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INTERMEDIATE MATURATION STAGES OF THE HUMAN 40S RIBOSOMAL SUBUNIT

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CERTIFICA:

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ABSTRACT

The formation of eukaryotic ribosomes is a multistep process that entails the sequential maturation of preribosome intermediates in the nucleolus, nucleoplasm and cytoplasm. The whole process is assisted by hundreds of ribosome biogenesis factors (RBFs) that, in the context of the preribosomes, mediate the numerous events required to process the rRNAs and assemble the ribosomal proteins. Most knowledge on ribosome formation comes from studies in yeast, and it has long been assumed that most features are similar in humans. However, although the basic aspects are conserved, there are rRNA processing steps and links to other cellular processes that are unique of higher eukaryotes. The mechanisms behind those idiosyncrasies are poorly understood because, unlike in yeast, there are no efficient methods to characterize preribosomal complexes in human cells. This thesis focused on the study of intermediate steps of maturation of the human 40S ribosomal subunit. The work presented here includes the optimization of a method to fractionate different pools of preribosomes, and the generation of genetically-modified cell lines to purify specific pre-40S maturation intermediates. Using these tools, combined with mass-spectrometry compositional analyses and fluorescence microscopy experiments, it was possible to dissect several steps of the 40S synthesis pathway and reveal the points of action of individual RBFs. The findings of this study unveil a previously-unknown process of preribosome compartmentalization inside the nucleolus and the presence of distinctive biochemical properties in nucleoplasmic intermediates. Most notably, it was identified a specific pre-40S maturation event, not present in yeast, that might have been acquired by human cells to regulate the amount and quality of 40S subunits that are delivered to the cytoplasm.

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INTRODUCTION

1. The ribosome

1. 1. General features

Ribosomes are the molecular machines in charge of protein synthesis in all living cells. They are RNA-based enzymes that convert the genetic information from messenger RNAs (mRNAs) into proteins. This process is called translation (see scheme in **Figure 1**). Although ribosomes are universally conserved, the number and size of their components is different between the distinct domains of life (**Table 1**). Eukaryotic 80S ribosomes are constituted by two subunits of uneven size, comprising a total of four different rRNAs and around 80 ribosomal proteins. The small subunit (commonly referred to as 40S subunit or SSU) contains one molecule of rRNA, the 18S, and 33 ribosomal proteins (RPS or S). The large subunit (60S or LSU) consists of three molecules of rRNA, the 25S/28S, 5.8S and 5S and, in most eukaryotic species, 47 ribosomal proteins (RPL or L) [1–4].

Table 1. Sedimentation coefficients and composition of bacterial and eukaryotic ribosomal subunits

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

The molecular weight of ribosomes in higher eukaryotes is approximately double compared to that of their bacterial counterparts (4.3 MDa and 2.3 MDa, respectively), although their rRNA/RP ratios are similar. This is thought to reflect the acquisition of more sophisticated functions or

regulatory mechanisms in higher eukaryotes. Eukaryotic cells also have mitochondrial ribosomes, which are smaller in size (2.7 MDa) and very specialized. The mitochondrial ribosomes translate about 13 different mRNAs while the cytoplasmic ones handle more than 100,000 templates[5].

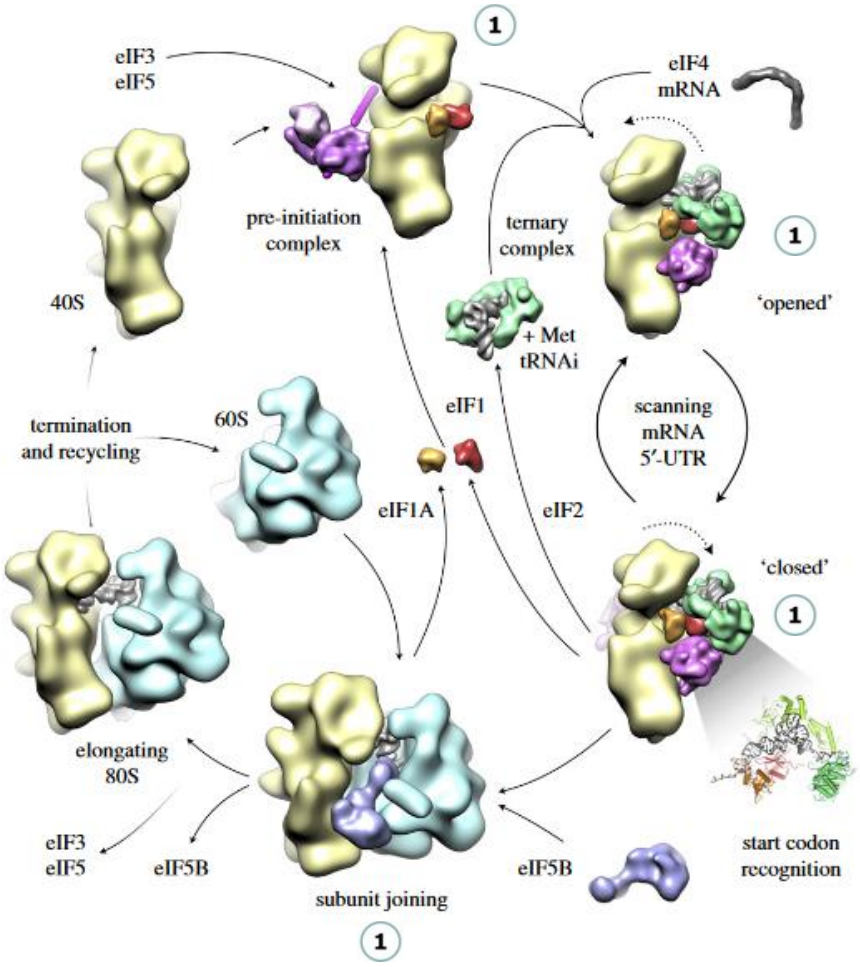


Figure 1. Translation cycle in eukaryotes showing different events of the initiation phase. The process of translation consists of four phases: initiation, elongation of the polypeptide chain, termination and recycling of ribosomes. The different steps are assisted by different proteins, which include initiation factors (eIFs), elongation factors (eEFs), release factors (eRFs) and recycling factors. In the initiation phase (steps marked with **1**), the Met-tRNA^{i} is bound to the small (40S) ribosomal subunit in the context of the initiation factor eIF2 to scan the 5' end of the mRNA until it reaches the AUG. Then, the large (60S) subunit is incorporated. The initial Met-tRNA^{i} and the AUG translocate to the P site (not shown) and the elongation phase begins. In each cycle of elongation, one aminoacyl-tRNA is positioned in site A of the ribosome, the peptide bond is formed and the ribosome translocates to the next mRNA codon. When the stop codon is reached, the ribosome releases the polypeptide in the termination phase. Figure taken from Aylett and Ban, 2017[6].

The main functions of the ribosome are the decoding of the mRNA and the formation of peptide bonds. Each subunit is in charge of each one of these two functions (**Figure 2A**). While the 40S subunit performs the decoding process, during which the correct aminoacyl-tRNA is selected according to the mRNA sequence, the 60S subunit is responsible of forming the peptide bonds between amino acids.

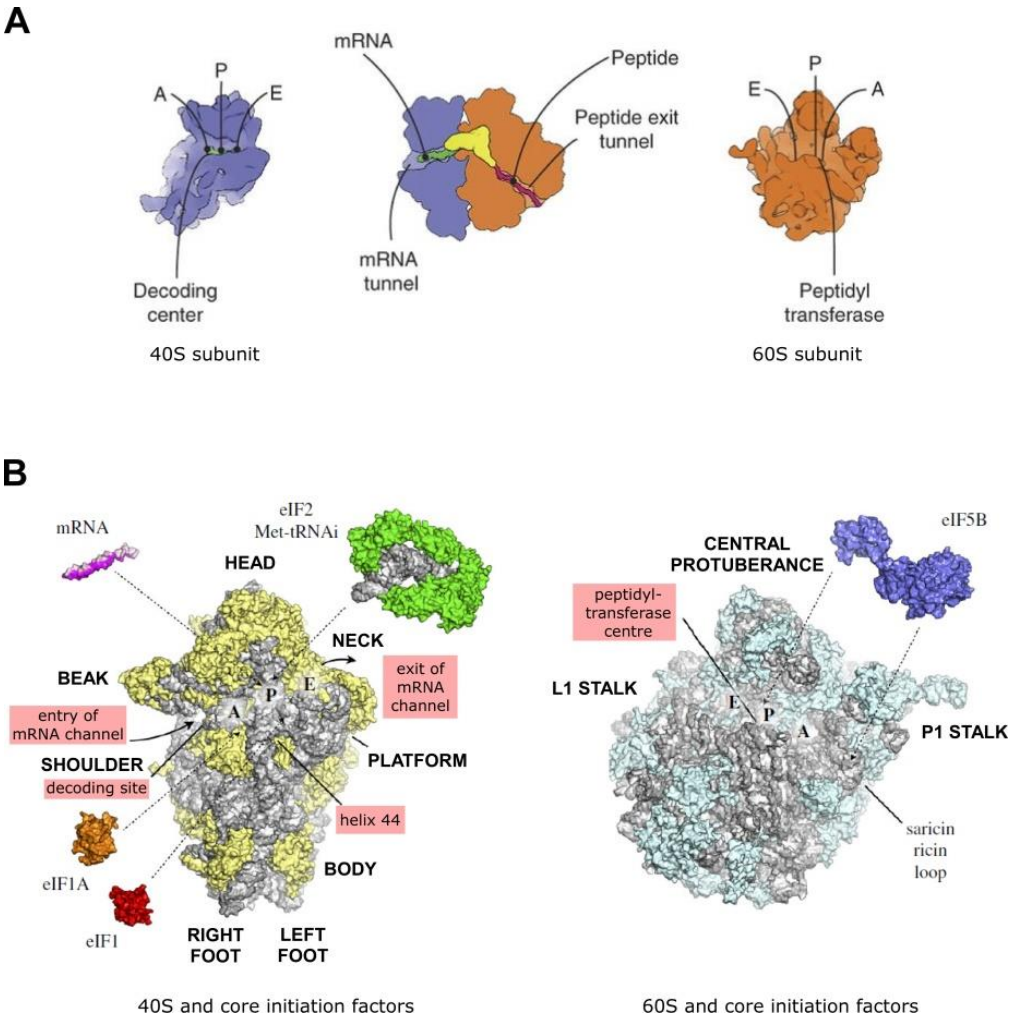


Figure 2. Main functional sites of the eukaryotic ribosomal subunits. (A) Scheme of the small (blue) and large (orange) subunits showing their contact interfaces and the locations of the decoding center and peptidyl transferase center. **(B)** Structural landmarks and functional sites of the 40S (left) and 60S (right) subunits of *S. cerevisiae*. The rRNA is represented in gray and the ribosomal proteins are shown in yellow (small subunit) and light blue (large subunit). The figure shows the interface sides of the two subunits with the main structural regions. Note that the main functional sites of the ribosome are regions of rRNA. The ribosomal proteins are scattered in the periphery. Figure A taken from Melnikov et al., 2012[4]. Figure B adapted from Aylett and Ban, 2017[6].

1. 2. Structural organization and functions of the ribosomal subunits

There are several functional regions on the subunits that have distinct roles (**Figures 2A** and **2B**). In the case of the small subunit these regions are: (1) the mRNA path, which guides the mRNA during translation; (2) the decoding center, where the pairing of the codon and anticodon takes place, and (3) the tRNA binding sites (A, P, E sites). The A (aminoacyl) site is the place occupied by the entering aminoacyl-tRNA, the P (peptidyl) site is the place that holds the tRNA carrying the nascent polypeptide chain (peptidyl-tRNA), and the E (exit) site is where dissociation of the tRNA from the ribosome takes place (**Figure 2B**, left). During translation, the tRNAs translocate from the A to the P site and from the P to the E site, and finally they exit the ribosome. The main functional domains of the large subunit are: (1) the tRNA binding sites (A, P and E); (2) the peptide exit tunnel, which extends over the body of the subunit, and (3) the peptidyl transferase center (PTC) (**Figure 2B**, right). The PTC is in charge of peptide bond formation and is situated at the beginning of the peptide exit tunnel, in a conserved region on the interface between the two subunits that is mainly constituted by rRNA.

The folding of the rRNAs into tertiary structures and their association with ribosomal proteins generate several characteristic morphological features in each subunit (**Figure 2B**). The main ones in the 40S subunit are the Head, the Neck, the Platform, the Body, the Left foot, the Right foot, the Shoulder and the Beak. Another distinctive structural region of the 40S subunit is helix 44 of the 18S rRNA, which houses at its base the decoding center. The main tRNA binding sites (A, P and E) are located in the interface (**Figure 2B**). The entry tunnel for the mRNA is located between the head and the shoulder. The exit channel, from where the 5' end of the mRNA egresses, is located between the head and the platform. The decoding center is positioned on the interface surface and includes three domains from the head, shoulder and the helix 44 of 18S rRNA. The main features of the large subunit are the Central Protuberance, the L1 Stalk and the P stalk (**Figure 2B**). The tRNA binding sites (A, P and E) are located on the interface side, along with the PTC. This later one is adjacent to the entrance to the exit tunnel, from which the nascent polypeptide chain emerges [7–9].

2. The formation of ribosomes

The building of a ribosome is a highly complex process that requires the synthesis and assembly of four rRNAs with 80 ribosomal proteins. It is also important to mention that during ribosome formation the rRNAs acquire covalent modifications on specific, highly conserved, residues. Such modifications include ribose methylations, base methylations and pseudouridylations, which play roles in the structural organization of the ribosome and in translation[10–12].

2. 1. General overview

The process of ribosome synthesis starts in the nucleolus with the transcription of a long pre-rRNA precursor (35S in yeast, 47S in human cells), which contains the sequences of the 18S, 5.8S and 25S/28S rRNAs, flanked by two external transcribed spacers (5' ETS and 3' ETS) and separated by two internal transcribed spacers (ITS1, between 18S and 5.8S; and ITS2, between the 5.8S and the 25S/28S) (**Figure 3**). These transcribed spacers must be eliminated through a complex series of cleavages to give rise to the mature rRNAs. Concomitantly, the 5S rRNA and the ribosomal proteins must be incorporated (**Figure 3**).

The processing of the pre-rRNAs and assembly of the ribosomal proteins requires the action of many assisting factors, known as ribosome synthesis factors, trans-acting factors or ribosome biogenesis factors (RBFs). In humans, there are more than 350 RBFs[13–15]. The successive steps of pre-rRNA processing and assembly of the ribosomal proteins occur within a series of large ribonucleoprotein (RNP) complexes, known as preribosomes or preribosomal particles. The initial one is the 90S preribosome, a gigantic RNP in which the initial folding and cleavages of the pre-rRNA take place (**Figure 4**). Upon cleavage of the pre-rRNA at site 2 on ITS1, two pre-rRNA species, one for the 18S and the other for the 5.8S – 28S are produced, and two particles (pre-40S and pre-60S) are generated. These two particles follow independent maturation routes[16], during which different pre-40S and pre-60S intermediates are produced in the nucleolus, nucleoplasm and cytoplasm. Within these preribosomes, pre-rRNA folding and processing are tightly coupled to the sequential assembly of ribosomal proteins[17]. Most ribosomal proteins are recruited in the first steps, but some continue to incorporate as maturation

proceeds. The major part of the assembly process occurs in the nucleolus but, in the case of the 60S subunit, there are also a good number of maturation events that take place in the nucleoplasm. Export-competent intermediates are actively transported from the nucleus across nuclear pore complexes to the cytoplasm, where they undergo some final maturation events to form the mature and translation-competent ribosomal subunits[18–23].

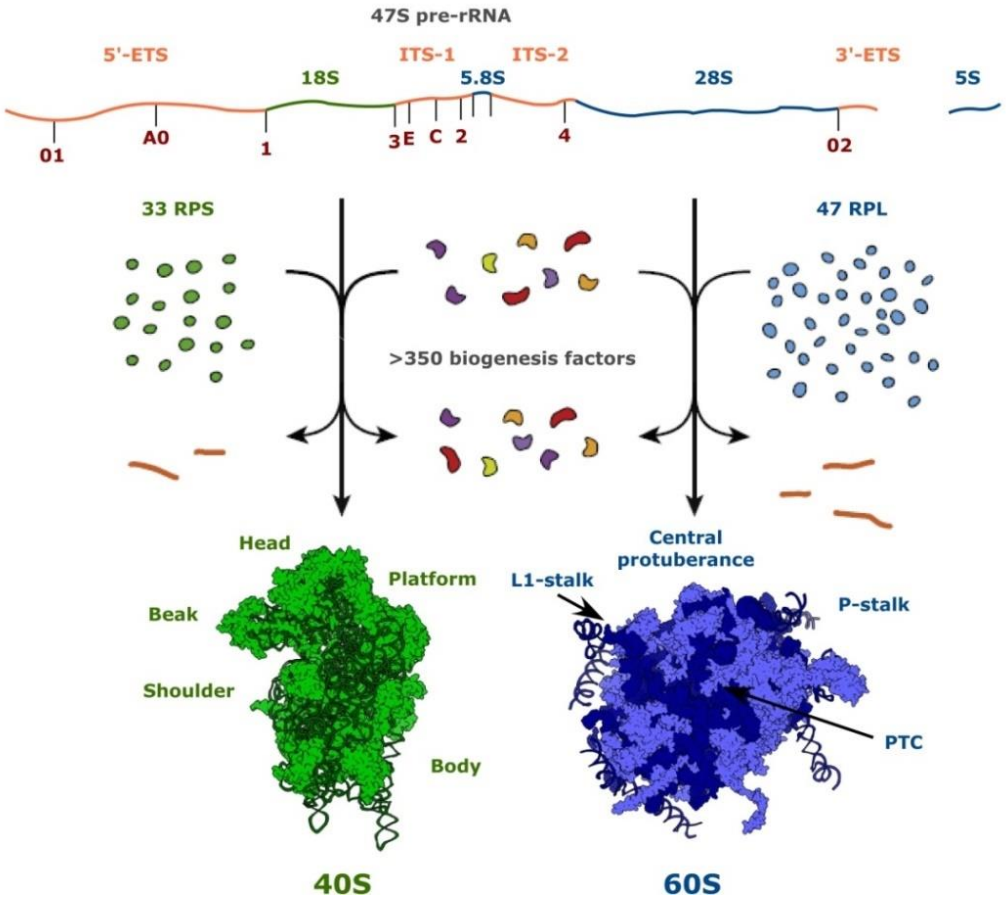


Figure 3. Simplified view of the components required for ribosome synthesis in human cells. Ribosome biogenesis encompasses the assembly of rRNAs and ribosomal proteins (RPS and RPL). This process is coordinated and catalyzed by more than 350 factors. The ribosomal proteins of the small subunit are shown in light green and the ones of the large subunit in light blue. RNA polymerase I synthesizes the 47S pre-rRNA precursor and RNA polymerase III produces the 5S rRNA (dark blue). Throughout the whole process, the external transcribed spacers (5'ETS and 3'ETS) and the internal transcribed spacers (ITS1 and ITS2) (orange) of the 47S pre-rRNA are eliminated to render the mature rRNAs: 18S (dark green), 5.8S, and 28S (dark blue). This maturation of the rRNA includes a series of endo- and exonucleolytic processing events at precise sites (indicated by red letters). The main structural features of the mature ribosomal subunits are indicated. Figure adapted from Kressler et al., 2017[24]. Surface representations of the mature 40S and 60S subunits from *H. sapiens*, showing the interface sides, were generated with ChimeraX software from PBD 4V6X[25].

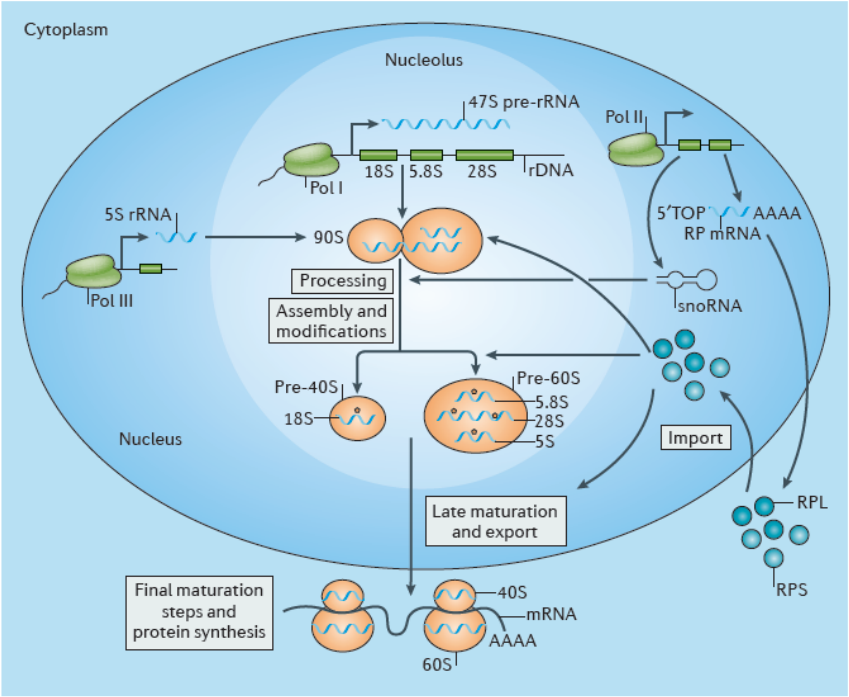


Figure 4. Overview of the ribosome biogenesis pathway in human cells. All three RNA polymerases are involved in the synthesis of ribosomes. The RNA polymerase I transcribes the 47S pre-rRNA from the rDNA repeats in the nucleolus. This precursor contains the sequences of the 18S, 5.8S and 28S rRNAs. The 5S rRNA is independently transcribed by RNA polymerase III. RNA polymerase II is required to transcribe the mRNAs of the RPSs, RPLs and RBFs (not indicated in the figure), which are translated in the cytoplasm and actively imported into the nucleus to be assembled into the maturing preribosomes. RNA polymerase II also transcribes snoRNAs that have roles in processing and modification of the rRNAs. For simplicity, the intermediate pre-40S and pre-60S preribosomes at different stages of maturation are not shown. See main text for further information. Figure taken from Pelletier et al., 2018[26].

In summary, after the transcription of the initial pre-rRNA by RNA polymerase I, the key processes in ribosome synthesis are: (1) the covalent modification of the rRNA regions of the pre-rRNA; (2) the processing of the pre-rRNA to give the mature rRNAs; and (3) the assembly of the rRNAs with the ribosomal proteins. In eukaryotes, additional steps include the import of the ribosomal proteins from the cytoplasm to the nucleus and the transport of preribosomes from the nucleolus to the nucleoplasm and then to the cytoplasm (**Figure 4**). All these processes take place thanks to the assistance of the RBFs, that transiently associate with preribosomes at specific maturation stages.

Given its complexity and dimension, the ribosome biogenesis route consumes a large percentage of the cellular energy and, therefore, occupies a central position in growth control[27,28]. Indeed, to adapt ribosome levels to cellular needs, ribosome production is subject to tight regulation in response to various cellular stimuli such as nutrient availability or activation by growth factors[29–31].

2. 2. Main steps of the ribosome synthesis pathway in yeast

Most current knowledge on ribosome biogenesis in eukaryotes has been obtained from studies in the budding yeast *Saccharomyces cerevisiae.* The main reasons for the use of this model have been that: (1) it is one of the simplest eukaryotic organisms, (2) it is amenable to genetic manipulation, and (3) it has been successfully used in high-throughput preribosome purification schemes[32,33].

The synthesis of ribosomes in yeast starts with the transcription by RNA polymerase I of the 35S pre-rRNA precursor. Early visualizations of actively transcribed chromatin on Miller spreads by electron microscopy revealed the presence of large terminal knobs associated with the 5'-ends of the nascent pre-rRNA transcripts[34]. These terminal knobs are the 90S preribosomes, which assemble co-transcriptionally onto the pre-rRNA[35,36] (**Figure 5**, steps **B-C**). The 90S preribosome contains the primary pre-rRNA, the early-recruited ribosomal proteins and more than 50 RBFs[37–39]. In this first complex, the pre-rRNA is cleaved at two sites in the 5'-ETS and at one site in the ITS1. The cleavage at ITS1 separates the pre-40S and pre-60S particles, whose subsequent maturation and processing proceeds independently[22,33,40]. The dismantling of the 90S preribosome liberates a complex containing assembly factors bound to the 5'-ETS that is recycled for successive assembly rounds (**Figure 5,** step **D**). A small number of export factors mediate the transport of pre-40S particles to the cytoplasm (**Figure 5**, step **F**), where they experience further maturation steps, including the formation of the beak region. Final 40S maturation takes place within 80S-like ribosomes and includes a final pre-rRNA cleavage and a quality control step that involves a transient binding to the 60S subunit[41] (**Figure 5**, steps **G-H**).

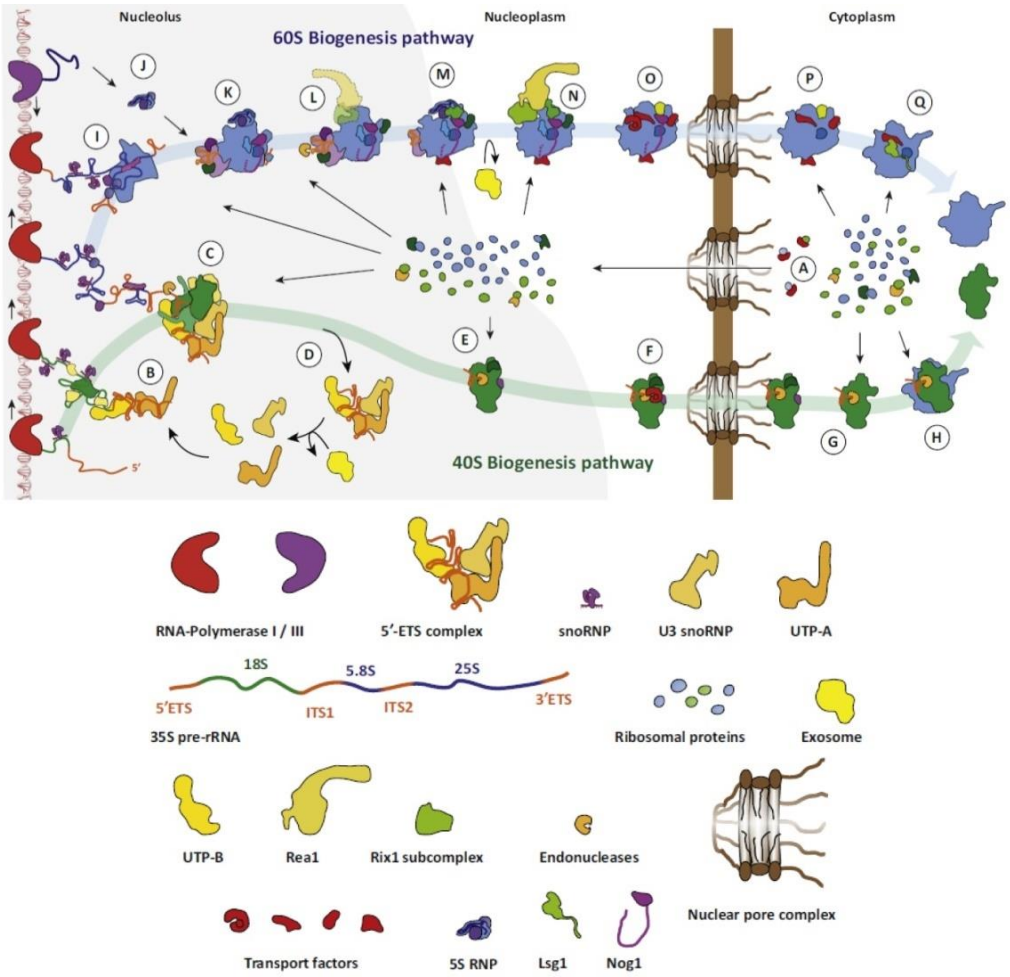


Figure 5. Steps of ribosome formation in *S. cerevisiae*. The 40S subunit maturation pathway is shown in green (B-H) and the 60S maturation pathway in blue (I-Q). On the left of the figure is represented the transcription of an rDNA repeat by RNA polymerase I. The main participants are shown in the bottom half of the figure. See main text for more details. Figure taken from Kressler et al., 2017[24].

The first components of the 60S particle begin to associate with the initiation of transcription of the pre-rRNA ITS2 segment. When the transcription of the whole pre-rRNA is completed, and the cleavage at ITS1 occurs, the early pre-60S particle is released (**Figure 5**, steps **I-K**). The 5S RNP, which contains the 5S rRNA and ribosomal proteins RPL5 and RPL11, associates with the early pre-60S particles (**Figure 5**, steps **J-K**). Subsequently,

several processing events in the ITS2 and concomitant rRNA remodeling steps occur to give rise to one of the architectural features of the 60S subunit, the foot structure, and to promote the accommodation of the 5S RNP in its mature position (**Figure 5**, steps **L-N**). Export-competent 60S preribosomes are actively transported to the cytoplasm (**Figure 5**, steps **O-P**) where they undergo final maturation to give rise to the mature 60S ribosomal subunits (**Figure 5**, step **Q**)[42].

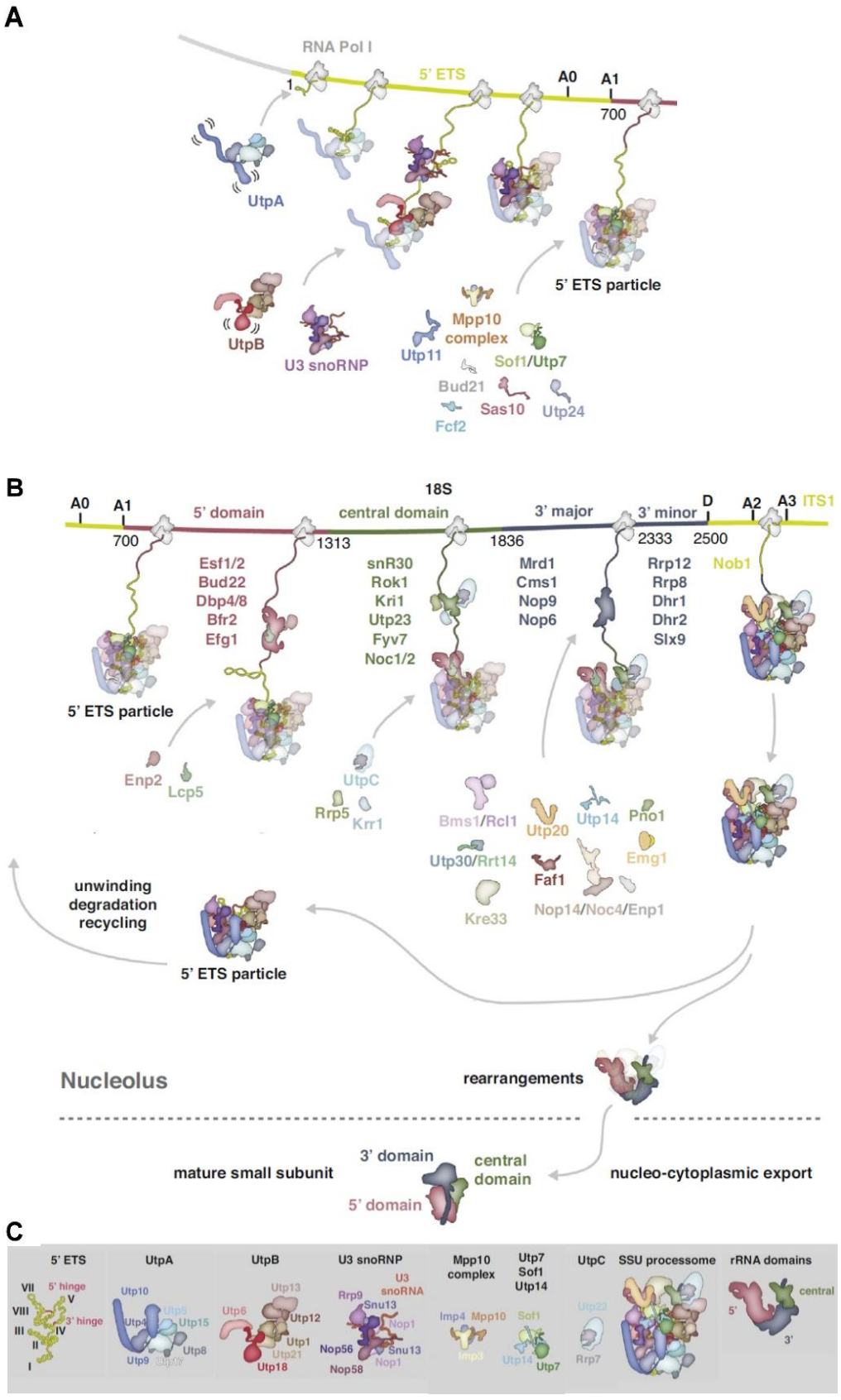
2. 3. Specific features of the pathway in human cells

Although the basic aspects of ribosome synthesis are conserved throughout eukaryotes, it is currently assumed that substantial differences must exist between higher and lower eukaryotes because in higher eukaryotes: (1) the organization of the rDNA is more complex[43], (2) there are more pre-rRNA processing steps[22,44], (3) the ribosomes are bigger, (4) the nucleolus has a more complex organization and houses more diverse cellular processes[45,46], and (5) the whole process is regulated by many different pathways[47] (and references therein). However, the molecular bases for those differences are mostly unknown because the knowledge of the mammalian pathway lags far behind. There is very little direct information about the characteristics of human preribosome intermediates because there are no good techniques to purify them. The identification of human ribosome synthesis factors has been possible only through systematic and high-throughput siRNA-based screenings that scored defects in the production of pre-rRNA intermediates or altered accumulations of ribosome or preribosome components in the nucleolus or nucleoplasm[13–15]. Such screenings have identified hundreds of proteins, including the orthologs of yeast ribosome synthesis factors, and some additional human-specific candidate proteins. It has been established that, as expected, the conserved factors participate in ribosome synthesis in human cells but, for most of them, it is unclear how conserved are their modes and times of action. The roles of the human-specific factors are mostly unknown. The advancement of knowledge on human ribosome synthesis requires that the techniques successfully used in yeast, such as preribosome purification, become available for studies with human cells.

3. The synthesis of the 40S ribosomal subunit in yeast

3. 1. The 90S preribosome

The 90S preribosome is assembled onto the pre-rRNA in a modular and hierarchical manner[48,36,49–51] (**Figure 6**). First, several protein factors are recruited in complexes (UtpA, UtpB and UtpC) that act as building blocks to conform the earliest preribosomal assembly intermediate, the 5' ETS particle (**Figure 6A**). Concomitant to the completion of the 5' ETS particle, the RNA polymerase I proceeds with transcription of the 18S rRNA region. The assembly of additional RBFs continues and the complete 90S particle is assembled (**Figure 6B**). The 90S preribosome undergoes several enzymatic and structural changes that lead to the building of the first pre-40S particle. The enzyme-driven changes include: (1) the Dhr1-promoted unwinding of RNA duplexes[52], (2) the cleavage at site A_0 in the 5' ETS, (3) the cleavage at site A_1 in the 5'-ETS by Utp24[53,54], (4) the release of the GTPase Bms1, whose place will be later occupied by Tsr1, and (5) the cleavage at site A_2 in ITS1. The structural changes include the rotational and translational movements of the central and 3' rRNA domains with respect to the 5' domain and the folding of the central pseudoknot and its surrounding elements, which is a step important for the overall 18S rRNA architecture and the decoding center[55].


A
RNA Pol I
5' ETS
A0
A1
1
700
UtpA
UtpB
U3 snoRNP
Utp11
Mpp10 complex
Sof1/Utp7
Bud21
Sas10
Utp24
Fcf2
5' ETS particle
B
18S
A0
A1
5' domain
central domain
3' major
3' minor
D
A2
A3
ITS1
700
1313
1836
2333
2500
Esf1/2
Bud22
Dbp4/8
Bfr2
Efg1
snR30
Rok1
Kri1
Utp23
Fyv7
Noc1/2
Mrd1
Cms1
Nop9
Nop6
Rrp12
Rrp8
Dhr1
Dhr2
Slx9
Nob1
5' ETS particle
Enp2
Lcp5
UtpC
Rrp5
Krr1
Bms1/Rcl1
Utp20
Utp14
Pno1
Utp30/Rrt14
Faf1
Emg1
Kre33
Nop14/Noc4/Enp1
unwinding
degradation
recycling
5' ETS particle
Nucleolus
rearrangements
mature small subunit
3' domain
central domain
5' domain
nucleo-cytoplasmic export
C
5' ETS
UtpA
UtpB
U3 snoRNP
Mpp10 complex
Utp7 Sof1 Utp14
UtpC
SSU processome
rRNA domains
5' hinge
3' hinge
Utp10
Utp5
Utp4
Utp15
Utp8
Utp9
Utp17
Utp13
Utp6
Utp12
Utp1
Utp21
Utp18
U3 snoRNA
Rrp9
Snu13
Nop1
Nop56
Snu13
Nop58
Nop1
Imp4
Mpp10
Imp3
Sof1
Utp14
Utp7
Utp22
Rrp7
central
5'
3'

3. 2. Export of pre-40S particles

After the cleavage of the primary 35S pre-rRNA at sites A_0, A_1 and A_2, within the 90S preribosome, the 20S pre-rRNA is generated (**Figure 7**). This species contains the 18S rRNA sequence and an extension of ITS1. The 20S pre-rRNA is the RNA component of the first pre-40S particle that emerges after the dismantling of the 90S preribosome. The pre-40S particles recruit several RBFs and are rapidly transported from the nucleolus to the cytoplasm, where they undergo a final cleavage at site D that renders the mature 18S rRNA (**Figure 7**).

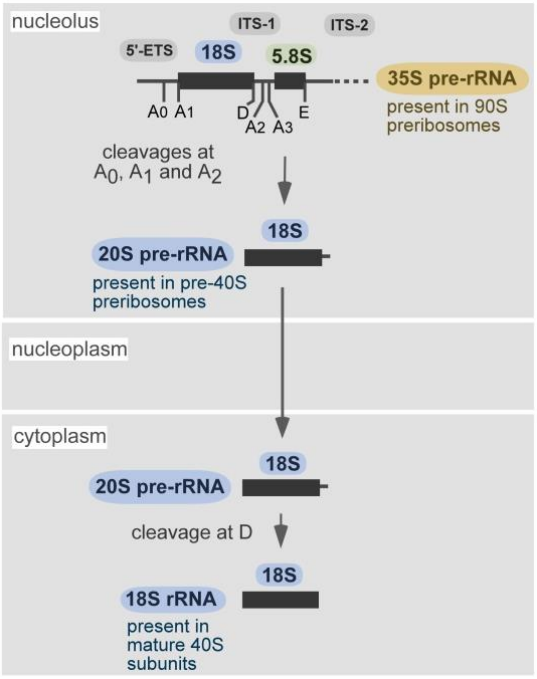


Figure 7. Main precursors of the 18S rRNA in yeast. The initial 35S pre-rRNA is cleaved at sites A_0, A_1 and A_2 within the 90S preribosome to give rise to the 20S pre-rRNA. This species, which contains a 3' end extension of ITS1, is the RNA component of immature pre-40S preribosomes. The 20S pre-rRNA is cleaved at site D in the cytoplasm producing the mature 18S rRNA.

Figure 6. Co-transcriptional assembly of the 90S preribosome. (A) A segment of the rDNA locus between 5'-ETS (yellow) and the beginning of the 5' domain of the 18S rRNA (red) is represented. The UtpA/tUtp (Utp= U three binding proteins) is the first complex that binds the pre-rRNA. Then the UtpB/Pwp2 complex and the U3 snoRNP (an essential chaperone for the steps to follow) are incorporated to form a stable intermediate. Next, it is promoted the association of the Mpp10 subcomplex, several individual assembly factors and Utp24, yielding the so-called 5' ETS particle. **(B)** The segment of the rDNA that follows the one in A is represented, showing the 5' domain (red), the central domain (green), the 3' domain (gray) of the 18S rRNA sequence and the beginning of ITS1 (yellow). The sequential incorporation of RBFs leads to the formation of the 90S preribosome. One of these RBFs is Krr1, which joins the 90S preribosome adjacent to the ribosomal proteins Rps14 and Rps1 and helps to recruit the UtpC module (Utp22 and Rrp7) to the platform region[56]. **(C)** The main components and subcomplexes of the 90S preribosome are shown. Figure adapted from Barandun et al., 2018[57].

The RBFs recruited by the pre-40S particle, before being exported, include one protein that comes from the nucleolus (Tsr1), and three proteins (Ltv1, Rio2 and the endonuclease Nob1) that come from the cytoplasm (**Figure 8**). These four proteins, together with three other ones (Enp1, Dim1 and Pno1), that had been recruited earlier, constitute the set of late maturation factors that accompany the pre-40S particle to the cytoplasm.

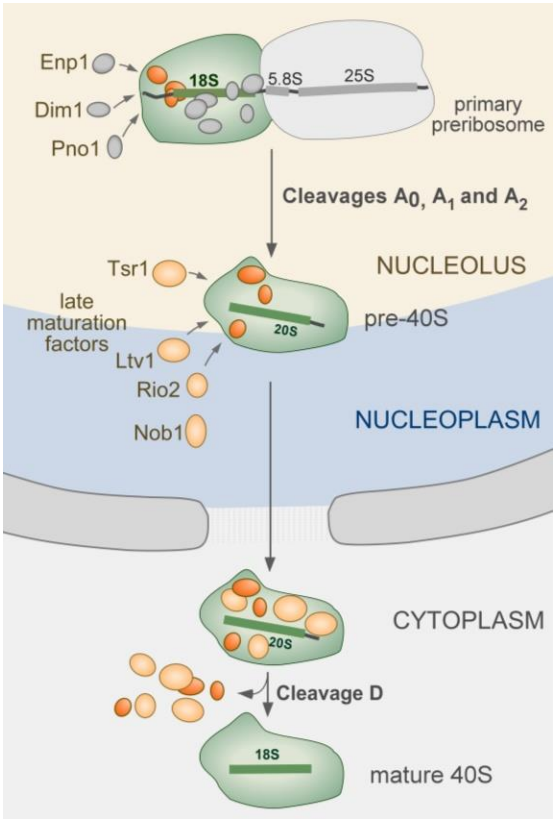


Figure 8. Scheme of the main preribosome intermediates of 40S subunits in yeast. The initial precursor particle is the 90S preribosome. The next one is the pre-40S particle, which recruits several RBFs (Tsr1, Ltv1, Rio2 and Nob1) for late maturation and cleavage of the 20S pre-rRNA in the cytoplasm. Three other RBFs (Enp1, Dim1 and Pno1) that accompany the pre-40S particle to the cytoplasm were recruited onto the 90S preribosome because they are already needed during the cleavages at sites A_0, A_1 and A_2.

Due to their large size, preribosomes are translocated through the NPC one at a time. The two essential factors for this step are the small GTPase Ran/Gsp1 and the general exportin Crm1/Xpo1. Crm1 belongs to the karyopherin-β-like family of export receptors, which bind proteins bearing nuclear export signals (NES) and promote their export to the cytoplasm in a Ran-GTP dependent manner[58–61]. At least three RBFs (Dim2, Ltv1, and Rio2), present in pre-40S particles, harbor predicted or functional NESs but their functions maybe redundant because none of them is essential for export. Several other factors have been implicated in the export of pre-40S subunits, although their exact functions in this process remain imprecise. One of them is

Rrp12, a HEAT-repeat protein that, together with Crm1, is recruited to the 90S preribosome and facilitates the processing of the 35S pre-rRNA and the elimination of the 5'-A_0 fragment [62,63]. The depletion of Rrp12, similarly to the depletion of Crm1, causes an accumulation of pre-40S particles in the nucleoplasm.

3. 3. Cytoplasmic events

As stated above, the pre-40S particle that reaches the cytoplasm contains seven RBFs that will promote late maturation events (**Figure 8**). The two major events that take place in the cytoplasm are the structural rearrangements that generate the beak structure, and the endonucleolytic cleavage of the 20S pre-rRNA at site D by the endonuclease Nob1. These events are tightly coupled to quality control mechanisms and proofreading of the functional centers, which ensure that the ribosomal subunits are translational competent[64]. The maturation of the beak is promoted by the release of RBFs and export factors, the stable incorporation of a small number of ribosomal proteins and the conformational switch that sets up the decoding site at the top of helix 44 of the 18S rRNA. This process requires a large structural rearrangement and starts with the phosphorylation by Hrr25 kinase of Ltv1 and Enp1, thus allowing their release and the mature positioning of the ribosomal protein Rps3. This change in conformation will promote the Nob1-dependent cleavage at site D[65–68].

The positioning of the RBFs in the cytoplasmic pre-40S particles has been established by cryo-electron microscopy[67,69,70] (**Figure 9**). Their localizations indicate that, in addition to their roles in the formation of the beak and the D cleavage, they block premature translation initiation (**Figure 9**). Ltv1 and Enp1 prevent the opening of the mRNA channel by directly binding Rps3 on its solvent-exposed side. Meanwhile, Dim1, Rio2 and Tsr1 are bound to the subunit interface, preventing the interaction with the 60S subunit and the initiation factor eIF1A. In a similar way, Nob1 and Pno1, that are critical for the 3' cleavage of the pre-rRNA, block the binding of eIF3. Tsr1 has additional roles in translation inhibition because it occupies the binding site of eIF5 (Fun12), and also interferes with the binding of Rio1 kinase, an RBF involved in a proof-reading step that requires the joining of the 60S subunit.

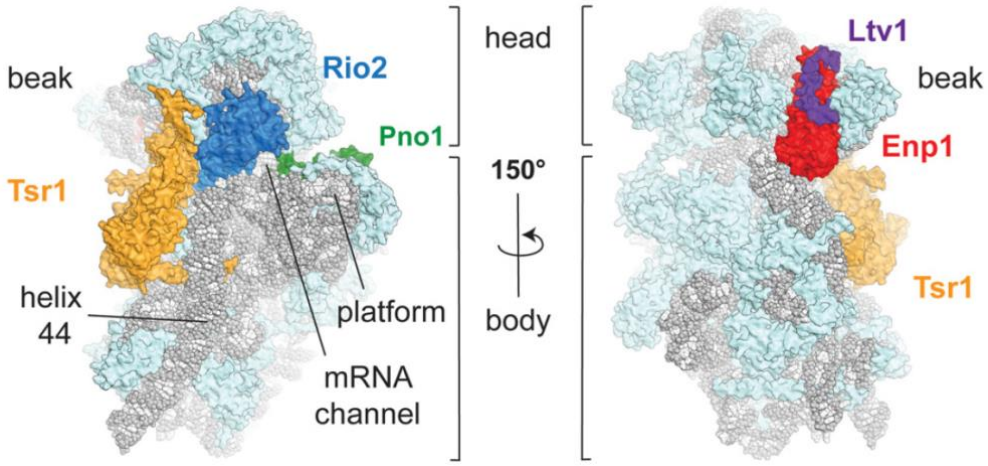


Figure 9. Structure of the cytoplasmic pre-40S preribosome from yeast. Front (left) and back (right) sides of the structure of the cytoplasmic pre-40S preribosome. The 20S pre-rRNA is shown in grey, the ribosomal proteins in light blue, Tsr1 in yellow, Rio2 in blue, Pno1 in green, Enp1 in red, and Ltv1 in purple. Figure taken from Scaiola et al., 2018[70].

During maturation of the pre-40S particle in the cytoplasm, the RBFs are sequentially released. First, upon maturation of the beak, Ltv1, Enp1, Rio2, Tsr1 and Dim1 are released. Then, it is joined Rio1 and finally, upon proofreading with the 60S subunit and 20S pre-rRNA processing, Nob1, Pno1 and Rio1 are released (**Figure 10**).

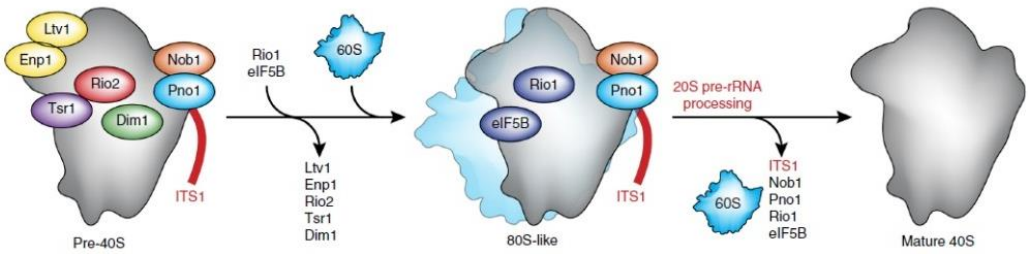


Figure 10. Late cytoplasmic pre-40S particle showing the associated RBFs and their orderly release. Figure taken from Peña et al., 2017[71].

4. The synthesis of the 40S ribosomal subunit in human cells

The 18S rRNA processing pathway involves more steps in human cells than in yeast[44,72](**Figure 11**). The first precursor of the human 18S rRNA, the 30S pre-rRNA, is formed when the primary 47S pre-rRNA is cleaved both at site 01 in the 5' external segment (5'-ETS) and at site 2 in the first internal segment ITS-1 (**Figure 11B**). The next processing steps include the removal of the 5' ETS-1 extension by two endonucleolytic cleavages and the partial removal of the 3' ITS-1 extension by exonucleolytic trimming, giving rise to the 21S and 21S-C pre-rRNAs. Then, it follows an endonuclease cleavage at site E that yields the 18S-E pre-rRNA. It is thought that, as it is being produced, the 18S-E species is rapidly exported to the cytoplasm, where it undergoes a final cleavage step at site 3. As mentioned above, in yeast there are only two major 18S pre-rRNA species, the 35S pre-rRNA (equivalent to the human 47S pre-rRNA) and the 20S pre-rRNA (equivalent to the human 18S-E pre-rRNA) (**Figure 11A**). The detection of more intermediates in humans indicates that the kinetics of pre-rRNA processing, folding and binding to ribosomal proteins is different to those in yeast. Furthermore, two recent studies have described that, upon release from the nucleolus, the 3' end of the 18S-E pre-rRNA is progressively trimmed by the exonuclease PARN[73,74]. The relevance of this step, which is not present in yeast, for the formation of the human 40S subunits is unclear. An even more intriguing finding is a recently-described regulatory mechanism that controls the levels of 40S subunits during diurnal rhythms in the liver through polyadenilation and degradation of the 18S-E pre-rRNA inside the nucleus[75]. When and how is the 18S-E pre-rRNA targeted for degradation is unknown.

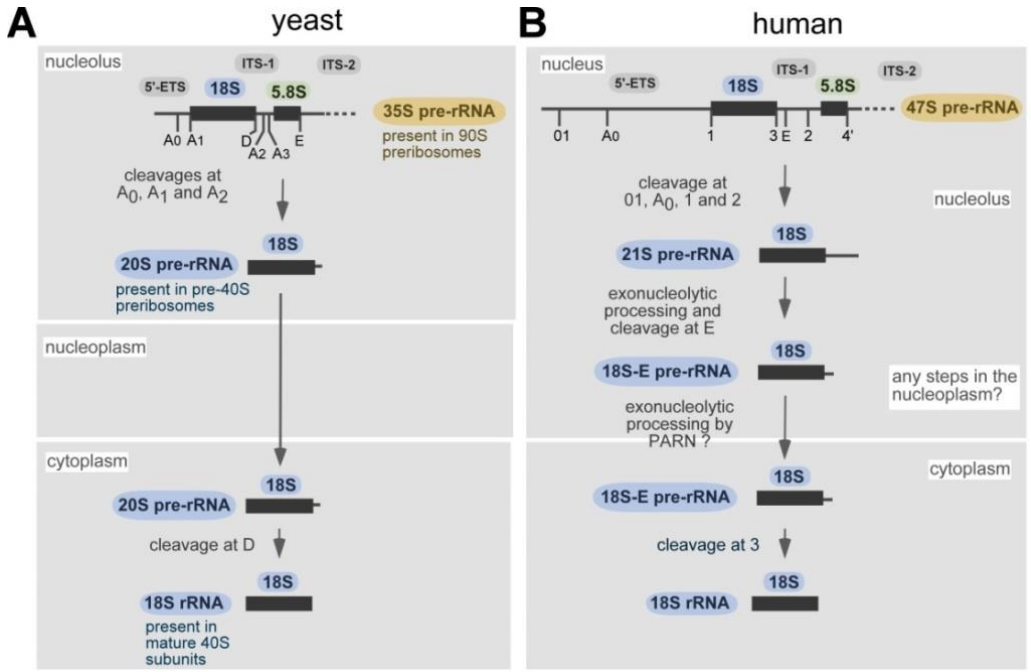


Figure 11. Differences in the steps of the 18S pre-rRNA processing in *S. cerevisiae* (left) and in *H. sapiens* (right). (A) In yeast, the two main precursors of the 18S rRNA are the 35S, that is cleaved at sites A_0, A_1 and A_2; and the 20S that, after cleavage at site D in the cytoplasm, generates the mature 18S rRNA. **(B)** In human cells there are more precursors of the 18S rRNA. First, the 47S yields the 30S (not shown), then this species gives rise to the 21S that, after processing and cleavage at site E produces the 18S-E pre-rRNA. This last precursor is subjected to cleavage at site 3 in the cytoplasm to yield the mature 18S rRNA.

As inferred from the presence of more pre-rRNA intermediates, the 40S synthesis pathway in human cells must involve at least two additional pre-40S precursors, those that contain the 30S pre-rRNA and those containing the 21S pre-rRNA (**Figure 12**). These precursors, and the possible intermediates have not been characterized. Only the cytoplasmic pre-40S particles have been amenable to compositional analyses using current techniques[76–78]. They were found to contain a set of final maturation factors similar to yeast, which includes BYSTIN (Enp1 in yeast), LTV1, RIO2, TSR1, PNO1 and NOB1. As mentioned above, the cytoplasmic maturation events in yeast include the formation of the 40S subunit beak, the stable incorporation of the remaining ribosomal proteins, the proof-reading with the 60S subunit and the pre-rRNA cleavage at the 3' end in the 18S rRNA sequence by the NOB1 endonuclease.

Although not studied directly, it is assumed that similar events must happen within the cytoplasmic pre-40S of human cells.

In summary, many basic questions about the 40S ribosome synthesis pathway still remain unanswered. The compositions, activities and transformations of all preribosome intermediates are poorly understood because most studies rely on indirect analysis or inferences from the yeast pathway.

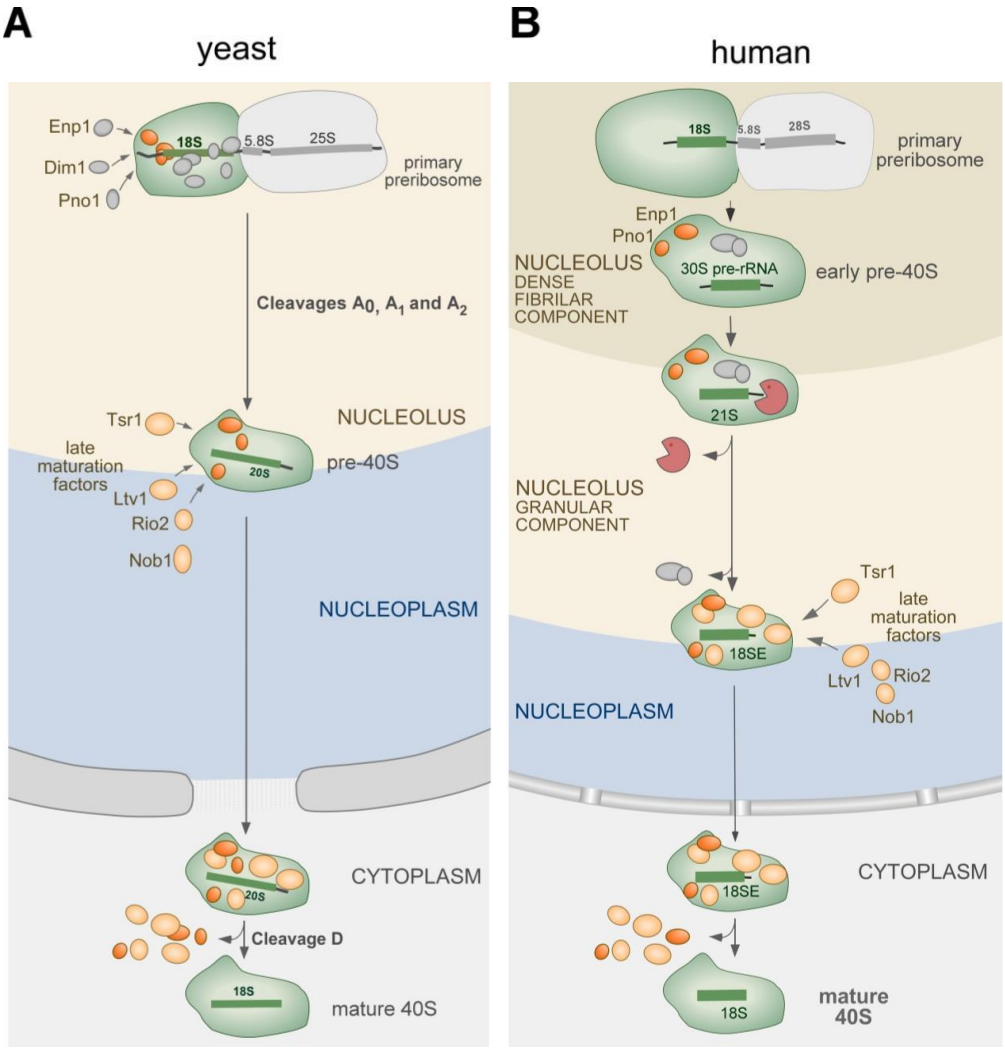


Figure 12. 40S preribosome intermediates in *S. cerevisiae* (left) and in *H. sapiens* (right). The stable accumulation of two additional pre-rRNAs (30S and 21S) in human cells indicates that there are at least two distinct steps of early maturation that are not seen in yeast. The similar compositions of the cytoplasmic pre-40S particles suggest that late maturation is similar in yeast and humans.

5. Technical reasons that slow the studies in human cells

Genetic approaches and *in vivo* preribosome purification schemes have been instrumental in yeast, not only to identify hundreds of ribosome biogenesis factors but also to define how the loss of each factor affects the composition and localization of distinct preribosomes[19,20,55]. After more than fifteen years of continuous advances, the research in yeast is currently being culminated with the visualization by cryoelectron microscopy of preribosome intermediates that display the numerous biogenesis factors in their structural contexts[24,42,48,57,77,79–81]. In contrast, to date, very few preribosomal particles have been isolated from human cells. Those were mostly obtained by immunoprecipitation of overexpressed epitope-tagged factors and, with the exception of cytoplasmic 40S precursors, the preparations have low yields or are highly heterogenous[73,74,76,82]. In fact, one major problem in human cells is that the nucleolar preribosomes are not efficiently solubilized, without disruption, with current extraction protocols. Two features that probably contribute to this problem are that the mammalian nucleolus is surrounded by thick regions of condensed chromatin, and has a tripartite organization that includes an internal compartment with a solid-like viscoelastic nature[46,83,84] (**Figure 13**). Another problem with the studies in human cells is that the preribosome purification baits are exogenously-expressed proteins and do not efficiently incorporate into the pathway.

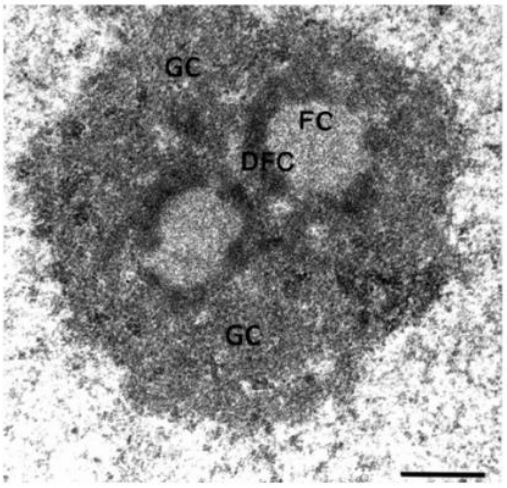


Figure 13. Electron micrograph from a HeLa cell showing the three subcompartments of the nucleolus: the fibrillar center (FC), the dense fibrillar component (DFC), and granular component (GC). Bar 0.5 µm. Figure taken from Sirri et al., 2002[85].

Due to these technical difficulties, most studies of human ribosome synthesis just rely on experiments that silence a specific RBF and analyze the changes in the production of pre-rRNAs species and subcellular localization of other RBFs. In this thesis we decided to set up new procedures to study the compositional changes and subcellular localization of preribosomes at different stages of maturation in human cells.

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PUBLICATIONS

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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.* 2014 Dec 4;10 (12): e1004836

APPENDIX

RESUMEN EN CASTELLANO

TÍTULO DE LA TESIS

Estadíos intermedios de maduración de la subunidad ribosómica 40S humana

ÍNDICE DE LA TESIS TRADUCIDO AL CASTELLANO

RESUMEN DE LA INTRODUCCIÓN

Los ribosomas son los complejos enzimáticos macromoleculares encargados de la síntesis proteica dentro de la célula. Traducen la información codificada por los ARN mensajeros (mRNAs) en cadenas polipeptídicas. Están compuestos por dos subunidades de distinto tamaño, la subunidad pequeña (40S en eucariotas) y la subunidad grande o 60S. Cada una de la subunidades, a su vez, está formada por ARN ribosómico (rRNA) y proteínas ribosómicas. En el caso de la subunidad 40S, la componen una molécula de rRNA, el rRNA 18S, y 33 proteínas ribosómicas (RPS), mientras que la subunidad 60S está formada por 3 moléculas de rRNA, 28S, 5.8S y 5S; y 47 proteínas ribosómicas (RPL). Cada una de las subunidades tiene una función diferente en la traducción. Por un lado, la subunidad 40S tiene función descodificadora, ya que es la encargada de asignar a cada triplete de nucleótidos el RNA de transferencia (tRNA) cargado con el aminoácido correcto. Por otro lado, la subunidad 60S es la encargada de la formación del enlace peptídico.

La síntesis de los ribosomas es un proceso muy complejo que empieza en el nucleolo de la célula, con la transcripción por la ARN polimerasa I de un precursor policistrónico, el pre-rRNA 47S, que contiene las secuencias de los rRNAs 18S, 5.8S y 28S, separadas por dos espaciadores externos (5’ ETS y 3’ ETS) e internos (ITS1 e ITS2). Este precursor primario tiene que ser procesado para eliminar esas secuencias ETS e ITS, y ello ocurre a lo largo de sucesivas etapas en el nucleolo, nucleoplasma, y finalmente, en el citoplasma, donde termina la maduración de los rRNAs. Este procesamiento del pre-rRNA está acoplado a la incorporación secuencial de las proteínas ribosómicas. También debe incorporarse el rRNA 5S, que es sintetizado por la ARN polimerasa III. Además de las cuatro moléculas de rRNA y de las 80 proteínas ribosómicas, en el proceso de formación de los ribosomas también participan más de 350 proteínas con funciones de ensamblaje y procesamiento, conocidos como factores de biogénesis de ribosomas (RBFs). A lo largo de toda la ruta de síntesis de ribosomas se forman distintos intermediarios, conocidos como preribosomas, que se caracterizan por poseer sus pre-rRNAs en diferente estadío de maduración, un número creciente de proteínas ribosómicas según va avanzando su maduración y distintos RBFs según el paso en el que nos encontremos. El primer intermediario que se forma es el preribosoma 90S. Lo hace de manera co-transcripcional a medida que se sintetiza el pre-rRNA 47S. Para ello, distintos subcomplejos formados por RBFs y RBFs aislados se asocian de manera secuencial, según va avanzando la transcripción. El pre-rRNA 47S debe ser escindido en el espaciador interno que está entre la secuencia de rRNA 18S y el 5.8S, conocido como ITS1. Este corte da lugar a dos pre-rRNAs distintos que conforman dos partículas, el primer preribosoma 40S (pre-40S) y el primer preribosoma 60S (pre-60S), que seguirán rutas de maduración

independientes. Después de sufrir algunas transformaciones en el nucleolo, los preribosomas llegan al nucleoplasma, donde siguen madurando y tienen que ser exportados de manera activa por la exportina CRM1 (también conocida como XPO1) de manera dependiente de RAN-GTP. En el citoplasma se producen los últimos eventos que darán lugar a las subunidades maduras y competentes para la traducción.

La mayoría del conocimiento que tenemos hoy en día de la ruta de síntesis de los ribosomas se debe a estudios en la levadura de gemación *Saccharomyces cerevisiae*. La elección de este organismo se debe a que la ruta está conservada evolutivamente y a que es muy fácil de manipular en el laboratorio para hacer modificaciones genéticas y experimentos de purificación de complejos proteicos. La ruta en células humanas ha sido mucho menos estudiada, pero se sabe que es más compleja y que en ella participan más factores que en levadura. Se ha asumido que los RBFs que están conservados entre humanos y levadura tienen las mismas funciones, pero en algunos casos se ha visto que no es así y tiene funciones adicionales o totalmente diferentes.

Uno de los principales motivos por los que la ruta de síntesis de ribosomas en células humanas es poco conocida son las dificultades técnicas para su estudio. Por un lado, los preribosomas nucleolares son difíciles de extraer debido a la naturaleza tripartita del nucleolo humano y a la capa de heterocromatina que lo rodea, haciendo que los preribosomas más tempranos, que están en las capas más internas del nucleolo, sean difíciles de solubilizar. Otro obstáculo a la hora de realizar estudios en células humanas es que las proteínas etiquetadas expresadas de manera exógena con el fin de realizar experimentos de purificación no se incorporan eficientemente en la ruta. Hasta ahora, la mayoría de los experimentos se han basado en silenciar un RBF y estudiar los defectos de producción de pre-rRNAs mediante Northern blot o en observar, mediante estudios de microscopía, los cambios en la localización de otros RBFs o proteínas ribosómicas etiquetadas. El problema es que estos experimentos no nos dan ninguna información sobre el papel exacto de esos RBFs en la ruta de síntesis de ribosomas.

En esta tesis se analizaron las funciones de algunos RBFs que intervienen en estadíos intermedios de la maduración de la subunidad ribosómica 40S en células humanas.