Cell. Mol. Life Sci. 65 (2008) 2334 – 2359
1420-682X/08/152334-26
DOI 10.1007/s00018-008-8027-0


Cellular and Molecular Life Sciences

# Review

# The post-transcriptional steps of eukaryotic ribosome biogenesis

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Received 16 January 2008; received after revision 29 February 2008; accepted 14 March 2008
Online First 14 April 2008

**Abstract.** One of the most important tasks of any cell is to synthesize ribosomes. In eukaryotes, this process occurs sequentially in the nucleolus, the nucleoplasm and the cytoplasm. It involves the transcription and processing of pre-ribosomal RNAs, their proper folding and assembly with ribosomal proteins and the transport of the resulting pre-ribosomal particles to the cytoplasm where final maturation events occur. In addition to the protein and RNA constituents of the mature cytoplasmic ribosomes, this intricate process requires the intervention of numerous protein and small RNA *trans*-acting factors. These transiently interact with pre-ribosomal particles at various stages of their maturation. Most of the constituents of pre-ribosomal particles have probably now been identified and research in the field is starting to unravel the timing of their intervention and their precise mode of action. Moreover, quality control mechanisms are being discovered that monitor ribosome synthesis and degrade the RNA components of defective pre-ribosomal particles.

**Keywords.** Pre-rRNA processing, pre-ribosomal particle, snoRNP, nuclear export, quality control.

## Introduction

Ribosome biogenesis is one of the most crucial and energy-consuming processes of any cell. In eukaryotes, it requires the activity of all three RNA polymerases and accounts for the vast majority of total cellular transcription [1, 2]. RNA polymerase II synthesizes the pre-mRNAs of ribosomal proteins and accessory factors involved in ribosome biogenesis, RNA polymerase III produces the precursor to 5S ribosomal RNA (rRNA), and RNA polymerase I (RNA Pol I) the common precursor to mature 5.8S, 18S and 25S (yeast)/28S (mammals) rRNAs. These rRNA precursors (called pre-rRNAs) undergo chemical modification of specific nucleotides and nucleolytic cleavages to remove so-called 'transcribed spacers', thus releasing the mature-size rRNA species (for complete schemes of the pre-rRNA processing pathways in yeast and human cells, see Fig. 1). Concomitantly with processing, pre-rRNAs assemble not only with ribosomal proteins retained within mature ribosomes, but also with so-called 'non-ribosomal' proteins and small nucleolar ribonucleoprotein particles (snoRNPs), which participate in various aspects of ribosome synthesis. Such assembly steps generate pre-ribosomal particles. Assembly in the nucleolus of the initial pre-rRNA precursors to the 5.8S, 18S and 25S/28S rRNAs with a subset of ribosomal and non-ribosomal proteins and snoRNPs yields 90S pre-ribosomal particles. These are then split into pre-60S and pre-40S pre-ribosomal particles. These particles are exported from the nucleoplasm to the cytoplasm where final assembly and maturation steps occur, yielding mature, functional ribosomes. Work over the past 10 years has uncovered probably

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most of the non-ribosomal proteins, which may total up to 200 different factors. Some of these non-ribosomal proteins were initially characterized using genetic screens in yeast [3]. Most of them, however, were identified a few years ago when purification of pre-ribosomal particles and identification of their protein components became a fairly routine task thanks to tandem affinity chromatography and novel highly sensitive mass-spectrometry techniques [4–6]. The stages at which these non-ribosomal proteins enter and leave the ribosome biogenesis pathway are now being defined. However, for most of them, we lack an understanding of their precise molecular functions. Recent research has uncovered that, quite unexpectedly in many instances, some of these factors also play central roles in other fundamental cellular processes such as pre-mRNA splicing, mRNA/tRNA turnover and cell cycle progression. Such sharing of factors may be a means to connect ribosome biogenesis with other central cellular pathways, although formal proof of this hypothesis remains elusive. As might be expected for such a crucial process, recent work has also shown that ribosome biogenesis is subjected to stringent quality control mechanisms. In this review, we will try to give the reader a flavour of these different aspects. For more specific reviews of the field, we direct the reader to some excellent previous essays [3, 7–14]. Since most information concerning ribosome biogenesis has been obtained in the yeast *Saccharomyces cerevisiae*, a large part of this review is devoted to the description of processes occurring in this organism. However, we will draw parallels to data obtained in other models whenever possible. As it turns out, trans-acting factors involved in ribosome biogenesis are highly conserved in eukaryotes [15] and pre-rRNA processing and assembly pathways seem very related, although not identical, between yeast and human.

## Formation and maturation of 90S pre-ribosomal particles

The primary precursor to 18S, 5.8S and 25S/28S rRNAs is generated by RNA Pol I in the nucleolus. The nascent primary transcript is 'delivered' in an electron-dense fibrillar region of the nucleolus [for a discussion of nucleolar structure, beyond the scope of this review, the reader is referred to refs 16, 17]. The primary pre-rRNA most probably starts to fold and to interact with small RNAs and proteins during ongoing transcription. Indeed, proper transcription elongation by RNA Pol I is required for efficient pre-rRNA processing and pre-ribosome assembly [18]. The initial co-transcriptional compaction of the Pol I transcripts can be visualized by electron microscopy on chromatin spreads (Miller spreads) as terminal balls, hence the term 'Christmas trees' to describe these spreads [19].

In many instances, RNA Pol I transcription carries on unabated until the 3′ external transcribed spacer is synthesized. Co-transcriptional cleavage within this spacer by RNase III [20, 21] releases the initial 90S pre-ribosomal particle containing the 35S pre-rRNA. In some cases in yeast, particularly when growth conditions are very favourable, the RNA Pol I transcript is cleaved co-transcriptionally in the internal transcribed spacer I (ITS1), thereby immediately releasing pre-40S particles without prior 90S particle formation [22].

### Formation of yeast 90S particles: stepwise addition of pre-formed 'modules'?

The protein composition of yeast 90S particles was investigated by tandem affinity purification (TAP) and mass-spectrometry identification of the partners of several nucleolar proteins required for pre-rRNA processing and sedimenting as components of 90S complexes on density gradients [23]. A second approach aimed at purifying the proteins associated with the box C/D U3 small nucleolar RNA, a crucial component of 90S particles (see below for more details), resulted in the identification of a large subset of the same proteins [24]. Several of the factors identified using these approaches were shown to interact with the yeast 35S pre-rRNA, thus confirming that they are *bona fide* components of early 90S pre-ribosomal particles.

Recent data suggest that these factors and the U3 snoRNP (defined as the U3 RNA tightly associated with Snu13p-15.5K, Nop56p, Nop58p, Nop1p-fribrillarin and Rrp9p-h55K) form separate modules that sequentially assemble on the nascent pre-rRNA to build up the earliest 90S particle [25, 26] (Fig. 2). The first protein complex to interact with the nascent pre-rRNA is probably the so-called 't-UTP' complex (U Three Protein complex required for transcription), made up of seven proteins (Utp10p, Nan1p, Utp4p, Utp8p, Utp5p, Utp9p, Utp15p), which can form independently from pre-rRNA transcription [27]. The tUTP complex seems to play a central role in the formation of the initial 90S particles for the following reasons. Depletion of any one of the tUTP factors inhibits both 40S and 60S ribosomal subunit formation [27]. Association of the tUTP complex with the 35S pre-rRNA is required for, but does not depend upon, the association of the U3 snoRNP, the Pwp2p/UTP-B or the UTP-C complexes with the 35S pre-rRNA [26, 27]. The latter two complexes, composed of the Pwp2p, Dip2p, Utp21p, Utp13p, Utp18p, Utp6p

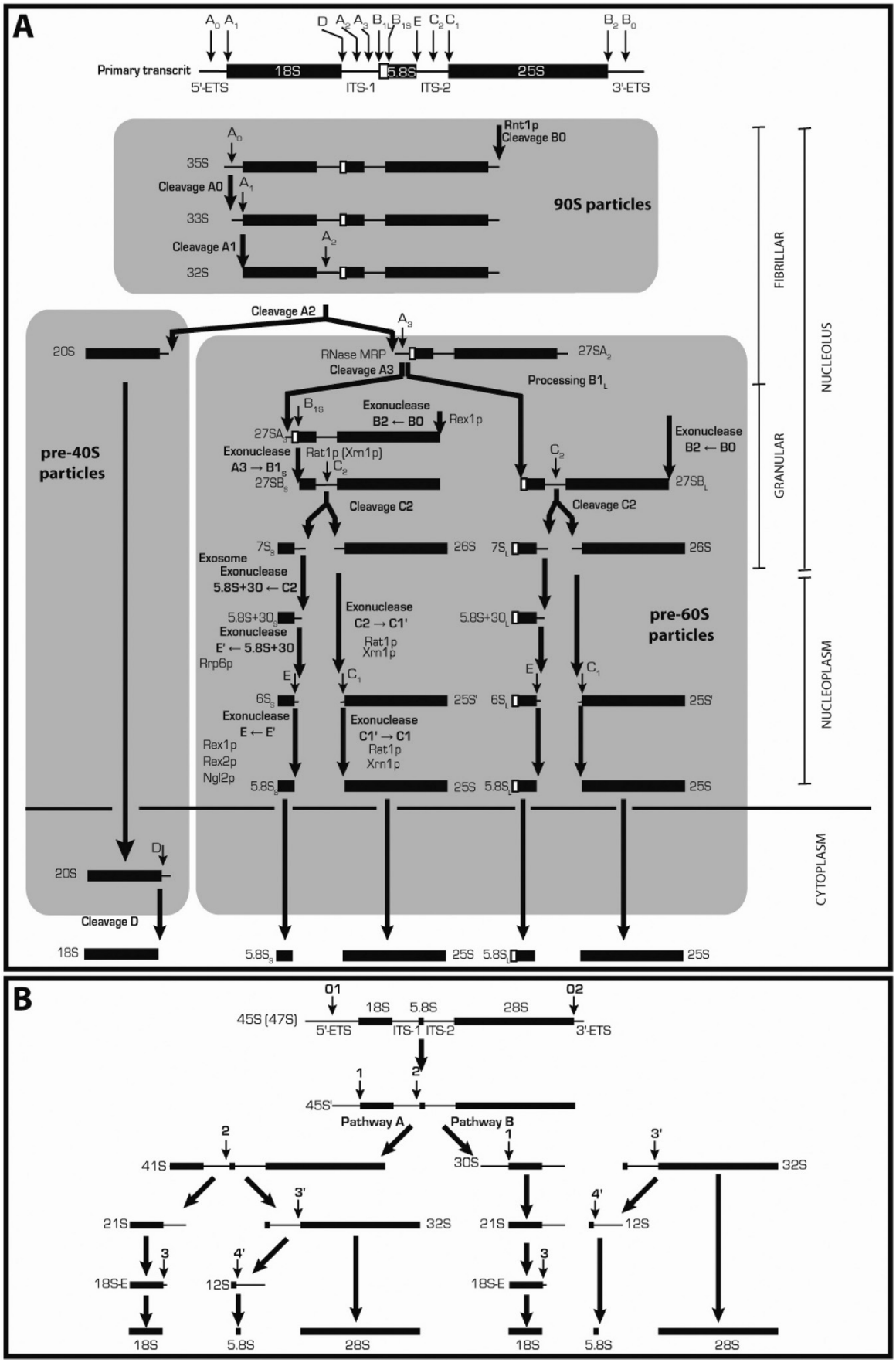


**Figure 1.** (*A*) Pre-rRNA processing scheme in *Saccharomyces cerevisiae.* (*B*) Pre-rRNA processing scheme in HeLa cells.

proteins and the Utp22p, Rrp7p, Cka1p, Cka2p, Ckb1p, Ckb2p proteins, respectively, can associate with the 35S pre-rRNA independently of each other [26]. Finally, the Rrp5p protein is required for the incorporation in pre-ribosomal particles of the UTP-C complex, but not the U3 snoRNP, the tUTP or the UTP-B complexes [26, 28]. Hence it is proposed that the tUTP complex binds nascent pre-rRNA first, followed by the UTP-B and UTP-C complexes, in an as yet unidentified order. There is also evidence suggesting that the assembly of the Pwp2p/UTP-B complex occurs concurrently with that of the U3 snoRNP and the Mpp10p complex (composed of Mpp10p, Imp3p and Imp4p; see below) [29–32]. Assembly of Rrp5p may precede that of the UTP-C complex since Rrp5p does not require components of this complex to be incorporated into pre-ribosomes [26].

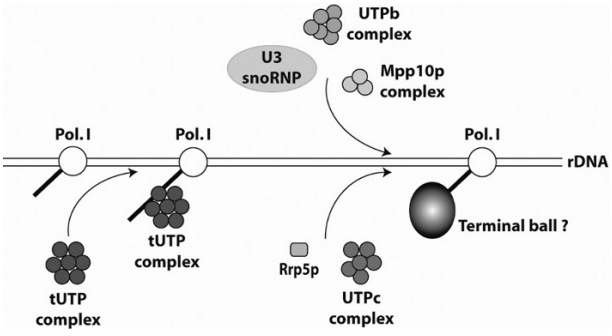


**Figure 2.** Model for the initial steps of 90S particle assembly in *Saccharomyces cerevisiae.*

## Does 5S rRNA assemble into 90S pre-ribosomal particles?

Recent data obtained with yeast suggest that 5S rRNA is incorporated into 90S particles as part of a small RNP complex. The timing of association of this 5S rRNA-containing RNP with 90S particles relative to the assembly of the small complexes described above is unknown. Both in yeast and mammalian cells, 5S rRNA is found associated with ribosomal protein Rpl5p in a ribosome-independent small RNP [33, 34]. Moreover, two-hybrid and GST pull-down experiments have revealed a network of protein-protein interactions between non-ribosomal proteins Rpf2p and Rrs1p and ribosomal proteins Rpl11p and Rpl5p [35–37]. Rpl5p can directly interact with Rpf2p, Rpl11p and Rrs1p [37]. In addition, a sub-complex composed of Rpf2p, Rrs1p, Rpl11p, Rpl5p and 5S rRNA can be purified from yeast cells defective for 60S ribosomal subunit assembly [37]. Hence these data suggest that Rpf2p, Rrs1p, Rpl11p, Rpl5p and 5S rRNA form a complex within pre-ribosomal particles. Whether these components assemble into pre-ribosomal particles as a preformed complex or as smaller entities is not known. It seems at least established that 5S rRNA is incorporated into pre-ribosomal particles as a 5S rRNA-Rpl5p complex [34]. Rpf2p and Rrs1p seem required for incorporation of Rpl11p, Rpl5p and 5S rRNA into pre-ribosomal particles [37]. Furthermore, Rpf2p, Rrs1p, Rpl11p and Rpl5p are found associated with pre-rRNAs possessing the 5′ external transcribed spacer (5′ ETS, the first part of the pre-rRNA transcript to be synthesized by RNA Pol I) [37]. Hence, Woolford and collaborators propose that Rpf2p and Rrs1p promote the recruitment of Rpl11p and Rpl5p-5S rRNA into 90S pre-ribosomal particles.

## Assembly of ribosomal proteins

Analysis of the ribosomal protein composition of pre-ribosomal particles has been hampered by the fact that many ribosomal proteins are detected in a substantial proportion of TAP purification experiments (including control experiments using untagged strains); hence it has been difficult to differentiate between background and *bona fide* associations. Some recent investigations have analysed directly the association of tagged yeast ribosomal proteins of the small subunit with various pre-rRNAs. These analyses demonstrate that most ribosomal proteins of the small subunit associate with 35S pre-rRNA, albeit weakly [38]. A subset of these ribosomal proteins are required for the early pre-rRNA processing events at sites termed A0 (within the 5′ETS), A1 (positioned at the 5′ end of the 18S rRNA sequence) and A2 (in the middle of ITS1) [39]. Strikingly, the bacterial homologues of several proteins of this subset are part of the so-called 'body' of the small ribosomal subunit, and three of them (bacterial S17, S4, and S15, corresponding to yeast Rps11p, Rps9p and Rps13p, respectively) are 'primary binders', i.e. proteins that assemble first during the *in vitro* reconstitution of *Escherichia coli* small ribosomal subunits [40]. How assembly of eukaryotic ribosomal proteins takes place relative to that of non-ribosomal factors remains ill-defined, except in a few cases, one of which will be discussed below. As 90S particles evolve to produce pre-40S particles, the integration of small subunit ribosomal proteins within pre-ribosomal particles becomes more stable, as judged from the greater co-precipitation with tagged ribosomal proteins of nuclear restricted 20S pre-rRNA compared to 35S pre-rRNA [38].

**Early steps of 90S pre-ribosome assembly and 35S pre-rRNA processing: the crucial role of the U3 snoRNA**

Work in yeast has demonstrated that formation of the aforementioned 'terminal balls' is lost upon depletion of U3 snoRNA [24]. These balls likely correspond to 90S pre-ribosomal particle assembly intermediates obtained following UTP complexes and U3 snoRNP loading. In addition, depletion of U3 snoRNA in yeast inhibits early endonucleolytic cleavages of the pre-rRNA at sites A0, A1 and A2 [41]. The upstream 23S pre-rRNA fragment released by direct cleavage of the pre-rRNA at site A3 (see Fig. 1) is, under those particular conditions, a dead-end intermediate that is not matured into 18S rRNA but is degraded. By and large, the role of U3 seems similar in higher eukaryotes. A cleavage in the 5′ETS of mammalian pre-rRNAs can be reproduced *in vitro* and the cleavage activity is abolished when U3 is depleted from the extracts [42]. Moreover, U3 is required for cleavages in the 5′ETS, at the 5′end of the 18S sequence and within ITS1 of Xenopus pre-rRNAs [43–46].

How does the U3 snoRNP function ? There is no evidence to suggest that the U3 snoRNA itself possesses endonucleolytic activity(ies) that would be directly responsible for the cleavages just enumerated. As reviewed below, it is more probable that the U3 snoRNA acts as a chaperone orchestrating the correct folding of the central core of 18S rRNA. Pre-rRNA folding and pre-ribosomal particle assembly are certainly intertwined and interdependent processes which have to proceed properly for efficient pre-rRNA processing. Ample evidence demonstrates that the U3 snoRNA interacts by direct base-pairing with the nascent pre-rRNA (Fig. 3). It has been proposed that U3 snoRNA from yeast to humans can establish two distinct base-pairing interactions via its so-called hinge regions with 5′ETS sequences of the pre-rRNA [47]. Compensatory mutation studies in yeast and Xenopus have demonstrated that these interactions are important for early pre-rRNA cleavage events and 18S rRNA production [45, 48, 49]. These interactions (helices V and VI) are probably involved in the initial docking of the snoRNA onto the nascent pre-rRNA. U3 has also been postulated to bind via its conserved A and A′ boxes to several other more downstream elements of the pre-rRNA, located within the 18S rRNA sequence [50, 51]. Strikingly, these sequence elements are involved in the formation of the universally conserved pseudo-knot that organizes the overall fold of the 18S rRNA (Fig. 3). The two 18S rRNA sequence elements that form the pseudo-knot are separated by more than 1 kb in the primary sequence and thus the more upstream part of the pseudo-knot will be transcribed well before the more downstream one. It is envisaged that binding of U3 to the more upstream part may prevent it from establishing illegitimate base-paring interactions prior to the transcription of its correct base-pairing partner sequence. Binding of U3 to the more downstream element of the pseudo-knot may then bring together the correct base-paring partners of this central pseudo-knot. U3 may thus function as a 'folding chaperone' of 18S rRNA. Although appealing, this model has yet to be experimentally demonstrated, which will not be a trivial endeavour. Compensatory mutation studies in yeast have confirmed that box A of U3 does bind to the upstream loop of the 18S pseudo-knot and that this interaction (termed 'helix II'; Fig. 3) is required for pre-rRNA processing and 18S rRNA production [52]. The other proposed interaction between U3 box A and the downstream part of the pseudo-knot (termed 'helix I'; Fig. 3) could not be validated by compensatory mutations [52], which may reflect the fact that the sequences involved play several distinct functions during processing.

If the U3 snoRNA is not itself an endonuclease, could some of its associated proteins in the context of 90S particles perform such a function? It has been suggested that one of the U3-associated proteins, Rcl1p, could function as an endonuclease [53], although this hypothesis has not yet been validated experimentally. Interestingly, Rcl1p interaction with the U3 snoRNA in the context of 90S particles requires Rcl1p binding to the Bms1p GTPase [54–57]. Bms1p binds directly to both U3 snoRNA and Rcl1p [56]. GTP, but not GDP, binding to Bms1p increases its affinity for Rcl1p, while Rcl1p binding to Bms1p increases its affinity for U3 snoRNA [56]. It has therefore been proposed [56] that Rcl1p binds to Bms1p in its GTP-bound form and that the resulting ternary complex docks onto 90S pre-ribosomal particles via a direct Bms1p-U3 snoRNA interaction. A conformational switch within 90S particles may induce GTP hydrolysis by Bms1p, resulting in Rcl1p dissociation from Bms1p, leading in turn to the release of Bms1p from U3 snoRNA.

U3 snoRNA hybridization to and dissociation from pre-rRNA are almost certainly modulated by factors possessing RNA-RNA annealing and RNA helicase activities. *In vitro* work suggests that the Imp3p and Imp4p proteins, which bind the U3 snoRNA directly, could stabilize the yeast 5′ETS/U3 hinge interactions [58]. In addition, Imp4p may unfold the helix trapping the U3 box A sequence allowing it to interact with the 5′ end of 18S rRNA [58]. Dhr1p, a putative helicase of the DEAH family associated with U3 and present in 90S particles, has also been proposed to modulate RNA-RNA interactions during formation of the 18S rRNA central pseudo-knot [59]. Finally, depletion of

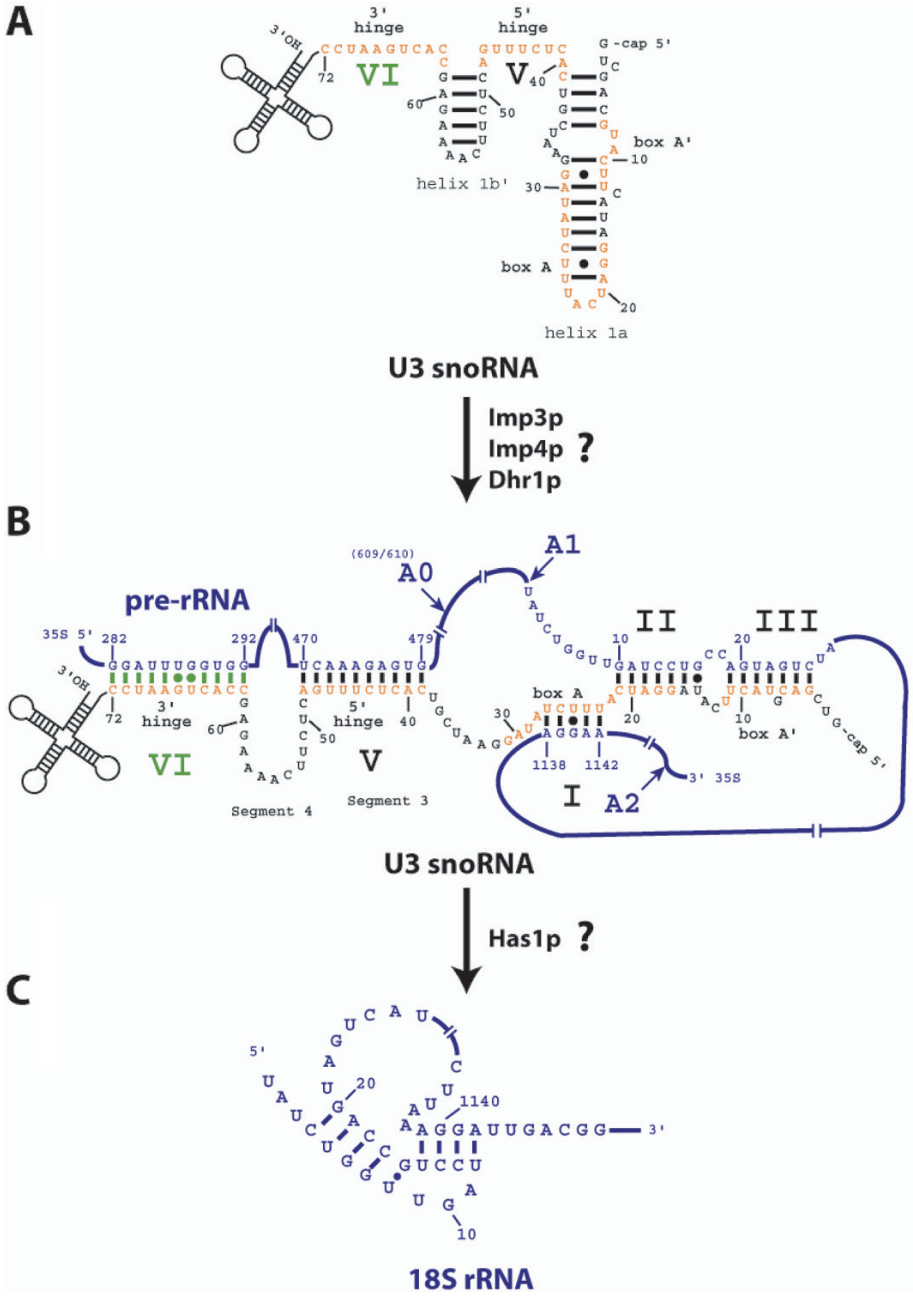


**Figure 3.** Yeast U3 snoRNA base-pairing with the 35S pre-rRNA is probably required for 18S rRNA pseudo-knot formation. Proteins potentially involved in promoting transitions between the three structures shown are indicated. (*A*) Secondary structure of free U3 snoRNA. (*B*) U3 snoRNA bound to 35S pre-rRNA. In yeast, formation of only two helices, helices II and V, has been confirmed by compensatory mutation studies. (**C**) Structure of the central pseudo-knot of 18S rRNA.

the putative helicase Has1p results in retention of U3 snoRNA in high-molecular-weight complexes, suggesting that Has1p intervenes in U3 release from pre-ribosomal particles [60].

**Other snoRNAs required for early cleavage steps of the pre-rRNA and 40S ribosomal subunit formation**

In addition to the U3 snoRNA, the U22 [61], U14 [62], snR30 (yeast)/U17 (mammals) [63] and snR10 [64] snoRNAs are required for early cleavage steps of the pre-rRNA necessary for 18S rRNA production. Like U3, snR30 and U14 are likely to perform their functions in the context of 90S particles and directly interact with the 35S pre-rRNA [63, 65, 66]. Compensatory mutation studies have demonstrated that the essential so-called domain A of U14 mediates a direct interaction with 18S rRNA required for 18S rRNA production [67, 68]. It is not known how snR30/U17 interacts with the 35S pre-rRNA. However, it has been shown that snR30/U17 contains two specific conserved sequence elements that are essential for its role in pre-rRNA processing and might constitute pre-rRNA binding regions [69]. Whether docking of U14 and snR30 snoRNAs onto the 35S pre-rRNA results in the assembly of snoRNA-associated protein factors within 90S particles and/

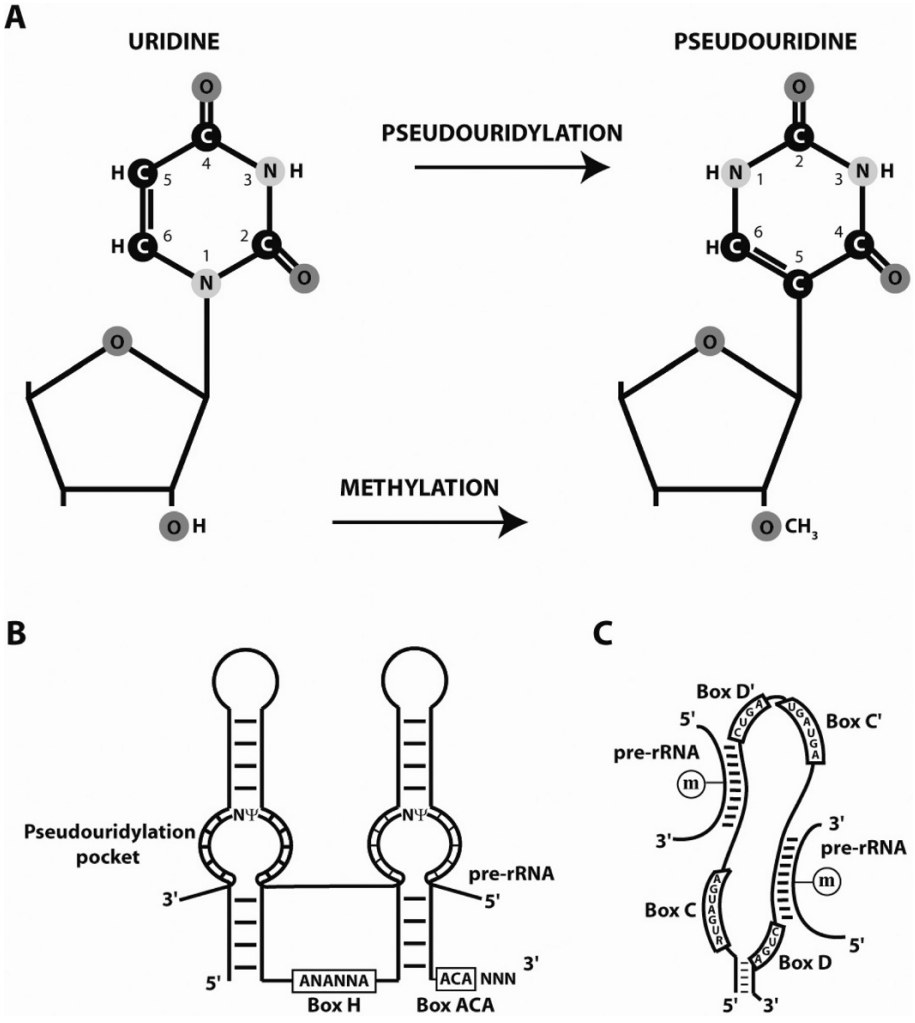


**Figure 4.** 2′O methylations and pseudouridylations of pre-rRNAs are guided by box C/D and box H/ACA snoRNAs. (*A*) Conversion of uridine into pseudouridine (top) and 2′O methylation event (bottom). (*B*) Schemes of the interactions established between the pre-rRNA and a box H/ACA snoRNA (left) or box C/D snoRNA (right). See text for details.

or modulates pre-rRNA folding remain as yet untested hypotheses.

**Modifications of pre-rRNA nucleotides by snoRNPs**

Pre-rRNAs undergo extensive chemical modifications. Most of these are of two types: isomerisation of uridines into pseudouridines and addition of a methyl group to the oxygen linked to the 2′ carbon of riboses (Fig. 4A). The bulk of these modifications probably occur in the context of nascent 90S pre-ribosomal particles within the fibrillar strands/dense fibrillar component of the nucleolus [70–73]. They are carried out by two groups of snoRNPs: box C/D snoRNPs perform 2′O methylations [74–76] while box H/ACA snoRNPs are responsible for pseudouridylations [77, 78] [for recent extensive reviews on the biogenesis and structure of these snoRNPs, see refs 79–81]. The only exceptions are the snoRNPs containing the box C/D snoRNAs U3, U8 [82], U22 and the box H/ACA snoRNA snR30/U17 that are required for pre-rRNA cleavage steps and seem devoid of nucleotide modification functions. Mixed cases are constituted by the snoRNPs containing the U14 box C/D snoRNA and the snR10 box H/ACA snoRNA: in addition to their roles in pre-rRNA cleavage steps, both these snoRNPs are responsible for one nucleotide modification each [83, 84].

The ‘box C/D’ and ‘box H/ACA’ terminology derives from the presence in the particles of the first group of a small RNA containing the conserved C and D sequence elements (‘boxes’) and in particles of the second group of a small RNA featuring the conserved H and ACA boxes (Fig. 4B). These RNAs are called ‘guide RNAs’ because they are responsible for the selection of the nucleotides that will be modified, through specific Watson-Crick base-pairing interactions with the pre-rRNAs. Box C/D snoRNAs contain long segments (10 to 20 nucleotides long) of perfect complementarity with pre-rRNA sequences, always positioned immediately upstream from their D and/or D′ (a more degenerate version of the D box) boxes. In the duplex resulting from the box C/D guide RNA/

pre-rRNA interaction, the pre-rRNA nucleotide that undergoes 2′O-methylation is always base-paired to the residue of the guide antisense sequence located five nucleotides upstream from the D (or D′) box [75] (Fig. 4B). Box H/ACA guide snoRNAs contain small sequences of complementarity with pre-rRNAs located on both sides of internal loops (so-called 'pseudouridylation pockets') within irregular stem-loop structures that bracket the H box. The antisense sequences of the pseudouridylation pocket bind immediately upstream and downstream from the targeted pre-rRNA uridine [77] (Fig. 4B). This uridine is thus exposed unpaired at the centre of a three-way RNA junction. Actual catalysis of the modification reactions is carried out by protein enzymes that interact with the guide RNAs. The methyl-transferase of box C/D snoRNPs is Nop1p (yeast)/fibrillarin (mammals) while the pseudouridine synthase of box H/ACA snoRNPs is Cbf5p (yeast)/dyskerin (humans).

All nucleotide modifications introduced on the pre-rRNAs are found within the sequences retained in the mature rRNAs and are mostly concentrated within the functionally important regions of rRNAs, in particular the decoding centre and the peptidyltransferase centre [85]. It has therefore been proposed that these modifications are required for full activity of the ribosome. Indeed, specific alterations of the S-adenosyl-methionine binding site of Nop1p in yeast inhibit both snoRNP-directed methylation of pre-rRNAs and growth, without major defects in ribosome subunit production [86]. Somewhat similarly, specific alterations of the catalytic centre of Cbf5p inhibit both growth and pseudouridylation, with no major pre-rRNA processing defects [87]. It is therefore believed that collectively, rRNA modifications introduced by snoRNPs are essential for ribosome function. Consistent with this proposal, Fournier and collaborators have shown that the absence of a subset of guide snoRNAs directing modifications of the peptidyltransferase centre or helix 69 of the large rRNA alter rRNA structure and ribosome fidelity and/or activity [84, 88].

Dissociation of guide snoRNAs from pre-rRNAs likely requires helicase activities, particularly in the case of box C/D snoRNAs possessing long segments perfectly complementary to pre-rRNAs. Recently, evidence has been provided for the involvement of the DEAD box helicases Dbp4p and Has1p in the removal of several snoRNAs from pre-rRNAs [60, 89].

### Are all 90S particles devoid of most factors required for large ribosomal subunit formation ?

Strikingly, the majority of identified 90S particle components (with some notable exceptions such as Rrp5p [90] and components of the tUTP complex) are only required for small ribosomal subunit (40S) synthesis. The paucity of factors required for large ribosomal subunit (60S) synthesis among the proteins identified as 90S particle components led to the suggestion that most components of early pre-60S pre-ribosomal particles assemble concomitantly with pre-rRNA cleavages within ITS1. However, Baserga and colleagues have argued that, at steady-state, most known 90S particle components are mainly associated with the 23S pre-rRNA, based on Northern blot data. Hence the TAP purification approaches used to purify 90S particles may have yielded primarily late '90S' complexes devoid of components required for 60S ribosomal subunit formation that may however have been present in earlier 90S particles [10]. Indeed, the recent use of a novel one-step very fast purification protocol [91] has revealed that the Nop15p protein required for 60S ribosomal particle biogenesis previously catalogued as a 'pre-60S particle component' [8] is in fact associated with numerous 90S particle components. Many other such cases will probably be found.

## Nuclear steps of pre-60S particle maturation

### A surprisingly complex pre-rRNA processing pathway leading to mature large subunit rRNAs

The intricacies of the pre-rRNA processing steps leading to the mature rRNAs of the 60S ribosomal subunits and the enzymes involved have been best characterized in the yeast *S. cerevisiae.* Cleavage of the 32S pre-rRNA (see Fig. 1A) at site A2 in the context of late 90S pre-ribosomal particles releases early pre-40S and pre-60S particles. The latter will evolve into mature 60S ribosomal subunits through an intricate process involving a surprisingly complex series of endo- and exonucleolytic cleavages. The RNA component of the earliest pre-60S particles, the 27SA2 pre-rRNA, can be processed in either of two mutually exclusive ways. It can be processed directly at site B1(L), corresponding to the 5′ end of the long form of mature 5.8S rRNA, leading to the production of the 27SB(L) pre-rRNA. This processing step is most likely an endonucleolytic cleavage performed by an as yet unidentified activity. Alternatively, the 27SA2 pre-rRNA can be cleaved by the endoribonuclease RNase MRP at site A3 [92–94]. RNase MRP is a ribonucleoprotein complex that probably evolved from RNase P [95]. Since bacterial RNase P is a ribozyme

[96], it is believed that the catalytic activity of eukaryotic RNase MRP lies in its RNA component. The 5′ end released by cleavage at site A3 is the entry site for the Rat1p and Xrn1p 5′ to 3′ exoribonucleases [97]. These exoribonucleases digest ITS1 until they reach the B1(S) site, corresponding to the 5′ end of the short form of 5.8S rRNA, resulting in the formation of the 27SB(S) pre-rRNA. Both 27SB(L) and 27SB(S) pre-rRNAs are then processed identically. They are cleaved by an unknown endonuclease at site C2 within internal transcribed spacer 2 (ITS2). The upstream fragments released, 7SB(L) or 7SB(S), are processed at their 3′ends by the successive intervention of distinct 3′ to 5′ exoribonucleases. The core exosome [98–100] intervenes first, to produce a 5.8S precursor containing a 30-nucleotide-long extension. This 5.8S + 30 precursor is then processed by the exosome-associated Rrp6p exoribonuclease to yield the 6S pre-rRNA that still contains 8 additional nucleotides compared to mature 5.8S [101, 102]. The exosome is assisted by the Dob1p/Mtr4p helicase during these digestion steps [101, 103]. The 6S pre-rRNA is then the substrate of the Rex1p and Rex2p exoribonucleases [104] which remove approximately 3 nucleotides, and the final trimming is performed by the Ngl2p nuclease [105], leading to the production of the short and long forms of 5.8S. It is worth pointing out at this stage the oft overlooked fact that eukaryotic cells contain therefore at least two different types of ribosomes, possessing either the long or short form of 5.8S. These different ribosomes may translate different sets of mRNAs [94]. Interestingly, the Rex1p exoribonuclease is also required for the production of the mature 3′ ends of 5S rRNA and 25S rRNA [104]. The mature 5′ end of 25S rRNA is produced by processing of the 25.5S pre-rRNA, resulting from the C2 cleavage, by the Rat1p and Xrn1p 5′ to 3′ exoribonucleases [106].

At least the exosome, Mtr4, RNase MRP, Rat1 and Xrn1 are conserved in higher eukaryotes and are likely involved in 5.8S and 28S rRNA production in these organisms as well [see for example refs. 107, 108]. One additional factor required for 5.8S and 28S rRNA synthesis in higher eukaryotes has been described, namely the box C/D U8 snoRNA, that seems absent in yeast. The U8 snoRNA is required for normal pre-rRNA processing and 5.8S and 28S rRNA accumulation in *Xenopus* oocytes [82]. The 5′ end of U8, which is well conserved from *Drosophila* to humans, is essential for pre-rRNA processing and probably mediates its direct base-pairing with pre-rRNAs [109, 110]. Finally, it has been reported that the multifunctional nucleolar B23 protein [111] can function as an endoribonuclease, preferentially cleaving within ITS2 of rat pre-rRNA *in vitro* [112]. Hence, B23 may be directly involved in mammalian ITS2 processing, although to our knowledge no *in vivo* data supporting this hypothesis has yet been published.

**Composition and intranuclear movement of nuclear pre-60S pre-ribosomal particles**

The intricate pre-rRNA processing events just described occur within a succession of pre-60S pre-ribosomal particles of highly dynamic protein composition [4, 113–117]. Some non-ribosomal protein factors may associate with a range of particles containing pre-rRNA components at different stages of maturation, while other factors display a much more restricted pattern of association. Overall, Hurt and collaborators have noticed that the number of non-ribosomal proteins present in the successive pre-60S pre-ribosomal particles declines as pre-rRNA maturation proceeds and the particles move from the nucleolus to the nucleoplasm and are then transported to the cytoplasm [115]. Putative enzymes are prevalent among non-ribosomal components of pre-60S particles, including helicases (Mak5p [118], Dbp2p [119,120], Dbp3p [121], Dbp6p [122], Dbp7p [123], Dbp9p [124], Dbp10p [125], Dob1p [103], Drs1p [126], Spb4p [127], Has1p [128,129], Prp43p [130–132]), ATPases (Rix7p [133], Rea1p [134]), GTPases (Nug1p [113], Nog1p [135], Nog2p/Nug2p [113,116]), methyl-transferases (Spb1p [136,137], Nop2p [138,139]) and peptidyl proline isomerases (Fpr3p [140], Fpr4p). Most of these components have been shown to be required for normal pre-rRNA processing and large subunit rRNA accumulation. However, except for the Spb1p methyltransferase [72], the Dob1p helicase [103] and the Rea1p ATPase, the precise roles and targets of the enzymes listed above remain elusive. Exonucleases cleaving the pre-rRNAs can only be retrieved using a very rapid one-step purification protocol, suggesting that their interactions with the pre-ribosomal particles are very transient and/or labile [37, 91].

Identification of the intracellular position of protein factors chiefly associated with a given pre-rRNA or required for a specific pre-rRNA processing step has helped define the localization of successive pre-60S particles. The Npa1p protein is mainly associated at steady-state with the 27SA2 pre-rRNA. As Npa1p is detected at the interface between the fibrillar strands and the granular component of the nucleolus, it is envisaged that at least a subset of pre-60S particles containing 27SA2 pre-rRNA are located there [65]. Inactivation of the pre-60S particle component Rlp7p leads to inhibition of C2 cleavage within ITS2 and to the accumulation of pre-rRNAs containing ITS2 within the nucleolus. As Rlp7p is detected in the granular component of the nucleolus, this is where C2 cleavage is likely to occur [141]. Finally, depletion of

the Nog2p protein, associated with pre-60S particles containing 27SB or 7S pre-rRNAs, leads to accumulation of 7S pre-rRNA that fails to be processed, and to retention of pre-60S particles within the nucleoplasm [116]. These data suggest that 7S processing occurs within nucleoplasmic pre-60S particles.

It is not clear what processes and factors drive the movement of pre-60S particles from the nucleolus to the nucleoplasm and through the nucleoplasm to the nuclear pores. The Noc1p, Noc2p and Noc3p proteins have been proposed to play an important role in these movements. Noc2p can interact either with Noc1p, which is mainly nucleolar, or with Noc3p, which is mainly nucleoplasmic [142]. Milkereit and collaborators envisage that the exchange of Noc1p for Noc3p in pre-ribosomal particles participates in their exit from the nucleolus [142]. How these Noc proteins might function in this transport step remains unclear. Their putative transport function may rely on their putative α-helical repeats displaying similarities to Huntington-elongation-A subunit-TOR (HEAT) repeats found in nucleocytoplasmic transport factors [143].

### Pre-60S pre-ribosomal particle assembly and structure: the importance of protein/protein interactions

One protein sub-complex present in many nuclear pre-60S pre-ribosomal particles has been identified in yeast and mammalian cells. The three protein components of this complex, called Nop7p (or Yph1p)/Erb1p/Ytm1p in yeast or Pes1/Bop1/WDR12 in mammals, are all required for pre-rRNA processing steps leading to the production of 5.8S and 25/28S rRNAs [4, 144–152]. Integrity of this sub-complex can be maintained outside the context of pre-60S pre-ribosomal particles. It probably exists in cells as a free complex and can also be released from pre-60S pre-ribosomal particles, for example when they are disrupted by inactivation of a protein component, by high-speed centrifugation or by chemical treatment [4, 25, 145–147, 153, 154]. Erb1p/Bop1 interacts directly with Nop7p/Pes1 [147, 153] and with Ytm1p [153]. As Nop7p and Erb1p interact with yeast 35S pre-rRNA but Ytm1p does not, it is envisaged that Nop7p and Erb1p assemble first with 90S pre-ribosomal particles and Ytm1p is then recruited at the pre-60S stage via a direct interaction with Erb1p [153]. The position of this sub-complex within pre-60S pre-ribosomal particles and its precise molecular functions remain unknown.

The formation of protein-only sub-complexes held together by specific high-affinity protein-protein interactions may be a common aspect of pre-ribosomal particle architecture and assembly, underscored by the fact that at least 22 components of pre-ribosomes contain, like Ytm1p and Erb1p, a WD40 motif thought to mediate direct protein-protein binding [8, 155]. Other pre-60S sub-complexes have been described, including Rlp24p/Nog1p [117], Rlp24p/Mak11p [155], the Dbp6p helicase-containing complex specific to early pre-60S particles [156] or the Rix1p-Ipi1p-Ipi3p [25] complex found in late nuclear pre-60S particles (see below).

### Does late nuclear remodelling of pre-60S particles involve ATP hydrolysis by the AAA-type ATPase Rea1p ?

Late nuclear pre-60S particles contain the largest protein of the yeast proteome, the AAA (ATPases associated with diverse cellular activities)-type ATPase Rea1p, and the Rix1p-Ipi1p-Ipi3p complex. Strikingly, Rea1p and Nug2p/Nog2p dissociate from these particles *in vitro* in the presence of exogenous ATP, but not ADP or a non-hydrolysable analogue of ATP. These data may indicate that ATP hydrolysis by Rea1p triggers a conformational change in pre-60S particles associated with its own release and that of Nog2p. Most interestingly, electron microscopy analyses of purified Rea1p-containing pre-60S particles show that they display a tadpole-like structure. Antibody cross-linking experiments demonstrate that Rea1p is located in the 'tail' of the structure, whereas ribosomal proteins are present in the 'head', which presumably will evolve into the mature 60S subunit [157].

## Nuclear steps of pre-40S particle maturation

The pre-40S particle released by cleavage of 32S pre-rRNA at site A2 contains far fewer non-ribosomal proteins than the pre-60S particle released by the same cleavage [158]. Most of these components, namely Dim1p, Dim2p, Enp1p, Hrr25p, Nob1p, Prp43p, Rrp12p and Tsr1p were already present within 90S particles [131, 158–162]. A few other non-ribosomal proteins, Ltv1p, Pfa1p/Sqs1p, Rio1p, Rio2p then join pre-40S particles following A2 cleavage [131, 158, 163–165]. Association of Ltv1p and Pfa1p with 20S pre-rRNA probably occurs in the nucleus since these proteins are found in the nucleus and since Ltv1p appears to be required for efficient export of pre-40S particles to the cytoplasm [166, 167]. Whether the yeast Rio1p and Rio2p proteins associate with pre-40S particles in the nucleus or the cytoplasm remains a debated issue. Rio2p is predominantly, and Rio1p exclusively, detected in the cytoplasm under normal conditions, yet both are shuttling proteins which accumulate in the nucleus of *crm1–1* mutant cells at non-permissive temperature [164]. Little is

known concerning the mode of recruitment of non-ribosomal pre-40S factors to pre-ribosomal particles, apart from the fact that association of Enp1p, Rio2p and Tsr1p with 20S pre-rRNA requires the Rps19p ribosomal protein [168].

One key maturation event/conformational rearrangement that likely takes place within nuclear pre-40S pre-ribosomal particles has recently been identified in yeast, concerning the Enp1p, Ltv1p and Rps3p proteins. These proteins probably form a complex within pre-40S particles as they are released bound to one another after high-salt washes of purified pre-40S particles [169]. All three proteins can be phosphorylated *in vivo* and Enp1p and Rps3p fail to become phosphorylated in Hrr25p-depleted cells, suggesting that Hrr25p, which is a casein kinase I isoform, directly phosphorylates Enp1p and Rps3p. Phosphorylation of Enp1p, Ltv1p and Rps3p is correlated with a decreased affinity of these proteins for the pre-40S particles *in vitro*. Phosphorylation of Enp1p, Ltv1p and Rps3p might be necessary for the pre-40S particles to adopt a structure compatible with efficient passage through the nuclear pore. Consistent with this hypothesis, depletion of Hrr25p inhibits nuclear export of pre-40S particles [169].

## Transport of pre-ribosomal particles

### Export of both the pre-40S and pre-60S pre-ribosomal particles requires the small GTPase Ran cycle and a specific subset of nucleoporins

Pre-ribosomal particles assembled in the nucleus are exported to the cytoplasm through the nuclear pore complexes (NPCs). Early analysis of the transport of injected radiolabelled 40S or 60S ribosomal subunits across the nuclear envelope of *Xenopus* oocytes revealed that this transport is unidirectional, occurring only from the nucleus to the cytoplasm, and energy dependent [170, 171]. These observations suggested that export of the pre-ribosomal particles is a facilitated diffusion process relying on the small GTPase Ran. The GTP- and GDP-bound forms of Ran are asymmetrically distributed across the nuclear envelope [172]. The Ran-GTP to Ran-GDP ratio is high in the nucleus and low in the cytoplasm. This gradient originates from the asymmetric localization of crucial components of the cycle of the Ran GTPase (called Gsp1p in *S. cerevisiae*): the GTPase-activating protein (RanGAP, Rna1p in yeast) is a cytoplasmic factor, whereas the nucleotide exchange factor (Ran-GEF, Prp20p in yeast) is localized to the nucleus. In the nucleus, Ran-GTP promotes the interaction between the macromolecules or complexes to be exported (cargoes) and so-called export receptors whose function is to facilitate translocation of the export-competent complexes (cargoes, export receptors, RanGTP) through the NPCs (see below). Once in the cytoplasm, GTP hydrolysis by Ran induces release of the cargo and Ran-GDP and the export receptors are then imported back to the nucleus. There, GDP is rapidly substituted for GTP in the active site of Ran to regenerate a pool of Ran-GTP available for new rounds of export [172]. In yeast, mutations in genes encoding components of the Ran GTPase cycle (*GSP1*, *RNA1*, *PRP20*, *YRB1*) affect the export of both the pre-60S [173–175] and the pre-40S pre-ribosomal particles [173, 176, 177], strongly suggesting that the Ran GTPase cycle is required for the export of both pre-ribosomal particles. This conclusion also seems to apply to higher eukaryotes since (i) depletion of RanGTP from *Xenopus* oocyte nuclei inhibits the export of the newly synthesized 18S and 28S rRNAs [178] and (ii) hamster cells lacking the RCC1 protein, the orthologue of yeast Prp20p, display processing and export defects of both the 18S and 28S rRNAs [179].

The NPCs are large structures embedded in the nuclear membrane forming selective channels through which large molecules or complexes exchange between the nucleus and the cytoplasm. NPCs are composed of different nucleoporins, among which several sub-complexes can be distinguished on the basis of specific physical interactions. Several laboratories have addressed the function of specific nucleoporins in the export of pre-ribosomal particles [173–177]. Only a specific subset of nucleoporins seem to be required for export of both the pre-40S and pre-60S pre-ribosomal subunits. Altering the function of Nup84p and Nup85p, for example, does not result in nuclear retention of pre-60S or pre-40S subunits, suggesting that these specific nucleoporins are not required for the export of pre-ribosomal particles [173, 174, 177]. However, mutations in the genes encoding the Nup82p sub-complex (Nup82p-Nup159p, Nsp1p) induce the accumulation of both the pre-40S and pre-60S particles in the nucleus but do not affect significantly the early processing steps of pre-rRNA, suggesting that the Nup82p complex is directly required for the export of pre-ribosomes [173]. In HeLa cells, the Nup214-Nup88 nucleoporin sub-complex, proposed to be the homologue of the Nup82p sub-complex in yeast, is required for export of the pre-60S pre-ribosomal subunits [180], suggesting an evolutionarily conserved function of this sub-complex in pre-ribosome export.

### Several nuclear receptors promote export of the pre-60S pre-ribosomal particles

Most nucleoporins contain phenylalanine-glycine (FG)-rich repeats thought to form a hydrophobic

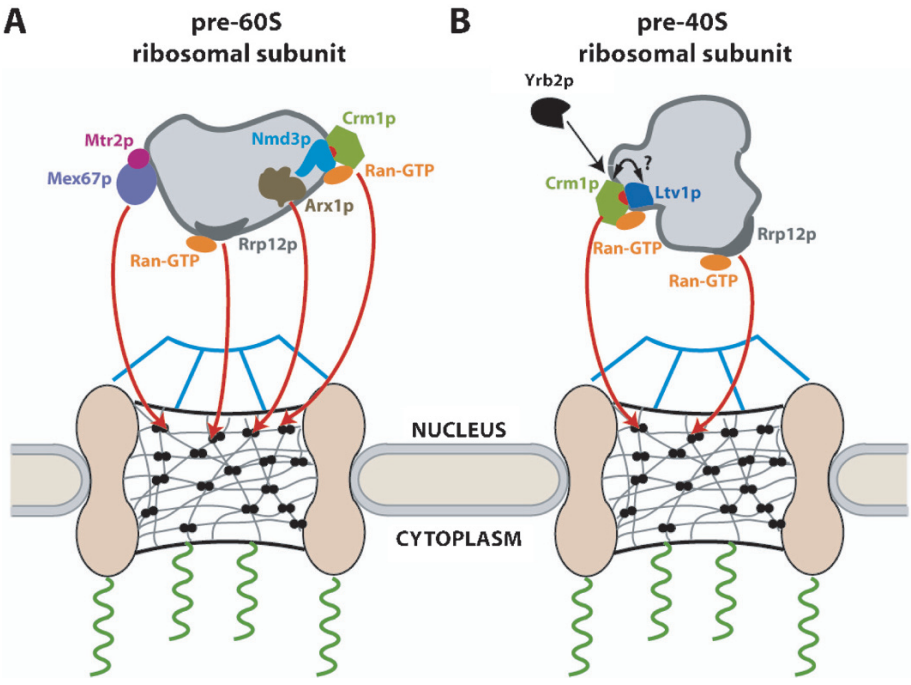


**Figure 5.** Schemes of export-competent pre-60S (*A*) and pre-40S (*B*) particles with associated factors relevant to nuclear export. The red protuberances on Ltv1p and Nmd3p represent the nuclear export signals mediating interaction with Crm1p/Xpo1p. Arrows between export receptors and the hydrophobic mesh of the NPCs refer to the reported ability of these factors to interact directly with the FG repeats of some nucleoporins. Ltv1p is not essential in yeast; the double-spiked arrow and question mark highlight the fact that the direct interaction between this protein and Crm1p is still hypothetical.

meshwork at the entrance and/or in the lumen of the NPC. This hydrophobic meshwork has been proposed to create a selective barrier preventing passage of large molecules that are not bound to transport receptors. Through their ability to interact with the FG-rich repeats of the nucleoporins, transport receptors are thought to help dissolve their cargoes within the hydrophobic mesh and thereby promote translocation through the NPC. Pre-ribosomes are large particles that very probably display exposed hydrophilic surfaces given their high RNA content, which may preclude passage through the hydrophobic NPCs. Growing evidence suggests that the export of the large pre-60S pre-ribosomal particles is mediated by several export receptors (see Fig. 5A) that associate with the particles in the course of their maturation in the nucleus, promote their translocation through the NPCs and dissociate from their cargoes once in the cytoplasm.

CRM1/XPO1 belongs to the karyopherin β family of proteins and has been characterized as a receptor that promotes export of cargoes containing leucine-rich nuclear export signals (NESs) both in yeast and *Xenopus laevi* oocytes [181, 182]. In *S. cerevisiae,* a specific point mutation in *CRM1*/*XPO1* results in the synthesis of a modified Crm1p/Xpo1p protein that is specifically inactivated by leptomycin B (LMB). Treatment of these mutant cells with LMB induces a strong nuclear retention of pre-60S pre-ribosomal particles [183, 184]. This phenotype occurs very rapidly after addition of LMB, strongly suggesting that it does not result indirectly from defects in the export of other Crm1p/Xpo1p-dependent cargoes such as mRNP particles. Instead, Crm1p/Xpo1p is most likely an export receptor mediating export of the pre-60S pre-ribosomal particles. The NES mediating the interaction between the particles and Crm1p/Xpo1p is provided in *trans* by Nmd3p, a protein component of the pre-60S particles. Nmd3p is a conserved protein found from archaea to mammalian cells. In yeast, *NMD3* is essential and mutations in this gene affect the production of the large ribosomal subunit and induce the accumulation of pre-60S pre-ribosomal particles in the nucleolus and the nucleoplasm [183, 185, 186]. These results suggest that Nmd3p assembles with pre-60S particles in the nucleus and carries out an evolutionarily conserved function in the maturation of these particles. In addition, the carboxy-terminal domains of eukaryotic Nmd3p orthologues contain both a nuclear localization sequence (NLS) and an NES, and deletion analyses in yeast showed that these signals drive the shuttling of Nmd3p across the nuclear envelope. Deletion of the C-terminal part of Nmd3p containing the NES is lethal, and overexpression of this truncated version of Nmd3p in wild-type cells induces a dominant negative phenotype correlated with a retention of pre-60S particles in the nucleus [184, 187]. Addition of a heterologous NES to this altered version of Nmd3p abrogates the pre-60S particle export defects and restores viability [183, 184]. Altogether, these results strongly suggest that in yeast, Nmd3p mediates the export of the pre-60S particles by providing the NES recognized by the export receptor Crm1p/

Xpo1p. Interestingly, a very similar mechanism drives nuclear exit of the pre-60S pre-ribosomal particles in higher eukaryotes. In *X. laevi* oocytes and HeLa cells, CRM1 is indeed required for export of the pre-60S particles and the NES mediating recognition of these particles by CRM1 is provided in *trans* through the association of the orthologue of yeast Nmd3p (hNMD3 in human) with the pre-60S particles [178, 188].

The Rrp12p protein is found associated with late precursors of the 60S ribosomal subunit [161]. *In vitro*, Rrp12p interacts with the FG repeats of the nucleoporins Nup100p and Nup116p [161], suggesting that Rrp12p functions in the export of the pre-60S pre-ribosomal particles. Indeed, yeast heterokaryon experiments showed that Rrp12p shuttles between nucleus and cytoplasm and in the absence of Rrp12p, nuclear export of pre-60S particles is inhibited [161]. Interestingly, Rrp12p belongs to the family of the HEAT repeat proteins characterized by the repetition of α-helical HEAT motifs, and secondary-structure predictions suggest that more than 60% of Rrp12p residues form α-helical domains. On this basis, Rrp12p has been suggested to function as a pre-60S particle export factor that helps dissolve them in the hydrophobic mesh of the NPC in two ways: by interacting with the FG repeats of the nucleoporins and covering the hydrophilic surfaces of the particles with its extended α-helical domains.

The Mex67p-Mtr2p heterodimer was initially characterized as a shuttling complex required for mRNA export. This complex does not belong to the karyopherin β family, and mediates export of mRNAs independently of the Ran GTPase cycle. It is recruited to export-competent mRNPs and promotes export of these cargoes through a direct interaction with the FG repeats of nucleoporins [189, 190]. In yeast, specific mutations in *MEX67* or *MTR2* genes induce synthetic lethal growth phenotypes when combined with mutations in *NMD3*, and overexpression of Mex67p-Mtr2p rescues the pre-60S export defects induced by the expression of truncated versions of Nmd3p lacking the NES [191]. This genetic evidence suggested a direct function of the Mex67p-Mtr2p complex in the export of pre-60S particles in addition to its role in mRNA export. Indeed, this complex is physically associated with late, export-competent pre-60S particles containing Nmd3p, and specific point mutations within *MEX67* or *MTR2* genes induce defects in nuclear exit of pre-60S particles but do not affect mRNA export significantly [191]. Interestingly, these specific mutations abolish the interaction of the Mex67p-Mtr2p complex with late pre-60S particles but do not affect the recruitment of Nmd3p to these export-competent particles [191]. The Mex67p-Mtr2p complex therefore functions as another export receptor for pre-60S subunits, in addition to Crm1p/Xpo1p, and facilitates the export of these large particles through the NPC by a Ran-independent mechanism. Interestingly, the surface of the Mex67p-Mtr2p complex proposed to interact with pre-60S particles was shown to form a versatile platform also mediating an association with the NPC, via a direct interaction with the nucleoporin Nup85, a component of the Nup84 complex embedded within the nuclear pore [192]. Mutations that perturb the interaction between Mex67p-Mtr2p and the Nup84 complex affect nuclear exit of mRNAs but have minor impact on pre-60S particle export. *In vitro*, pre-60S particles and the Nup84 complex bind the Mex67p-Mtr2p platform competitively, and *in vivo*, mutations in *NUP85* that weaken the association between Mex67p-Mtr2p and the Nup84 complex alleviate pre-60S particle export defects resulting from mutations in *NMD3*. These recent results suggest that the mRNA and the pre-60S particle export pathways may communicate via the Mex67p-Mtr2p complex [192].

In yeast, Arx1p is a non-essential protein component of late pre-60S particles [115]. Deletion of *ARX1* displays synthetic lethal or synthetic enhanced growth phenotypes in combination with specific mutations in genes encoding components of the NPC or components of the pre-60S particle export apparatus such as *NMD3*, *MTR2* or *MEX67* [193]. In some of these double-mutant strains, a specific defect in nuclear exit of the pre-60S particles is observed [193]. Given that the absence of Arx1p does not prevent recruitment of Nmd3p or the Mex67p-Mtr2p complex to late pre-60S particles, these results suggest a direct and specific function of Arx1p in the export of pre-60S particles. *In vitro*, recombinant Arx1p interacts physically with the FG repeats of some nucleoporins, and when injected into *X. laevi* oocytes or HeLa cells, Arx1p has the ability to shuttle across the nuclear envelope [193]. Interestingly, Arx1p is homologous to methionine aminopeptidases (MetAPs) that cleave the initial methionine from newly synthesized proteins. Three-dimensional structural modelling suggests that Arx1p contains a MetAP structural fold, but this domain is inactive *in vitro* [193]. Specific amino acid substitutions in this domain inhibit the interaction between Arx1p and the FG repeats of nucleoporins *in vitro* and induce defects in the export of pre-60S particles *in vivo* when combined with otherwise benign mutations in the genes encoding NPC components [193]. These experimental data strongly suggest that Arx1p functions as an additional export receptor for pre-60S ribosomal particles by mediating their interaction with specific nucleoporins via an atypical MetAP structural domain. Interestingly, the Arx1p protein

might also function as an 'anti-association' factor. Hung and Johnson [194] propose that Arx1p, because of its relatedness to MetAPs that act on nascent peptides as they exit from the so-called 'polypeptide exit tunnel', binds in the nucleus to pre-60S subunits in the vicinity of this tunnel. In so doing, it might prevent the nuclear association with pre-60S particles of SRP and of the 'nascent chain-associated complex' that bind 60S subunits near the tunnel during translation. Preventing such premature association, that would substantially increase the size of the particles, might be necessary for passage of the pre-60S subunits through the nuclear pores.

**Nuclear receptors required for the export of the pre-40S subunits**

When yeast cells expressing an LMB-sensitive version of Crm1p/Xpo1p are treated with LMB, a rapid nuclear retention of pre-40S pre-ribosomal particles is observed [177]. In *X. laevi* oocytes and HeLa cells, alteration of CRM1 function induces a rapid defect in nuclear export of precursors of the small ribosomal subunit [178, 188, 195], indicating that the function of CRM1 in pre-40S particle export is conserved in eukaryotes. An NES recognized by Crm1p/Xpo1p on the pre-40S particles seems to be provided by the non-essential protein Ltv1p (Fig. 5B). Yeast Ltv1p is a component of late pre-40S particles [158, 169] and is required for optimal production of the mature small ribosomal subunits [163]. More specifically, depletion of Ltv1p results in the accumulation of an aberrant 21S pre-rRNA precursor and induces a partial defect in the nuclear export of the pre-40S particles [167]. Although one cannot formally exclude that the latter phenotype is a consequence of the former, the following results favor a direct function of Ltv1p in pre-40S particle export. In yeast cells, Ltv1p shuttles between the nucleus and the cytoplasm and the protein strongly accumulates in the nucleus upon LMB treatment [167], suggesting that the nuclear export of Ltv1p requires Crm1p/Xpo1p. In agreement with this observation, Ltv1p interacts with Crm1p/Xpo1p in two-hybrid assays [196] and contains a leucine-rich NES similar to that located at the C terminus of Nmd3p. Fusing this NES to a reporter nuclear protein is sufficient to delocalize the reporter to the cytoplasm, suggesting that this NES mediates the export of Ltv1p by a Crm1p-dependent mechanism. Nevertheless, a direct binding of Ltv1p to Crm1p in the context of late pre-40S particles has not been reported so far. Interestingly, Ltv1p interacts with the ribosomal protein Rps3p [169, 196]. Ltv1p may therefore be loaded onto the pre-40S particle through a direct interaction with the ribosomal protein Rps3p. Together, these results suggest a model in which Ltv1p, a component of late 40S pre-ribosomal particles, provides a NES that mediates a Crm1p/Xpo1p-dependent export of the particles. However, Ltv1p is not essential for cell viability, and deletion of the encoding gene affects only partially the export of pre-40S subunits. These observations suggest that other components of the pre-40S particles may provide an NES recognized by Crm1p/Xpo1p and/or that other export receptors, such as the Rrp12p protein, may facilitate nuclear exit of pre-40S particles. Indeed, Rrp12p is physically associated with pre-40S particles and depletion of the protein induces a nuclear retention of the 20S pre-rRNA [161]. On this basis, Rrp12p has been proposed to facilitate the export of precursors of the small ribosomal subunit by a mechanism similar to that described for pre-60S particles (Fig. 5).

Yrb2p is another non-essential protein that has been reported to function in the export of pre-40S particles [197, 198]. Deletion of *YRB2* induces a decrease in the production of the 40S ribosomal subunits correlated with a nuclear retention of the pre-40S pre-ribosomal particles [177], which suggested a function of Yrb2p in the export of pre-40S particles (Fig. 5B). Deletion of *YRB2* induces synthetic lethal growth phenotypes when combined with a mutation in *CRM1*/*XPO1* [199] and with mutations in genes encoding components of the small GTPase Ran cycle [197, 198]. In addition, Yrb2p physically interacts with Crm1p/Xpo1p *in vivo* [199]. These data support a role of Yrb2p in the Crm1p/Xpo1p-dependent export of the pre-40S particles. However, Yrb2p does not seem to shuttle across the nuclear membrane [199], suggesting that Yrb2p facilitates the interaction between the NES-containing pre-40S particle, Crm1p/Xpo1p and/or the GTPase Ran but does not escort the export-competent particles through the NPCs. In agreement with this model, deletion of *YRB2* induces a synthetic enhanced growth defect when combined with deletion of *LTV1* [167] but deletion of both genes is not lethal, strongly suggesting that the export of the pre-40S particles is not completely inhibited in the absence of Yrb2p and Ltv1p. These two factors likely form part of a more complex pre-40S export apparatus that needs further investigation to be fully understood. The ribosomal protein Rps15p may be an additional component of this apparatus both in yeast and mammalian cells. Indeed, depletion of Rps15 (by transcriptional repression in yeast or RNA interference in HeLa cells) induces a strong accumulation of late precursors to the mature 18S rRNA but no major defect in the processing of earlier intermediates [195, 200]. These late 18S precursors are retained in the nucleoplasm in the absence of Rps15 as assessed by fluorescent *in*

*situ* hybridization, and in yeast, the nuclear restricted pre-40S particles contain most known non-ribosomal factors. Incorporation of Rps15p within the pre-40S particles may therefore be required for the acquisition of export competence.

## Cytoplasmic steps of ribosome biogenesis

### Cytoplasmic steps of large ribosomal subunit synthesis

Nascent pre-60S particles entering the cytoplasm are not immediately incorporated into polysomes [201]. This lag reflects the occurrence of final cytoplasmic maturation steps necessary for the acquisition of translational competence. During these final stages, the few non-ribosomal components (including Alb1p, Arx1p, Nmd3p, Nog1p, Rlp24p, Tif6p) that bind to the pre-60S particles in the nucleus and are exported to the cytoplasm while bound to the particles, will be removed sequentially in a carefully controlled fashion. This sequential dissociation process requires the interplay of several strictly cytoplasmic factors, including ATPases and GTPases that presumably perform proofreading functions and induce conformational rearrangements upon nucleotide hydrolysis. The removal process is also coupled with incorporation of the last ribosomal proteins, including Rpl24p. Inactivation of the cytoplasmic factors required for these removal steps has two major consequences: (i) nuclear pre-rRNA processing steps and pre-60S subunit export are inhibited due to failure to recycle shuttling pre-60S components back into the nucleus and (ii) stalled nearly mature 60S subunits are unable to associate with 40S subunits to engage in translation.

### Release of the export adaptor Nmd3p

Dissociation of Nmd3p from cytoplasmic pre-60S particles and its recycling back to the nucleus is crucial for subsequent rounds of nucleo-cytoplasmic transport of nascent pre-60S subunits. This removal requires the ribosomal Rpl10p protein, its binding partner Sqt1p and the cytoplasmic putative GTPase Lsg1p. Arlen Johnson and colleagues propose that the Sqt1p-assisted integration of Rpl10p into pre-60S subunits in the cytoplasm is a prerequisite for the Lsg1p-driven removal of Nmd3p [202, 203]. Whether Rpl10p associates with the 60S subunits in the cytoplasm remains a controversial issue, however. Indeed, Rpl10p may have a nuclear phase since it contains a functional NLS [183], some altered Rpl10p proteins accumulate in the nucleus [204] and even wild-type Rpl10p concentrates in the nucleus in exosome mutant *rrp44/dis3–1* cells [183].

### Removal of the export receptor Arx1p and the putative anti-association factor Tif6p

In addition to Lsg1p and Sqt1p, several non-ribosomal cytoplasmic factors required for the cytoplasmic maturation of pre-60S particles have been identified, including Drg1p, Efl1p, Jjj1p, and Rei1p. The cytoplasmic AAA-ATPase Drg1p probably intervenes at an early stage of this process [205, 206]. Indeed, Drg1p dissociates from pre-60S particles before Lsg1p and Rei1p associate with them and is needed for Rei1p binding to the particles [205]. Inactivation of Drg1p inhibits the release of Arx1p, Nog1p, Rlp24p and Tif6p from pre-60S particles. In the case of Arx1p and Tif6p at least, their retention on pre-60S particles is unlikely to be the direct result of Drg1p inactivation because in wild-type conditions they remain bound to the particles after Drg1p dissociation [205]. Lack of Rei1p in turn inhibits the recycling of Alb1p, Arx1p and Tif6p, but not Rlp24p, back into the nucleus [194, 207]. In addition, lack of Jjj1p also inhibits the nuclear recycling of Alb1p and Arx1p, but not of Tif6p, due to stalling of Alb1p and Arx1p on pre-60S particles [208, 209]. Jjji1p could act together with the Ssa1p chaperone, since Jjj1p is able to stimulate the ATPase activity of Ssa1p *in vitro* [209]. In contrast, according to Lebreton and colleagues, lack of Rei1p does not prevent the release of Alb1p and Arx1p as a complex from pre-60S particles but inhibits their nuclear recycling as such [207]. However, the team of A. Johnson detects a retention of Arx1p on pre-60S particles when Rei1p is absent [194]. The reason for this discrepancy is unknown. Lack of Rei1p is also associated with the retention of Tif6p on pre-60S particles [207]. Strikingly, in strains lacking Rei1p and either Alb1p or Arx1p, Tif6p is not stalled on pre-60S particles and is recycled normally into the nucleus. Thus, Lebreton and colleagues propose that it is the accumulation of the Alb1p-Arx1p complex in the cytoplasm due to the absence of Rei1p that inhibits Tif6p release from pre-60S particles [207]. Release of Tif6p also depends upon the activity of Sdo1p [210] and of the Efl1p GTPase [211]. Efl1p is likely to directly target Tif6p, since in *in vitro* assays, recombinant Efl1p can remove recombinant Tif6p bound to purified 60S subunits in the presence of GTP [211]. The available data thus suggest that Tif6p removal requires the proper execution of several prior maturation steps in the cytoplasm, probably to ensure that only carefully monitored 60S subunits fully competent for translation are capable of shedding Tif6p (Fig. 6). Indeed, premature removal of Tif6p might cause incorporation of immature 60S subunits into polysomes, as Tif6p binding to 60S subunits prevents their association with 40S subunits [211–213].

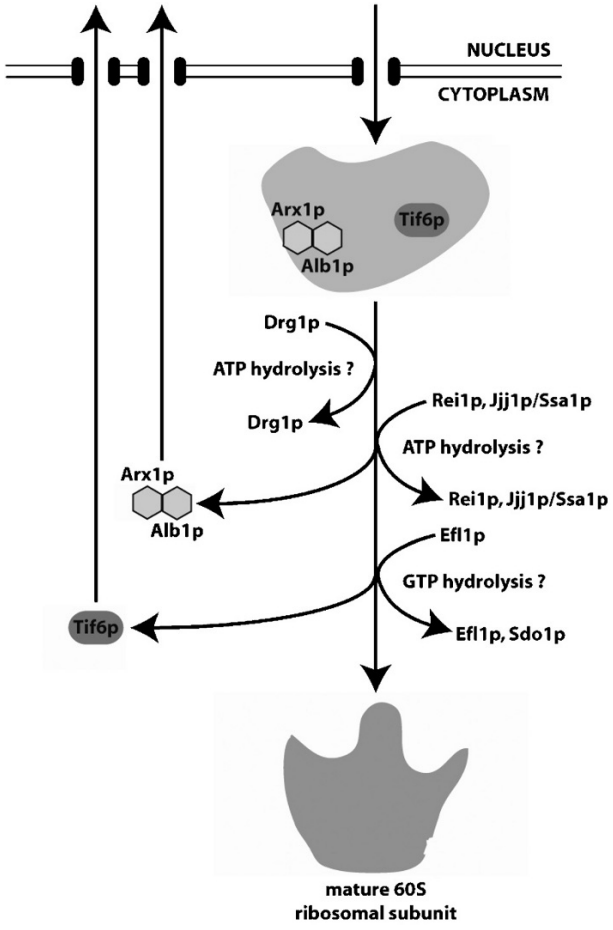


**Figure 6.** Cytoplasmic events required for the release of the anti-association factor Tif6p from yeast pre-60S pre-ribosomal particles.

## Cytoplasmic steps of small subunit synthesis

**Cytoplasmic dimethylation of 18S rRNA.** In *S. cerevisiae*, the exported 20S pre-rRNA undergoes dimethylation of two adjacent adenine bases (*S. cerevisiae* A1779 and A1780) at the very 3′ end of 18S rRNA [71]. These base modifications are conserved from bacteria to eukaryotes and are catalysed by the Dim1p dimethylase in yeast [214]. Interestingly, although the dimethylation is proposed to occur in the cytoplasm, Dim1p associates in the nucleolus with the 90S particle and is required for the early pre-rRNA cleavages at sites A1 and A2 [215]. Altered Dim1p proteins inactive in 18S rRNA dimethylation are still able to support normal pre-rRNA processing and growth [215, 216]. Thus, it is probably the involvement of Dim1p in ribosome assembly that renders this factor essential. The 18S rRNA dimethylations are believed to be required for optimal 40S ribosomal subunit function since extracts from strains lacking these modifications are inactive in *in vitro* translation assays [216]. The K homology (KH)-domain-containing protein Dim2p/Pno1p is also required for 20S pre-rRNA dimethylation [162]. Depletion of this factor leads to the same pre-rRNA processing defect as that observed in Dim1p-depleted cells [162]. Since Dim2p and Dim1p can be co-precipitated in cell extracts and since the KH domain is a well-characterized RNA-binding domain, Dim2p has been proposed to recruit Dim1p to 90S particles via a direct interaction with the 35S pre-rRNA.

Since adenine dimethylation of 20S pre-rRNA is a cytoplasmic event, association with dimethylated 20S pre-rRNA can be tested to assess the presence of given pre-ribosomal proteins within cytoplasmic pre-40S particles. Using this assay, it has been established that Dim1p, Dim2p, Enp1p, Hrr25p, Nob1p, Rio2p, Rrp12p and Tsr1p are associated with cytoplasmic pre-40S particles [158, 160–162].

## Cytoplasmic production of the 3′end of mature 18S rRNA

In yeast, the last pre-rRNA processing step leading to mature 18S rRNA is the cytoplasmic endonucleolytic cleavage of the 20S pre-rRNA at site D, at the junction between the 3′ end of 18S rRNA and ITS1. It has long been held that in mammalian cells in contrast, all processing steps generating mature 18S rRNA occur within the nucleus. However, Rouquette and collaborators have shown that in human and murine cells, similar to the situation in yeast, the so-called 18S-E precursor to 18S rRNA, containing a 20 to 30-nucleotide-long ITS1 extension at the 3′ end, is converted to mature 18S rRNA in the cytoplasm [195].

The endonuclease directly responsible for 20S pre-rRNA cleavage at site D has not been identified with certainty, although Nob1p has been proposed as the best candidate so far. Nob1p, which is required for the conversion of 20S pre-rRNA to 18S rRNA [160], contains a PIN domain, which is related to $Mg^{2+}$-dependent 5′ to 3′ exonucleases [217, 218]. Moreover, integrity of conserved amino acids in the PIN domain of Nob1p, predicted to be crucial for nuclease activity, is essential for 20S pre-rRNA processing [217, 218]. However, no *in vitro* assay is yet available to demonstrate the direct involvement of Nob1p in 20S pre-rRNA cleavage at site D.

Fap7p also plays a crucial role in D site cleavage and it too has been suggested to be the elusive endonuclease responsible for this cleavage [219]. Fap7p depletion specifically inhibits cleavage of 20S pre-rRNA, leading to its massive accumulation in the cytoplasm. Fap7p only transiently interacts with pre-40S particles and its depletion does not inhibit their assembly or transport. Contrary to Nob1p, Fap7p fails to display any obvious homology with known nucleases; rather,

it belongs to a family of P-loop kinases related to ribonucleoside kinases [219]. Although Fap7p fails to display any enzyme activity *in vitro*, alteration of its Walker A and B motifs inhibits growth and 20S pre-rRNA processing. Thus, it is probable that D site cleavage requires NTP hydrolysis by Fap7p.

Two other key players in 20S pre-rRNA processing, Rio1p and Rio2p, belong to a conserved family of atypical protein kinases, the RIO proteins, and show an *in vitro* protein kinase activity [220–226]. Depletion of each of these proteins in yeast leads to cytoplasmic accumulation of 20S pre-rRNA [164, 165]. Despite some sequence and structural homology [226], Rio1p and Rio2p are not functionally redundant [164]. Rio2p stably associates with the pre-40S particle whereas Rio1p is not detected in TAP studies, presumably because it interacts more transiently with the pre-40S particles [158, 227]. Interestingly, a variant of Rio2p displaying reduced kinase activity *in vitro* does not fully support 20S pre-rRNA processing *in vivo* and the kinase activity of Rio1p is essential *in vivo* [221, 222]. Thus it is likely that phosphorylation events catalysed by the RIO kinases are necessary for site D cleavage, but the *in vivo* substrates of the RIO kinases remain unknown. At least some aspects of the final pre-rRNA maturation step leading to 18S synthesis are conserved from yeast to humans, since depletion of RIOK2, the human counterpart of yeast Rio2p, results in 18S-E accumulation in the cytoplasm. [195]. The involvement of RIOK1, the human Rio1p homologue, in small-subunit maturation has not yet been tested. Interestingly, a RIO3 protein sub-family, more related to the RIO1 than RIO2 proteins, appears in multicellular organisms but no data are available about its involvement in small ribosomal subunit biogenesis [226].

Several small subunit ribosomal proteins, including Rps0p, Rps2p, Rps3p, Rps10p, Rps20p, Rps21p, Rps26p and Rps28p are also implicated in the cytoplasmic maturation of the 20S pre-rRNA [39, 228, 229]. In addition, alterations to the C-terminal extension of Rps14p result in a massive accumulation of 20S pre-rRNA in the cytoplasm [230]. Interestingly, depletion of Rps14p leads to early pre-rRNA processing defects and loss of 20S pre-rRNA accumulation [39]; thus, Rps14p is required for both early and late stages of pre-rRNA processing. The C-terminal domain of Rps14p, as well as Rps0p and Rps2p seem to lie in the vicinity of the 3′-end of 18S rRNA [231]. As no known nuclease domain is present within these ribosomal proteins, they are probably implicated in securing the proper 20S pre-rRNA conformation for D site cleavage and/or in the recruitment of necessary transacting factors. Indeed, the *in vitro* detection of a direct interaction between Rps14p and Fap7p suggests that the former recruits the latter to pre-40S particles [219].

**Maturation events leading to the stable incorporation of ribosomal proteins within the mature small ribosomal subunit.** Rps3p can be released from purified pre-40S particles by high-salt washes, whereas it remains bound to mature 40S ribosomal subunits under the same conditions [169]. This suggests that Rps3p is not integrated in the same fashion in the two types of particle. Strikingly, electron microscopy images of purified pre-40S and mature 40S particles show that the former lack a distinct 'beak' structure, conspicuous in the latter. This structure is formed by a protrusion of helix 33 of 18S rRNA, at the basis of which Rps3p is placed. Lack of this beak structure in nuclear pre-40S particles might facilitate their nuclear export. Schäfer and colleagues propose that, following translocation through the nuclear pore, pre-40S particles acquire the beak structure, an event linked with the final stable positioning of Rps3p within the 40S subunit [169]. Schäfer and colleagues further suggest that this conformational rearrangement requires the dephosphorylation, by an unknown phosphatase, of Rps3p, previously phosphorylated in the nucleus by Hrr25p (Fig. 7). Data supporting this model can be summarized as follows [169]. Purified pre-40S particles can acquire the beak structure *in vitro* if they are first incubated with ATP, leading to Rps3p phosphorylation, followed by treatment with a phosphatase. In addition, these successive phosphorylation/dephosphorylation steps lead to the salt-resistant association of Rps3p with the particles. Rps3p is probably phosphorylated by Hrr25p, since Rps3p associated with pre-40S particles purified from Hrr25p-depleted cells fails to be phosphorylated under the conditions just described. Finally, Rps3p does not achieve stable integration within 40S subunits in Hrr25p-depleted cells. Although these experimental data support the aforementioned model, no data are currently available that actually demonstrate that phosphorylation of Enp1p/Ltv1p/Rps3p occurs in the nucleus and that the dephosphorylation step occurs in the cytoplasm *in vivo*.

Several other small-subunit ribosomal proteins may likewise undergo late restructuring events leading to their stable integration within mature 40S subunits. Indeed, Ferreira-Cerca and collaborators have noticed that several small subunit ribosomal proteins interact in a more stable fashion with 18S rRNA than with 20S pre-rRNA [38].

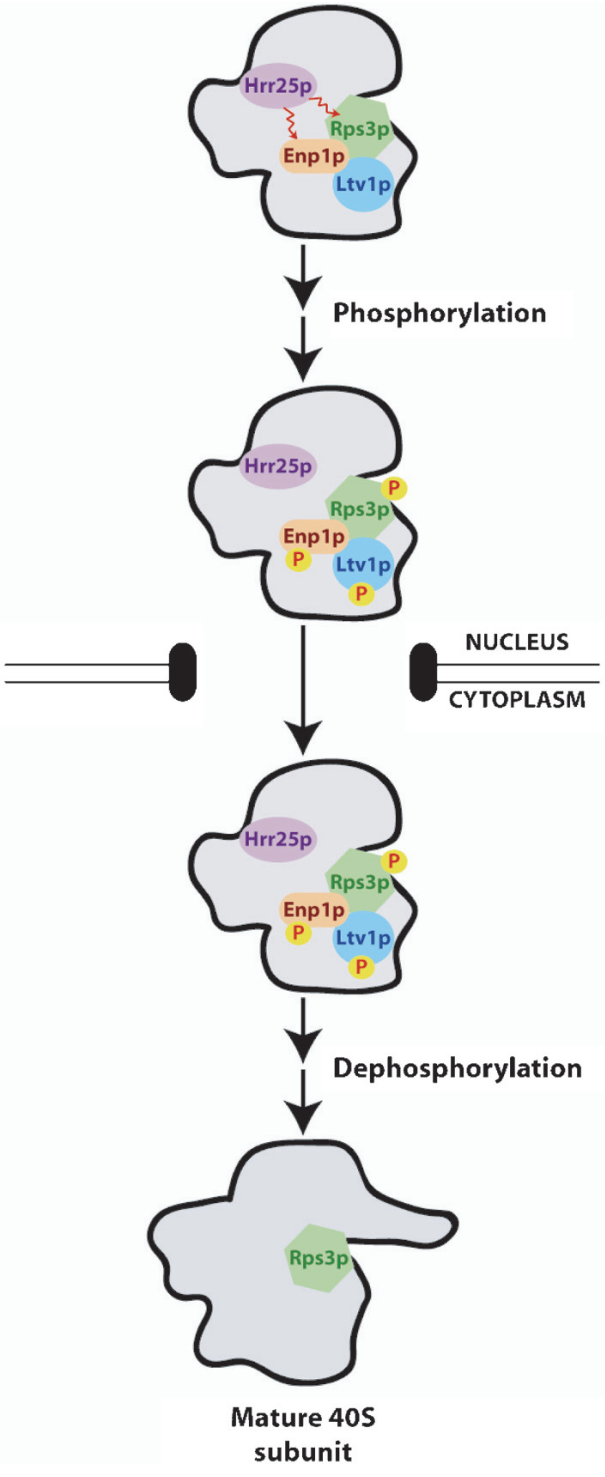


**Figure 7.** Model of the phosphorylation/dephosphorylation events that regulate stable integration of Rps3p into mature yeast 40S subunits. The lightning arrow indicates that Hrrp25p probably directly phosphorylates Rps3p and Enp1p within pre-40S pre-ribosomal particles. The identity of the phosphatase responsible for the dephosphorylation of Rps3p is unknown.

## Quality control of ribosome biogenesis

Mutations that impair a specific step of eukaryotic ribosome biogenesis do not lead to massive accumulation of the corresponding pre-rRNAs, with the exception of mutations that impair the cytoplasmic conversion of 20S pre-rRNA to mature 18S rRNA. It was thus hypothesized that quality control mechanism(s) function in the nucleus to detect and degrade faulty and/or stalled pre-ribosomal particles [232]. Convincing evidence for such nuclear quality control has recently been provided by Dez and collaborators [233]. They demonstrate that inactivation of the Sda1p protein, a component of late pre-60S pre-ribosomal particles, leads to a very quick nuclear retention of these particles and to the decreased accumulation of their RNA components, presumably reflecting their degradation. Indeed, normal accumulation of these RNA components (27S pre-rRNA and 25S rRNA) is restored in strains defective for both Sda1p and the exosome component Rrp6p, strongly suggesting that the exosome is involved in their turnover. Under these conditions, moreover, increased levels of polyadenylated 27S pre-rRNAs and 25S rRNAs are detected. Normal accumulation of 27S pre-rRNAs and 25S rRNAs is also restored in a strain defective for both Sda1p and Trf4p, the polyA polymerase of the Trf4p-Air2p-Mtr4p-polyadenylation (TRAMP) complex; in this case however, no polyadenylated 27S pre-rRNAs and 25S rRNAs are observed. The TRAMP complex has been shown to stimulate the degradation by the exosome of aberrant pre-tRNAs, pre-snRNAs, pre-snoRNAs and cryptic RNA Pol II transcripts [234–238]. One step required for such degradation is the polyadenylation of these substrates by the polyA polymerase component of TRAMP. Hence the most straightforward interpretation of the data of Dez and collaborators is that the RNA components of pre-60S pre-ribosomal particles retained in the nucleus are polyadenylated by the Trf4p component of TRAMP and degraded by the exosome. Soon after Sda1p inactivation, components of pre-60S pre-ribosomal particles, TRAMP, and the exosome accumulate in a sharp nucleolar focus. This accumulation is not seen when TRAMP or exosome components are absent. Therefore, it is likely that this nucleolar focus, termed the 'No-body', is the site where the RNA components of the stalled pre-ribosomal particles are degraded. The protein components of the particles may be degraded elsewhere or recycled, since the No-body does not seem to be enriched for proteasome components. One remaining key question is how TRAMP and the exosome distinguish between normal and defective pre-ribosomes, to only eliminate the latter.

Interestingly, mature cytoplasmic ribosomal RNAs seem also to be under surveillance. LaRivière and collaborators have recently shown that 18S rRNAs or 25S rRNAs bearing point mutations in the decoding center or in the peptidyltransferase center, respectively, are synthesized normally but display markedly decreased stability [239]. The components of this cytoplasmic surveillance mechanism remain unknown.

## Perspectives

In the past decade, we have seen an enormous increase in the number of known factors dedicated to the synthesis of eukaryotic ribosomes. We are now faced with the task of understanding how each of them fits in the jigsaw puzzle: when and how do they associate with pre-ribosomal particles and leave, and more importantly, what are their actual molecular functions. Experiments described in this review demonstrate that we have started to tackle these issues for a limited number of pre-ribosomal factors. Understanding their roles will require determination of their three-dimensional structure coupled with *in vivo* mutational studies and biochemical assays to assess the functional importance of seemingly key residues in the structure. It will also involve the determination of pre-ribosomal particle 'neighborhoods'. This has classically been done by genetic and biochemical approaches. However, landmark studies by the Hurt team show that it is now possible to analyse the structure of pre-ribosomal particles by electron microscopy and even to reconstitute a pre-ribosomal particle maturation step *in vitro* [157, 169]. Have most of the factors involved in ribosome biogenesis been identified? It is possible that components of pre-ribosomal particles with very low steady-state accumulation (such as 90S particles containing 33S pre-rRNA) or components displaying very transient or labile association with pre-ribosomal particles have been missed. The novel, one-step fast purification protocol [91] may prove ideal to identify such factors and, who knows, may even allow the identification of the elusive yeast endonuclease(s) cleaving sites A0, A1, A2 and C2.

Given the crucial importance of manufacturing efficient and reliable ribosomes, it comes as no surprise that cells have evolved quality control mechanisms to monitor ribosome biogenesis. Yet it is only very recently that the existence of such mechanisms has been proven experimentally. One key and as yet totally unexplored issue is how quality control mechanisms recognize faults in ribosomes. Equally little investigated is the probable link between late cytoplasmic steps of ribosome biogenesis and translation initiation. Translation itself is most probably fundamental to the quality control of cytoplasmic ribosomes, the mechanistic details of which are totally unknown.

Ribosomes are not all identical. For example, they may differ in the nature of their 5.8S [97] and 5S [240] rRNA components, and in *S. cerevisiae* at least, they may contain different combinations of ribosomal protein paralogues. Moreover, it is likely that ribosomal proteins and rRNAs display some diversity in their pattern of post-translational and post-transcriptional modifications, respectively. Some experimental evidence supports the hypothesis that different ribosomes translate different subsets of mRNAs [94, 241]. If so, to what extent is the manufacture of different ribosomes a controlled process or merely the result of stochastic events? Does the cell modify ribosome biogenesis pathways to adjust manufacture of different subtypes of ribosomes to meet varying translational needs?

*Acknowledgements.* We dedicate this review to the memory of Hélène Richard-Foy. We thank C. Dez, O. Gadal and P.-E. Gleizes for critical reading of the manuscript. J. Soudet is a recipient of a graduate fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche. M. Gérus and S. Lebaron are recipients of a graduate fellowship from the Ligue Nationale Contre le Cancer and the Association pour la Recherche sur le Cancer, respectively. Our research is supported by the CNRS, the Ligue Nationale Contre le Cancer (équipe labellisée), the Agence Nationale de la Recherche, the International Human Frontier Science Program Organization (to A. H.) and the Université Paul Sabatier.

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