor de síntesis de subunidades 40S que tiene una función esencial en el ensamblaje o maduración de partículas pre-40S.

Con la siguiente serie de experimentos se pudo demostrar que Rrp12 no participa en el ensamblaje de partículas pre-40S sino que es esencial para el paso de exportación al citoplasma. Por un lado, mediante ensayos de co-inmunoprecipitación proteína-RNA y de caracterización por espectrometría de masas de diferentes pre-ribosomas purificados, se comprobó que, en ausencia de Rrp12, se forman unos complejos que contienen pre-rRNA 20S y que tienen incorporados a la práctica

totalidad de los factores no ribosómicos típicos de partículas pre-40S citoplásmáticas. Por otro lado, mediante estudios de microscopía de fluorescencia, se demostró que los pre-ribosomas pre-40S que se forman en células que no contienen Rrp12 se quedan atrapados dentro del núcleo. En su conjunto, todas estas evidencias experimentales apoyan la idea de que Rrp12 no es un factor de maduración, sino un factor de exportación de pre-ribosomas pre-40S.

Además del bloqueo en el procesamiento de 20S pre-rRNA, causado por el defecto de exportación al núcleo, las células que no contienen Rrp12 presentan alteraciones transitorias en el procesamiento de otros pre-rRNAs. Así, en ausencia de Rrp12, se produce una acumulación anormal del pre-rRNA 33S y del fragmento 5'-A_0, un subproducto de procesamiento generado cuando el pre-rRNA 35S se corta en el sitio A_0. Tras realizar distintos experimentos de sedimentación en gradientes de sacarosa y análisis proteómicos de pre-ribosomas 90S, demostramos que Rrp12 es necesaria para el corte rápido del pre-rRNA 35S en el sitio A_2 y para la degradación eficiente del fragmento 5'-A_0, dos eventos que tienen lugar en una partícula 90S tardía. Estos datos permiten concluir que Rrp12 es necesaria para la salida de partículas pre-40S al citoplasma y para que el último paso de maduración de las partículas 90S, el que da lugar a las partículas pre-40S en el nucleolo, se produzca de forma eficiente.

En la última parte de la tesis se investigó si el paso de exportación nuclear de partículas pre-40S está asociado al paso final de maduración del pre-ribosoma 90S. Para ello, se analizó si la inhibición de la exportina Crm1 producía algún defecto en la dinámica de maduración del pre-ribosoma 90S. El análisis de la producción y procesamiento de pre-rRNAs en una cepa con una versión mutante de Crm1 ($Crm1^{T359C}$) que puede inhibirse por leptomicina B, evidenció que la inhibición del transporte de complejos pre-40S provoca los mismos defectos de maduración de 90S observados tras la eliminación de Rrp12. Además, mediante estudios de co-immunoprecipitación, se pudo demostrar que Crm1 se asocia a la partícula 90S, lo cual indica que este factor de exportación, al igual que Rrp12, se incorpora a la ruta en el nucleolo en un paso que justo antecede a la exportación.

Toda la información obtenida en esta tesis, cuando se analiza de forma conjunta, indica que el ensamblaje de subunidades 40S y su exportación al citoplasma

no son procesos separados. De este trabajo surge una nueva visión de la ruta de síntesis de subunidades 40S, en la que el ensamblaje de partículas pre-40S, la degradación de productos de procesamiento, y la adquisición de competencia para la exportación nuclear tienen lugar de forma integrada, en un paso común, en partículas pre-ribosómicas 90S tardías.

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CONCLUSIONES

Las principales conclusiones de esta tesis son las siguientes :

1. La proteína Rrp12 es esencial para la formación de las subunidades ribosómicas 40S, pero es dispensable para la formación de las subunidades 60S.

2. Rrp12 es un componente estable de los complejos pre-40S nucleares que es absolutamente necesario para su trasporte al citoplasma.

3. Rrp12 se incorpora a la ruta de síntesis de subunidades 40S en el nucleolo, y allí es importante para el procesamiento del pre-rRNA primario y la eliminación del fragmento 5'-A_0 en el contexto de un pre-ribosoma 90S transitorio.

4. La proteína Crm1, una exportina que transporta complejos pre-40S fuera del núcleo, también es necesaria para los eventos de maduración dentro del pre-ribosoma 90S en los que participa Rrp12.

5. En la ruta de síntesis de subunidades 40S hay factores de exportación nuclear que participan en pasos de maduración en el nucleolo anteriores al paso de exportación.

6. Surge un nuevo modelo de la ruta de síntesis de subunidades 40S en el que el ensamblaje de partículas pre-40S, la degradación de subproductos de procesamiento, y la adquisición de competencia para la exportación nuclear tienen lugar de forma integrada en partículas pre-ribosómicas 90S tardías.