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A Puzzle of Life: Crafting Ribosomal Subunits

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The biogenesis of eukaryotic ribosomes is a complicated process during which the transcription, modification, folding, and processing of the rRNA is coupled with the ordered assembly of ~80 ribosomal proteins (r-proteins). Ribosome synthesis is catalyzed and coordinated by more than 200 biogenesis factors as the preribosomal subunits acquire maturity on their path from the nucleolus to the cytoplasm. Several biogenesis factors also interconnect the progression of ribosome assembly with quality control of important domains, ensuring that only functional subunits engage in translation. With the recent visualization of several assembly intermediates by cryoelectron microscopy (cryo-EM), a structural view of ribosome assembly begins to emerge. In this review we integrate these first structural insights into an updated overview of the consecutive ribosome assembly steps.

Synopsis of Eukaryotic Ribosome Assembly

Ribosomes are the molecular machines that translate the genetic information from the intermediary mRNA templates into proteins [1]. Eukaryotic 80S ribosomes comprise two unequal subunits that contain four different rRNAs and around 80 r-proteins (Figure 1). The small 40S subunit (SSU) comprises the 18S rRNA and 33 r-proteins (referred to as RPS or S). The large 60S subunit (LSU) comprises the 25S/28S, 5.8S, and 5S rRNA and, in most eukaryotic species, 47 r-proteins (RPL or L); a notable exception is budding yeasts, which lack eL28 [1–4].

The act of building a ribosome begins in the nucleolus, where the ribosomal DNA (rDNA) is transcribed into a long pre-rRNA precursor (35S pre-rRNA in yeast) and involves the ordered assembly of the r-proteins with the pre-rRNA, which is concomitantly processed into the mature rRNA species [5–8] (Figure 1 and Box 1). These assembly and processing events are tightly coupled and occur within **preribosomal particles** (see Glossary) that travel, as maturation progresses, from the nucleus across nuclear pore complexes (NPCs) to the cytoplasm, where they are ultimately converted into translation-competent ribosomal subunits [5,9–13] (Figure 2, Key Figure). Given the gargantuan complexity of this process, it is unsurprising that the assembly of eukaryotic ribosomes strictly requires the assistance of a plethora (>200) of mostly essential ribosome **biogenesis factors**, which are also called *trans*-acting or assembly factors [5,14,15].

Our current knowledge has been mainly obtained by studying ribosome biogenesis in the yeast *Saccharomyces cerevisiae*. Research conducted in the 1970s defined the r-protein composition of ribosomes, revealed the major **pre-rRNA processing** intermediates, and uncovered the existence of the 90S, 43S, and 66S preribosomal particles. The following 25 years witnessed the identification of numerous biogenesis factors and attributed functional roles

Trends

Cryoelectron microscopy analyses of the 90S preribosome show that the biogenesis factors create a casting mold that encloses the nascent pre-40S subunit.

Structures of nuclear pre-60S particles reveal that accommodation of the 5S ribonucleoprotein into its final position involves major structural rearrangements of the central protuberance. Moreover, within the 'foot' region the internal transcribed spacer (ITS) 2 rRNA is bound by several biogenesis factors that coordinate ITS2 processing.

An increasing number of biogenesis factors can be viewed as checkpoint factors sensing the successful completion of assembly events before licensing preribosomes for the subsequent maturation steps.

Supplying all ribosomal building blocks in stoichiometric amounts is a highly regulated process. Several dedicated chaperones contribute to the production of assembly-competent ribosomal proteins (r-proteins). Excess r-proteins are degraded via the excess r-protein quality control pathway.

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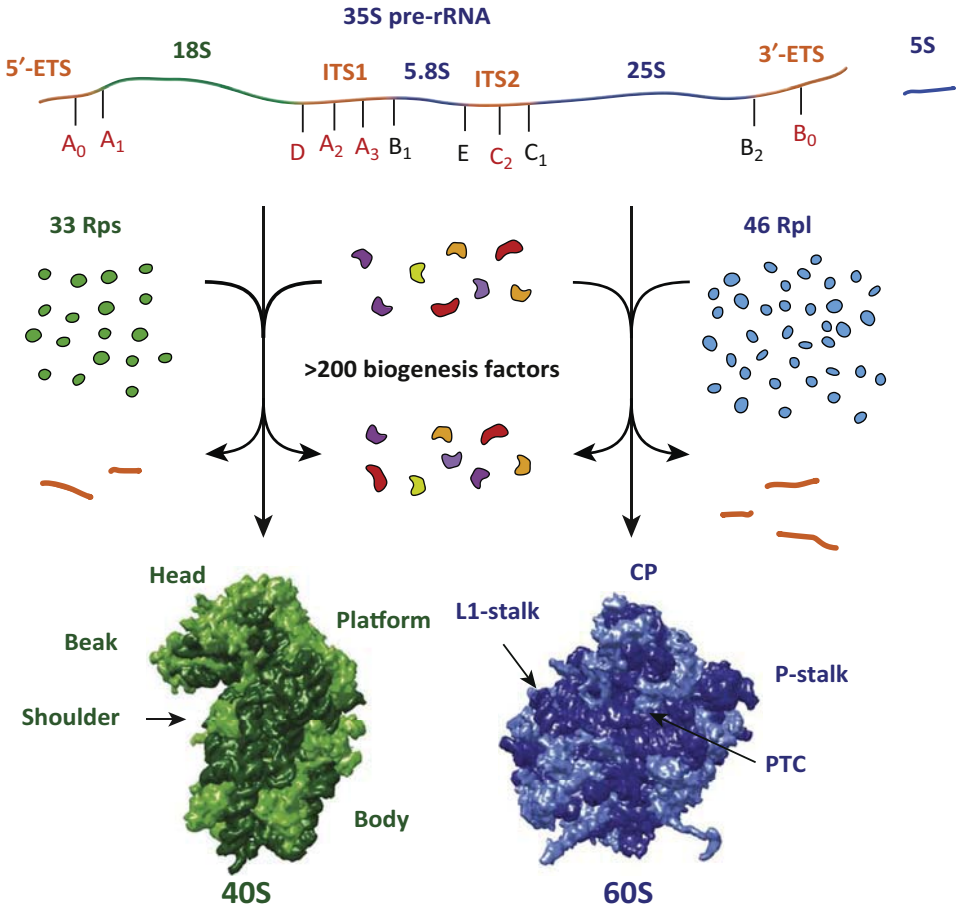


Figure 1. Schematic Representation of Ribosome Biogenesis in *Saccharomyces cerevisiae*. Ribosome biogenesis comprises the assembly of rRNA (top) and ribosomal proteins (r-proteins), which is coordinated and catalyzed by more than 200 biogenesis factors. r-Proteins of the 40S (light green) and 60S (light blue) subunits are synthesized from mRNA transcripts produced by RNA polymerase II. The 35S pre-rRNA and the 5S rRNA (dark blue) are synthesized by RNA polymerase I and RNA polymerase III, respectively. During ribosome biogenesis the external transcribed spacer (ETS) and internal transcribed spacer (ITS) (orange) are removed from the 35S pre-rRNA to obtain the mature 18S (dark green), 25S, and 5.8S (dark blue) rRNAs. These rRNA maturation events include a series of endo- and exonucleolytic processing reactions whose cleavage and trimming sites are indicated by red and black letters, respectively. The mature ribosome comprises two asymmetric subunits: the small 40S subunit contains 33 r-proteins (Rps; light green) and the 18S rRNA (dark green) while the large 60S subunit comprises 46 r-proteins (Rpl; light blue) and the 5.8S, 25S, and 5S rRNAs (dark blue). Structural hallmarks of the two mature ribosomal subunits are indicated, including the central protuberance (CP), which comprises the 5S rRNA and its associated r-proteins. Formation of new peptide bonds occurs in the peptidyl transferase center (PTC) of the 60S subunit. Surface representations of the mature 40S and 60S subunits from *S. cerevisiae*, shown in the interface view, were generated with Chimera using PDB 4V88 [113].

to these in the assembly and export of preribosomal subunits as well as the modification and processing of pre-rRNAs [16–18]. At the beginning of this millennium, the implementation of powerful *in vivo* affinity-purification approaches permitted the isolation and characterization of preribosomal particles [19,20]. Subsequently, the purification of additional preribosomal particles provided a detailed spatiotemporal picture of the preribosomal intermediates and their composition [5,9,14] (Figure 2). A few of these preribosomal particles were visualized at relatively low resolution by EM [21–24]. Recent progress in cryo-EM has enabled a quantum jump by permitting the visualization of preribosomal particles at atomic or near-atomic resolution [25–33]. These studies not only provided a detailed view of the overall architecture of

Glossary

Biogenesis factors: the efficient and accurate assembly of ribosomal subunits is promoted by more than 200 proteins that are transiently associated with preribosomal particles; these proteins are collectively referred to as biogenesis factors, or alternatively as *trans*-acting or assembly factors.

***Chaetomium thermophilum*:** a thermophilic filamentous ascomycete whose optimal growth temperature is around 50–55 °C. Proteins and macromolecular complexes of this eukaryotic organism often exhibit improved properties that can be exploited for biochemical and structural studies.

Decoding center: functional center of the SSU where decoding of mRNA occurs by selection of the correct base pairing between the codon and the anticodon of the cognate aminoacyl-tRNA.

External and internal transcribed spacer (ETS/ITS): the 35S pre-rRNA is flanked by 5′-ETS and 3′-ETS sequences and contains the internal ITS1 and ITS2, which separate the mature rRNA sequences; See also 'Pre-rRNA processing' and Figure 1.

Peptidyl transferase center (PTC): the active site of the LSU where the formation of a new peptide bond is catalyzed.

Preribosomal particle: maturation of ribosomal subunits occurs within preribosomal particles (also termed preribosomes) comprising (pre-) rRNA, r-proteins, and biogenesis factors (Figure 2).

Pre-rRNA processing: series of reactions that generate the mature rRNA species (18S, 5.8S, 25S, and 5S rRNA) from the large 35S pre-rRNA and the pre-5S rRNA. During processing of the 35S pre-rRNA, the ETS and ITS are removed by the action of endo- and exonucleases (Figure 1).

P stalk: characteristic feature of 60S subunits comprising a pentameric module of acidic r-proteins; contributes to the binding of translational GTPases.

Ribosomopathies: a group of inherited diseases caused by mutations in r-proteins or biogenesis factors that affect ribosome biogenesis or translation efficiency.

U3 small nucleolar ribonucleoprotein particle

Box 1. Basic Steps of Eukaryotic Ribosome Assembly

- Transcription of rDNA into pre-RNA and modification of the nascent pre-rRNA by snoRNPs (ribose methylation and formation of pseudouridines) in the nucleolus.
- Cytoplasmic synthesis of r-proteins and biogenesis factors and, for the majority, subsequent transport to the nucle(ol)us.
- Spatiotemporal association of biogenesis factors and r-proteins with the pre-rRNA results in the formation of preribosomal particles.
- Processing of the pre-rRNA (endo- and exonucleolytic removal of ETS and ITS sequences) separates the maturation pathways of the two subunits and is coordinated with the progression of ribosome assembly.
- Export of preribosomes from the nucleus across the nuclear pore complex to the cytoplasm.
- Final maturation of both preribosomal subunits occurs in the cytoplasm and includes the incorporation of the last r-proteins as well as the release and recycling of biogenesis factors.
- Quality control steps, involving several nuclear and cytoplasmic checkpoints, ensure that only fully functional ribosomal subunits can participate in the translation process.

(snoRNP): the U3 snoRNP contains the C/D-box snoRNA U3, all core C/D-box snoRNP proteins, and the U3-specific protein Rrp9. The U3 snoRNP is a structural component of the 90S preribosome and is essential for the early pre-rRNA processing reactions.

UTP-A/UTP-B/UTP-C: the UTP (U three protein) complexes are three independent assembly modules whose components were originally identified as factors associated with the U3 snoRNA. The letters A, B, and C denote their order of assembly into the 90S preribosome.

preribosomal particles but also displayed numerous biogenesis factors in the structural context of preribosomal particles. Here we outline the current view of the ribosome assembly process in yeast in the context of the recent functional and structural advances.

Providing a Balanced Supply of Ribosomal Components

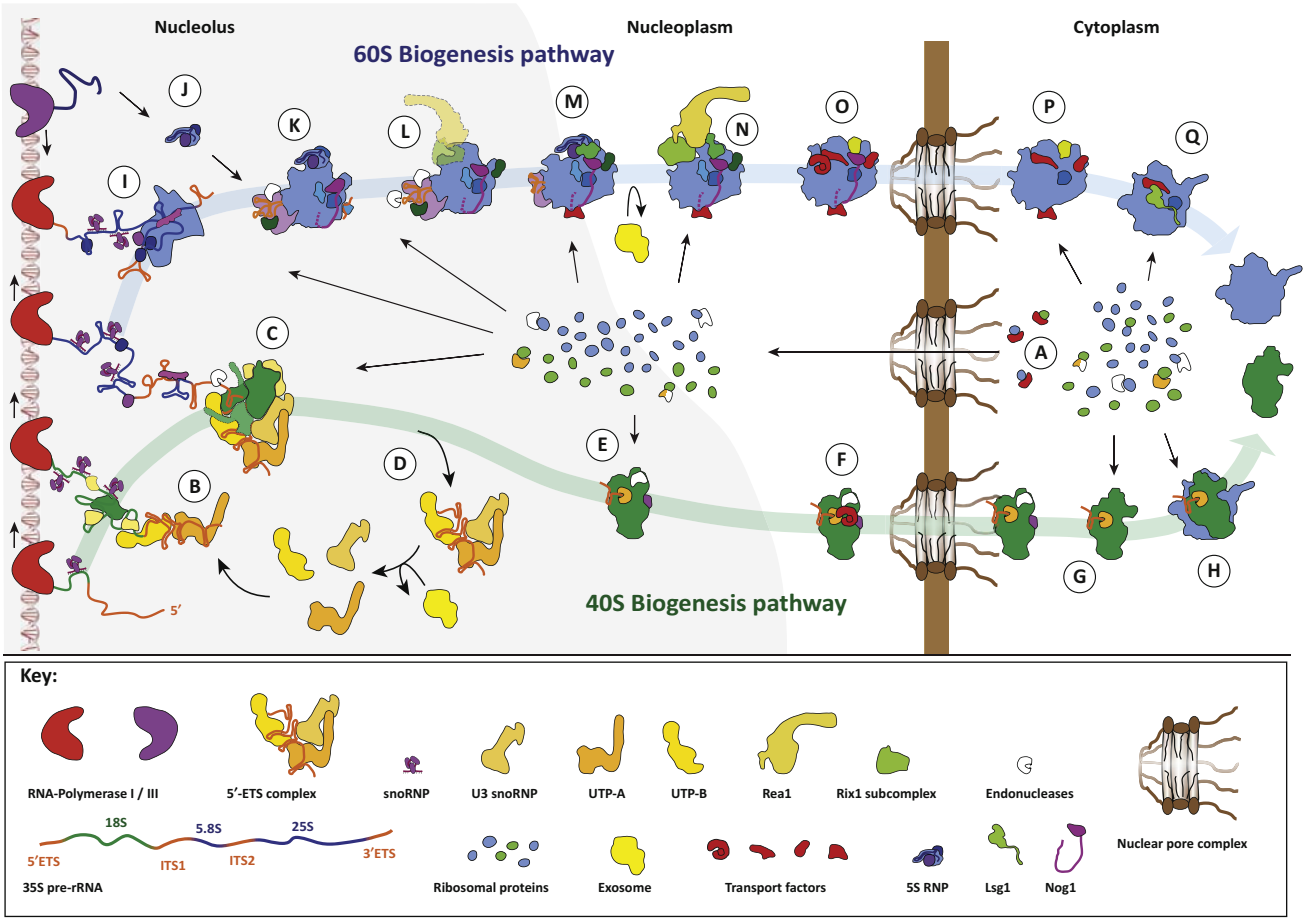
To guarantee the efficient assembly of ribosomes, cells are faced with the enormous logistic challenge of producing equal amounts of the four rRNAs and the 79 r-proteins (Figure 1). The transcription of rDNA and r-protein-encoding genes (RPGs) is connected to growth conditions, which are relayed via the TORC1 kinase [34], but a balanced production is complicated because the transcription of these components includes all three RNA polymerases [5,34]. While common promoter elements ensure the harmonized transcription of RPGs [34], a recent study revealed how a negative feedback loop links early assembly events with RPG transcription. A shortage of nascent pre-rRNAs leads to an increased free pool of the 90S biogenesis factor Utp22, which then sequesters the transcriptional activator Ifh1 from RPG promoters [35]. However, both the quantitative and qualitative production of r-proteins clearly depends also on several post-transcriptional events, including the stability and splicing efficiency of RPG mRNAs as well as the folding and intrinsic stability of r-proteins and, for most of these, their transport into the nucleus [6,36]. Therefore, the constant production of r-proteins in somewhat greater quantity than required seems advantageous for avoiding a shortage of these building blocks during ribosome assembly.

Prominent features of a large number of r-proteins are unusual overall folds and a high percentage of basic amino acids, which often cluster in long extensions and internal loops that are, in many instances, involved in rRNA binding [1]. Owing to these properties, r-proteins are especially prone to aggregation and general ribosome-associated chaperone systems contribute to their soluble expression [37,38]. In addition, a heterogeneous class of proteins, collectively referred to as dedicated chaperones, specifically protects individual r-proteins and safely guides them to their assembly site on preribosomal particles [37] (Figure 2A). So far, dedicated chaperones have been shown to associate with eight of the 79 yeast r-proteins [37,39]. Notably, the majority of these already capture their r-protein clients during translation [40,41].

The ubiquitin–proteasome system rapidly degrades unincorporated r-proteins, thereby antagonizing their aggregation bias [42,43]. To avoid targeting preribosomes for degradation, this nuclear quality control system, termed excess r-protein quality control (ERISQ), specifically ubiquitinates those lysine residues of r-proteins that are no longer accessible after their assembly into preribosomes [43]. Kinetic competition for r-proteins between ERISQ and the preribosome might constitute a mechanism to selectively eliminate excessive r-proteins [43]. Furthermore, association with dedicated chaperones and/or importins may prevent

Key Figure

Dynamic Maturation of Preribosomal Particles



Trends in Biochemical Sciences

Figure 2. The majority of ribosomal proteins (r-proteins) (A) need to be transported from the cytoplasm to their assembly sites within the nucleus. The assembly pathways leading to the formation of mature 40S [green; lower pathway from left to right (B–H)] and 60S subunits [blue; upper pathway from left to right (I–Q)] originate from a common pre-rRNA (35S pre-rRNA) that is transcribed by RNA polymerase I. Distinct assembly modules, biogenesis factors, and r-proteins associate cotranscriptionally with the nascent pre-rRNA (B) to form a 90S preribosome (C). Dismantling of the 90S preribosome and pre-rRNA cleavages liberate the 5′-external transcribed spacer (ETS) complex, whose components are recycled for further assembly rounds (D), and the first pre-40S particle (E). Export factors mediate the transport of pre-40S ribosomes to the cytoplasm (F) where they undergo further maturation steps, including beak formation (G). Final 40S maturation occurs within 80S-like ribosomes and couples pre-rRNA cleavage with a quality control step (H). The first pre-60S particle, whose components begin to associate with the nascent pre-rRNA, is likely to be formed after internal transcribed spacer 1 (ITS1) cleavage and termination of transcription (I). Then, the preformed 5S ribonucleoprotein particle (RNP) (J) associates with early pre-60S particles (K), which already contain the ITS2-associated biogenesis factors. Subsequently, ITS2 processing is initiated and the first Rea1-dependent remodeling step occurs (L). The Nog2-purified pre-60S particle exhibits the prominent ‘foot’ structure and the 5S RNP in its pre-mature position (M). The second Rea1-dependent remodeling step coincides with rotation of the 5S RNP into its mature position and occurs in concert with the release of the GTPase Nog2 (N), which is a prerequisite for recruitment of the export adaptor Nmd3 (O) and translocation through the nuclear pore complex. In the cytoplasm final maturation and quality control steps yield mature 60S subunits (P,Q). See main text for detailed descriptions of the highlighted assembly steps.

r-proteins from being ubiquitinated [37,43], thereby enabling their storage until they are incorporated into preribosomes.

r-Proteins are important not only for the structure and function of the ribosome but also for its assembly [6]. Whereas severe mutations in r-proteins will produce non-functional ribosomes, milder mutations may cause biogenesis and/or translation defects. In humans, reduced translation efficiency or accuracy may lead to **ribosomopathies**, whose clinical characteristics often include anemia or developmental defects [44–47]. Moreover, defects in ribosome assembly increase the levels of free r-proteins, acting as a signal for ribosome biogenesis stress. Accordingly, in multicellular organisms, elevated levels of uL5 and uL18 are functionally linked to the E3 ubiquitin ligase MDM2 leading to accumulation of its substrate p53, thereby blocking cell division [48,49]. Taking these findings together, the levels of free r-proteins are well regulated and monitored by various mechanisms, such as transcriptional regulation, association with binding partners, and degradation, to embed ribosome assembly into other cellular pathways that exert growth control.

Assembly of the SSU

Building the 90S Preribosome

Before folding, the nascent pre-rRNA is extensively modified by 2′-*O*-ribose methylation and isomerization of uridines to pseudouridines (Figure 2B). The modified sites, which cluster in functional regions of the ribosome, are identified by complementary base pairing between guide small nucleolar RNAs (snoRNAs) and rRNA [50]. These snoRNAs fall into two classes based on their integration into C/D-box or H/ACA-box small nucleolar ribonucleoprotein particles (snoRNPs), which have methyltransferase or pseudouridine synthase activity, respectively [50]. In yeast, 42 C/D-box snoRNAs and 28 H/ACA-box snoRNAs target around 100 different nucleotides for modification [51]. To allow accurate folding of pre-rRNA and its assembly with r-proteins, snoRNAs must dissociate from the rRNA. These processes involve DExD/H-box RNA helicases, which can generally be viewed as RNA chaperones or RNP remodelers [50,52,53].

Already during rDNA transcription, the first biogenesis factors and r-proteins associate with the nascent 35S pre-rRNA to form the 90S preribosome/SSU processome [5,9,54]. Most of these biogenesis factors form well-defined complexes, including the **U3 snoRNP** and the **UTP-A, UTP-B, and UTP-C** modules, which assemble in a stepwise manner with the pre-rRNA [5,55] (Figure 2B). Several recent studies have significantly advanced our understanding of these initial assembly events. Biochemical reconstitution and structural approaches revealed the architecture of the UTP-A and UTP-B modules [56–60]. Crosslinking analyses supported the idea that UTP-A is the first module to associate with the 5′-**external transcribed spacer (ETS)** region of the nascent 35S pre-rRNA [27,58] (Figures 1, 2B, and 3). Subsequently, the UTP-B complex binds downstream of UTP-A, but still within the 5′-ETS region. Thus, the UTP-A and −B modules might predominantly contribute to the structural nucleation of the evolving 90S preribosome (Figures 2B,C and 3). To obtain more insight into the timing of 90S assembly, two groups expressed various 3′ truncations of the pre-18S rRNA and analyzed the associated proteome, thereby revealing the gradual binding of distinct clusters of biogenesis factors [61,62]. Notably, relatively few biogenesis factors dissociate or show reduced association as formation of the 90S preribosome reaches completion [61]. If one hypothesizes a strict hierarchical order, these isolated complexes should represent the early, intermediate, and late stages of 90S ribosome assembly.

Architecture of the 90S Preribosome

The 90S preribosome appears to represent the first stable preribosomal particle (Figures 2C and 3). Accordingly, *in vivo* purification of UTP-A or −B components permits the isolation of

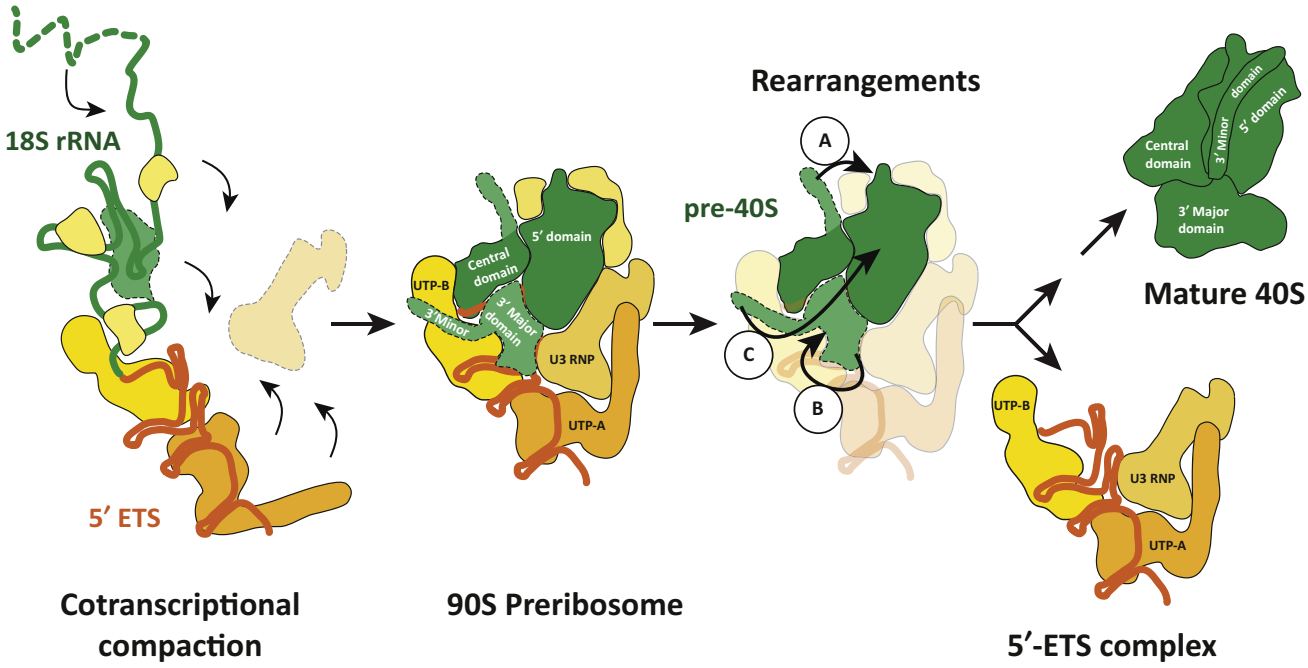


Figure 3. Model of 90S Formation and Its Conversion into Pre-40S Ribosomes. The UTP-A, UTP-B, and U3 small nucleolar ribonucleoprotein particle (snoRNP) modules associate cotranscriptionally with the 35S pre-rRNA. Presumably, subsequent compaction leads to formation of the 90S preribosome, where the biogenesis factors (yellow, orange) form a casting mold for the nascent 18S rRNA (green). While the overall fold of the 5′ domain of the 18S rRNA is already similar to its final conformation, several major structural rearrangements within the central, 3′ major, and 3′ minor domains are required to transform the pre-40S moiety of the 90S preribosome into the mature 40S subunit. (A) The eukaryote-specific expansion segment ES6 needs to be completely folded and moved towards the 5′ domain. (B) The 3′ major domain requires further stabilization and folding to form the characteristic head structure, which still has to be rearranged to acquire its final position. (C) The 3′ minor domain, mainly comprising the long helix H44, has to undergo a dramatic movement to be accommodated at its binding site on the 5′ domain. However, the timing and mechanisms of these rearrangements remain to be determined. Release of the 5′-external transcribed spacer (ETS) complex and endonucleolytic cleavage within internal transcribed spacer 1 (ITS1) (not depicted) liberate the first pre-40S particle, which is converted in further biogenesis steps into the mature 40S subunit.

preribosomal particles comprising a 5′-ETS-containing pre-18S rRNA, about 20 SSU r-proteins, 65 biogenesis factors, and the U3 snoRNP [27,29]. A major recent breakthrough is the determination of cryo-EM structures of the 90S preribosome derived from ***Chaetomium thermophilum*** and *S. cerevisiae* [27,29,31] (Figure 4A). All three structures unveil the features of the nascent 40S moiety, revealing how most of the pre-rRNA, about 30 biogenesis factors, and 15 SSU r-proteins are arranged within this ~5-MDa assembly intermediate [27,29,31]. The UTP-A and UTP-B modules are bound to the rRNA helices of the 5′ ETS and form, together with the U3 snoRNP and additional biogenesis factors, a stable building block of the 90S preribosome. Strikingly, the biogenesis-factor ensemble forms a casting mold that engulfs the nascent pre-18S rRNA (Figures 3 and 4), thereby protecting it from degradation and enabling its accurate processing and controlled folding. The 5′ domain of the 18S rRNA exhibits significant similarity to its mature conformation and already contains the majority of its ultimately associated r-proteins. Most of the central domain is properly positioned relative to the 5′ domain and already harbors some r-proteins at their cognate binding sites. The 3′ major domain is completely buried in the center of the 90S preribosome and its conformation is significantly distinct from that in the mature state, clearly indicating a hierarchical, 5′-to-3′-oriented assembly process. The cryo-EM structures further revealed how the essential U3 snoRNA engages in base-pairing interactions with the 5′ ETS and the pre-18S rRNA (Figures 3 and 4). An interesting hypothesis is that the U3 snoRNP not only stimulates pre-rRNA folding and processing but also locks the 90S preribosome in an intermediate folding state. This may provide a necessary time

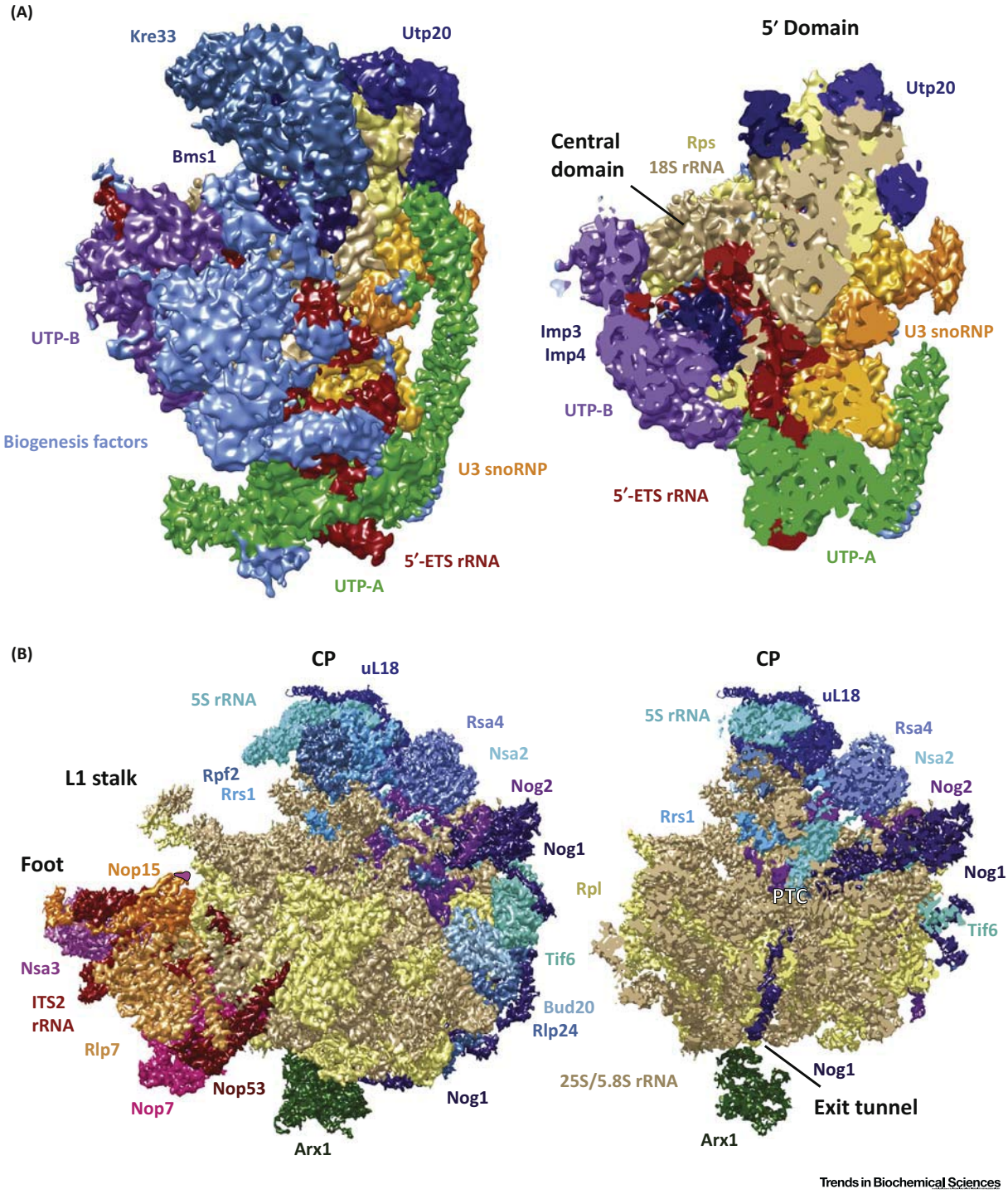


Figure 4. A Structural View of Selected Preribosomal Particles. (A) Cryoelectron microscopy (cryo-EM) structure of the 90S preribosome from *Chaetomium thermophilum* (EMD 8143) [27]. The indicated assembly modules and biogenesis factors are highlighted in different colors. The remaining biogenesis factors are colored in light blue. The 5′ external transcribed spacer (ETS) (red), the 18S rRNA (tan), and the ribosomal proteins (r-proteins) (Rps; khaki) are also highlighted. A cross-section of the 90S preribosome reveals the interior of the cage-like structure (right panel). The positions of the 5′ domain and the central domain of the 18S rRNA within the nascent pre-40S moiety are indicated. (B) Cryo-EM structure of the Nog2-purified pre-60S ribosome from *Saccharomyces cerevisiae* (EMD 6615) [25]. Characteristic features, including the 'foot', L1 stalk, and central protuberance (CP), are indicated. The prominent foot structure, comprising several biogenesis factors (Nop7, Nop15, Nop53, Nsa3, and Rlp7) and the internal transcribed spacer 2 (ITS2) rRNA, is highlighted. In its pre-mature orientation, the 5S ribonucleoprotein particle (RNP) (5S rRNA and r-proteins uL18 and uL5) contacts the Rpf2–Rrs1 heterodimer. The 25S/5.8S rRNAs are colored in tan and the r-proteins in khaki. A cross-section of the pre-60S ribosome (right panel) shows the insertion of Nog1's C-terminal extension (dark violet) into the polypeptide exit tunnel and the interconnection of biogenesis factors (Nsa2, Nog1) in proximity to the peptidyl transferase center (PTC).

window for biogenesis events to occur before the release of the 5′ ETS and the subsequent formation of the central pseudoknot structure that interconnects the four 18S rRNA domains.

Dismantling of the 90S Preribosome Releases an Early Pre-40S Particle

Despite our detailed knowledge of the pre-rRNA processing pathway in yeast and human cells [7,63], the enzymes that cleave the pre-rRNA at sites A_0, A_1, and A_2 have not yet been unambiguously assigned. While all three cleavages depend on the U3 snoRNP, processing at sites A_1 and A_2 appears to be coupled and occurs independently of A_0 cleavage [7]. Recent evidence strongly suggests that the 90S-associated Utp24 is the endonuclease responsible for cleavages at sites A_1 and A_2 [64]. This PIN-domain endonuclease is positioned in proximity to the A_1 cleavage site [27,29,64] and cleaves a pre-rRNA substrate at site A_2 *in vitro* [64]. A second 90S-associated endonuclease with *in vitro* A_2 cleavage activity is Rcl1 [27,29,31,65]. Unfortunately, the positions of the rRNA segments around sites A_0 and A_2 could not be assigned in the current 90S structures [27,29]. To clarify and understand the direct contribution of Utp24 and Rcl1 to these early cleavages, it will be instrumental to generate improved 90S cryo-EM structures. Such structures might also reveal how these early cleavages are coupled with progression to the first pre-40S particle. This transition requires the release of the 5′-ETS complex, which mainly comprises 5′-ETS rRNA, UTP-A and UTP-B modules, and U3 snoRNP [27] (Figures 2D and 3). Exosome-mediated degradation of the 5′-ETS rRNA may initiate the disassembly of the 5′-ETS complex, whose recycled components can then participate in a new round of 90S assembly [27,66]. Dismantling of the 90S preribosome is likely to depend on the RNA helicase Dhr1, since pre-40S intermediates trapped by catalytically inactive Dhr1 still contain the U3 snoRNP [67]. Moreover, Dhr1, stimulated by its recruiting cofactor Utp14, can unwind U3 rRNA duplexes *in vitro* [67,68]. However, other helicases associated with the 90S preribosome might also be required for the dismantling process that releases the early pre-40S particle. Clearly, further studies will be required to obtain a complete picture of these early biogenesis events.

Final Maturation of Pre-40S Particles

Cleavage of the pre-rRNA at site A_2 results in the separation of the biogenesis pathways of the SSU and LSU (Figure 2C), which will only meet again in the cytoplasm. The next biochemically characterized pre-40S intermediates following the 90S preribosome contain the 20S pre-rRNA, which corresponds to an 18S rRNA extended to site A_2, and around ten biogenesis factors [69]. Notably, most of these biogenesis factors are exclusively associated with pre-40S particles; therefore, the transition from the 90S to the first 40S preribosome is hallmarked by an enormous exchange of biogenesis factors [69]. Moreover, several r-proteins, including the early binders of the 3′ major domain, need to be recruited at this stage. However, since no transition intermediates have been purified so far, the 90S-to-pre-40S conversion appears either to be a kinetically fast process or to involve unstable interjacent particles (Figure 2E).

Rapidly after their formation, pre-40S particles are exported through the NPC to the cytoplasm (Figure 2F). Due to the large size of the pre-40S ribosome, it is predicted that multiple export factors are required to facilitate its rapid translocation [70]. Pre-40S export depends on the GTPase Gsp1/Ran and Crm1/Xpo1, the general exportin for substrates harboring nuclear export signal (NES) sequences [5,10,71,72]. At least three biogenesis factors (Dim2, Ltv1, and Rio2) contain predicted or functional NESs that are, possibly due to their redundancy, individually not required for pre-40S export [5,10]. Additionally, several other factors have been implicated in the export of pre-40S subunits [10], but their direct role in this process remains vague.

In agreement with a hierarchical assembly pathway, the cytoplasmic maturation events mainly concern the 3′ major and 3′ minor domains. Moreover, the release of biogenesis factors, which

occupy surfaces that later engage in interactions with translation factors [73,74], must be coordinated with the accommodation of the last r-proteins and the cleavage of the 20S pre-rRNA [6]. First, formation of the characteristic beak structure involves the stable incorporation of uS3, which is triggered by the phosphorylation-dependent release of Ltv1 [22,75,76] (Figure 2G). The Rio2 ATPase is strategically positioned between the body and the maturing head in proximity to the **decoding center** and subsequently may act as a self-releasing checkpoint factor [73,77]. Recruitment of the ATPase Rio1, presumably requiring the prior dissociation of Tsr1 [78], yields late pre-40S ribosomes that are competent to join 60S subunits [79–81]. Within these 80S-like ribosomes, Rio1 and the GTPase eIF5B stimulate the Nob1-catalyzed cleavage at site D of the 20S pre-rRNA into mature 18S rRNA [74,80,82–84] (Figures 1 and 2H). Coupling pre-40S maturation to such a quality control step ensures that only properly assembled 40S subunits enter the pool of translating 80S ribosomes.

Taken together, structural investigations of the 90S preribosome have unraveled its detailed architecture; however, many functional and enzymatic aspects remain to be explored. Subsequent pre-rRNA processing and dismantling releases the first pre-40S particle, which is rapidly exported to the cytoplasm where final maturation and functional proofreading occur.

Assembly of the LSU

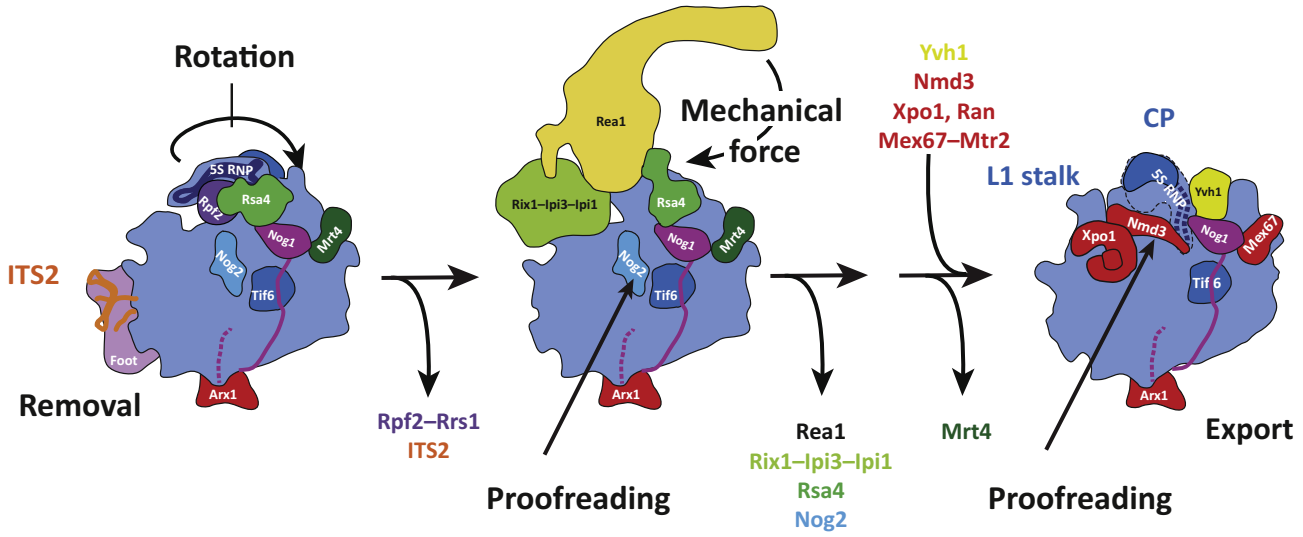
Nuclear 60S Biogenesis Events

Cleavage at site A_2 within **internal transcribed spacer 1 (ITS1)** liberates the SSU moiety from the pre-rRNA transcript and sets the stage for the assembly of the LSU (Figure 2C). Contrary to the relatively simple 40S assembly route, which proceeds along the sequential, 5′-to-3′-oriented folding of its four distinct 3D domains, 60S assembly appears more complex as its six secondary-structure rRNA domains are elaborately intertwined in the mature 60S ribosome [2,4]. Moreover, evidence gathered over the past 15 years has revealed that LSU assembly occurs within several distinct and successive pre-60S intermediates of partially overlapping composition (Figure 2). The initial steps of LSU assembly remain poorly characterized, but it appears that the formation of the first pre-60S particle depends on termination of transcription and the association of early-binding LSU r-proteins that connect the 5′ and 3′ regions of the 27S pre-rRNA [6,11] (Figure 2I). Evidence suggests that rRNA modification may not exclusively occur cotranscriptionally since the presumably earliest pre-60S precursor, isolated via the Npa1 bait protein, still contains C/D- and H/ACA-box snoRNPs [85]. Moreover, the presence of eight RNA helicases in this Npa1-defined particle, which mainly comprises the $27SA_2$ pre-rRNA, indicates that extensive rRNA folding is occurring.

The next nucleolar pre-60S particle, the Ssf1-defined particle, exhibits a complex composition: it contains a 27S pre-rRNA intermediate, more than 30 biogenesis factors, and already around half of the LSU r-proteins [86–88]. The following pre-60S ribosomes (Erb1- or Nsa1-associated particles) mainly comprise the 27SB pre-rRNAs and have already lost some early biogenesis factors [86,89] (Figure 2K). This loss might be due to the trimming of the 27S pre-rRNA at its 5′ and 3′ termini ($A_3 \rightarrow B_1$ and $B_0 \rightarrow B_2$; Figure 1). Moreover, the biogenesis factors that associate around the ITS2 region, forming the characteristic ‘foot’ structure (see below), have already been recruited at this stage [86]. A specific feature of LSU assembly is the incorporation of a preformed 5S RNP (Figure 2J) comprising the 5S RNA and the r-proteins uL5 and uL18. Formation of the 5S RNP is facilitated by the transport adaptor Syo1, which couples nuclear import of uL5 and uL18 with 5S rRNA association [90,91]. The subsequent incorporation of the 5S RNP module involves the Rpf2–Rrs1 heterodimer and has already occurred within the Nsa1-defined particle [25,86,92–94] (Figure 2K). However, the exact timing and the mechanistic details of the initial 5S RNP docking to the pre-60S ribosome remain to be determined.

During the subsequent maturation steps, the 27SB pre-rRNA is processed within the ITS2 region: consecutive endo- and exonucleolytic cleavages catalyzed by the Las1 complex result in the formation of the 7S and 25S′ pre-rRNAs [95] (Figure 2L). Interestingly, cryo-EM structures of pre-60S ribosomes (e.g., the Nog2 particle) show in great detail how several biogenesis factors are associated with the 7S pre-rRNA to form the foot structure [25,96–98] (Figures 2M and 4). It has been proposed that these ITS2-associated factors protect the pre-rRNA and coordinate the endo- and exonucleolytic processing events [25]. Notably, these pre-60S ribosomes exhibit further structural hallmarks. The 5S RNP is rotated by roughly 180° compared with its mature orientation [25,97]. In addition, the nascent **peptidyl transferase center (PTC)** is covered with biogenesis factors, including the GTPases Nog1 and Nog2, whereas the placeholder Mrt4 occupies the site of **P-stalk** assembly (Figures 4B and 5) (see below).

While navigating towards the nucleoplasm, the stepwise exonucleolytic 3′-end maturation of the 7S pre-rRNA is initiated by the Nop53-mediated recruitment of the exosome-assisting RNA helicase Mtr4 to the foot structure [66]. This phase is also marked by the association of the Rix1 subcomplex and the AAA-ATPase Rea1 [21,33,99] (Figures 2N and 5). Interestingly, the interaction between Rix1 and Rea1 may be regulated, as indicated by a recent study with the orthologous human proteins showing that their association is controlled by a dynamic SUMO conjugation/deconjugation cycle [100]. The cryo-EM structure of the Rea1-containing Rix1 pre-60S particle, compared with the upstream Nog2-defined pre-60S particle [25,97], revealed several major differences [33], among them the absence of the foot structure. Most notably, recruitment of the gigantic Rix1–Rea1 machinery coincides with the release of Rpf2–Rrs1 and rotation of the 5S RNP into its mature position [33] (Figure 5). Once properly positioned the ATPase activity of Rea1 powers the removal of Rsa4 [21], an event that occurs



Trends in Biochemical Sciences

Figure 5. Model of Nuclear Pre-60S Maturation Events. Simplified representation of successive pre-60S intermediates that have been structurally investigated. The nucle(ol)ar Nog2-purified pre-60S particle contains the characteristic 'foot' structure [internal transcribed spacer 2 (ITS2) in orange] and the 5S ribonucleoprotein particle (RNP) is in its pre-mature conformation (left). During the next maturation steps, the exonucleolytic degradation of the ITS2 is coupled with the removal of the foot structure. Recruitment of the Rix1–Ipi3–Ipi1 subcomplex and the AAA-ATPase Rea1 induces the rotation of the 5S RNP, accompanied by the release of the Rpf2–Rrs1 heterodimer. It is proposed that Rea1 and the Rix1 subcomplex sense the correct 5S RNP position, thereby allowing the activation of Rea1's ATPase activity (middle). The resulting mechanical force towards the Rsa4–Nsa2 heterodimer induces a rRNA rearrangement at the peptidyl transferase center (PTC). Intimately coupled with this process is the activation and release of the GTPase Nog2, which appears to monitor the correct conformation around the PTC and thus provides an additional proofreading step. Release of Nog2 is a prerequisite for the recruitment of the nuclear export signal (NES)-containing export adaptor Nmd3, which is recognized by the exportin Crm1/Xpo1 in a RanGTP-dependent manner (right). The recruitment of the export factor Mex67–Mtr2 is coupled with maturation of the P stalk. Mrt4, the placeholder for uL10, is released by Yvh1, which allows Mex67–Mtr2 binding in proximity to the nascent P stalk.

in concert with the activation and release of the GTPase Nog2 [101]. These reactions are involved in arranging the rRNA elements forming the PTC and allow recruitment of the NES-containing export adaptor Nmd3 [30,101,102] (Figures 4B and 5). Accordingly, Rea1 and Nog2 can be viewed as checkpoint factors that sense the correct assembly status of pre-60S ribosomes; their subsequent activation then triggers remodeling events that irreversibly license these particles for export to the cytoplasm (Figures 2O and 5). A further checkpoint monitors the peptide exit tunnel, which is occupied by the C-terminal extension of the GTPase Nog1 in nuclear pre-60S particles [25]. However, the functional relevance of this intriguing observation needs to be tested in future studies.

Nuclear Export and Cytoplasmic Maturation of Pre-60S Ribosomes

Several export-mediating factors interact with different surfaces of pre-60S ribosomes to mediate their efficient translocation across the NPC [5,10,14,103] (Figures 2O and 5). Following the release of Nog2, the export adaptor Nmd3 binds in the A-, P-, and E-tRNA-binding sites of the LSU and recruits the exportin Crm1/Xpo1 via its essential NES sequences [10,14,30,32,101]. Thus, Nmd3 appears to sense the correct conformation around the PTC and prevents premature tRNA binding [30,32]. In addition, Nmd3 brings together some peripheral elements (L1 stalk, rRNA helix H38), thereby compacting the preribosome and possibly facilitating the translocation process [30]. The Mex67–Mtr2 heterodimer, another important export factor, binds pre-60S ribosomes at two distinct sites: in the neighborhood of the nascent P stalk and within the 3′ region of the 5.8S rRNA [104,105]. A recent study has demonstrated that recruitment and release of Mex67–Mtr2 is also intimately coupled to maturation of the emerging P stalk [104]. Besides these, several additional factors contribute to the export process [5,10,14,103], most likely by shielding the charged ribosomal surface against the hydrophobic environment within the NPC channel.

After export, removal of the remaining biogenesis factors is coordinated with the incorporation of the missing r-proteins [5,6,14,30,103] (Figure 2P). First, the AAA-ATPase Drg1 releases the GTPase Nog1 and its binding partner Rlp24, thereby enabling recruitment of the r-protein eL24 [99,106]. Subsequently, Arx1 is dissociated by Jjj1 and Rei1 [103]. Interestingly, as shown by high-resolution cryo-EM, Rei1 and the closely related Reh1 probe the exit tunnel in a manner similar to Nog1 [25,28,30]. During P-stalk assembly, recruitment of uL10 is coupled with the release of Yvh1, which has previously displaced Mrt4 from pre-60S particles [103,104]. Release of the export adaptor Nmd3 is promoted by the GTPase Lsg1 and connected to the stable incorporation of uL16 [40,103,107] (Figure 2Q). The last biogenesis factor to be dissociated appears to be Tif6 [26,30,103,108]. Removal of this antiassociation factor is triggered by the GTPase Efl1 and is a prerequisite for subunit joining [26,108]. Interestingly, Efl1 and its cofactor Sdo1 largely cover the PTC region [26]; they are therefore strategically positioned to ensure that previous biogenesis events had been successfully executed. However, some of the abovementioned structures have been determined with *in vitro* reconstituted particles derived from mature 60S ribosomes; thus, important information about the interdependence of the cytoplasmic events might have been overlooked.

Taking these findings together, a unique feature of 60S biogenesis is the incorporation of a preassembled 5S RNP. Structural and functional investigations have already revealed fascinating insights into rRNA processing and conformational rearrangements. Finally, nuclear export and cytoplasmic maturation events are coupled with quality control checkpoints that monitor the functional integrity.

Concluding Remarks and Future Perspectives

Recent progress in the structural understanding of preribosomal particles has greatly fueled our knowledge about ribosome biogenesis. However, our structural view remains far from

Outstanding Questions

The mechanistic aspects of the early biogenesis events remain poorly understood. How are the earliest pre-ribosomal particles formed? What are the molecular mechanisms that promote the transition from the 90S pre-ribosome to the first pre-40S intermediate? Does the first distinct pre-60S particle form an rRNA-folding cage in a manner similar to the biogenesis-factor ensemble of the 90S pre-ribosome? When and how is the preassembled 5S RNP incorporated into nascent pre-60S particles?

Will it be possible to obtain a structural view of additional preribosomal particles? Of special interest are high-resolution structures of early pre-60S, nuclear and cytoplasmic pre-40S, and 80S-like intermediates. These are expected to shed light on ITS1 and ITS2 processing, the transition from 90S to pre-40S particles, and 40S quality control prior to 20S processing.

Several energy-consuming biogenesis factors are essential for ribosome assembly; however, many of these are involved in checkpoint regulations rather than in powering energetically unfavorable reactions. Is the driving force for ribosome assembly contained in the rRNA itself? Does correct folding of the rRNA create a lower-energy state that is guided, monitored, and regulated by the associated biogenesis factors?

Ribosome assembly in prokaryotes involves only few biogenesis factors and can be reconstituted *in vitro*. Why is the assembly process in eukaryotes much more complicated than in prokaryotes? Will it be possible to recapitulate eukaryotic ribosome biogenesis *in vitro*?

Ribosomes are essential for cell growth and division. Is ribosome biogenesis an appropriate target to restrict the deregulated growth of cancer cells? Can future studies yield additional small-drug inhibitors that may have the potential to be used in medical applications?

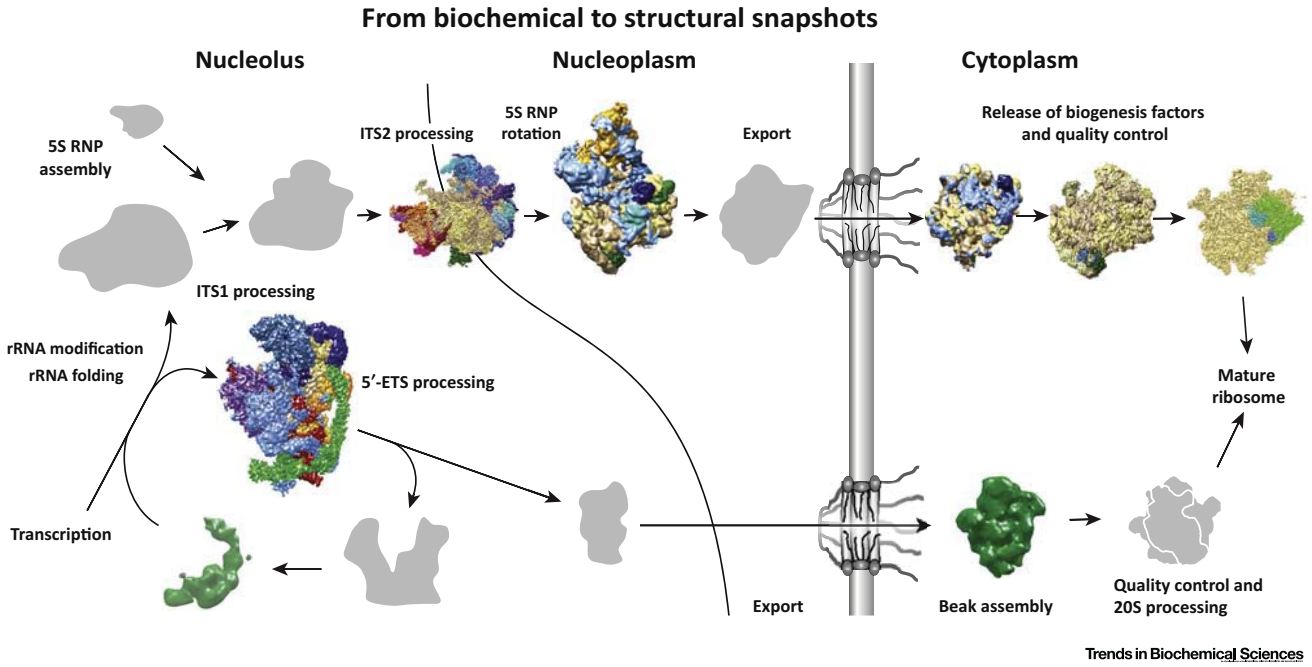


Figure 6. Towards a Structural View of Ribosome Assembly. Recent progress in cryoelectron microscopy (cryo-EM) has provided us with several high-resolution structures of preribosomal particles. The structures of the 90S preribosome (EMD 8143, PDB 5jpq; EMD 8473, PDB 5tzs; and EMD 6695, PDB 5WYJ), the Nog2 pre-60S particle (EMD 6615, PDB 3jct), the Rea1-containing Rix1 pre-60S particle (EMD 3199, PDB 5fl8), the Yvh1 pre-60S particle (EMD 4096), and the *in vitro*-reconstituted 60S–Arx1–Alb1–Rei1 (EMD 3151, PDB 5apo) and 60S–eIF6–SBDS–EFL1 (EMD 3146, PDB 5anb) particles are shown in the context of the ribosome assembly pathway (Figure 2). For simplicity, the cryo-EM structures of the cytoplasmic, Nmd3-containing pre-60S ribosome (EMD 9569, pdb 5h4p and EMD 8362, PDB 5T62) have been omitted. Moreover, lower-resolution structures of the UTP-B module (EMD 8223) and pre-40S particles (EMD 1927; see also EMD 8346) are shown. Evidently, the structures of many important assembly modules and preribosomal intermediates remain to be determined (shown in gray).

complete and many preribosomal particles await structural determination by cryo-EM, such as the first distinct pre-40S and pre-60S intermediates (Figure 6; see Outstanding Questions). It would also be interesting to visualize 80S-like ribosomes as they subject SSUs to final quality control before their engagement in translation. We expect that future structural, enzymatic, and reconstitution approaches will continue to benefit from the utilization of thermophilic proteins and preribosomal particles from the eukaryotic organism *C. thermophilum* [27,59,109]. Further mechanistic and structural insights into ribosome biogenesis may promote the identification and design of small-molecule inhibitors like diazaborine or the ribozinoindoles, which act as specific inhibitors of the AAA-ATPases Drg1 and Rea1, respectively [110,111]. Similar to inhibitors of RNA polymerase I, such molecules are expected to have great potential for the development of novel strategies in cancer treatment [112]. In addition, solving the puzzle of ribosome assembly, by the combination of functional and structural approaches, will be instrumental for future medical advances, including our understanding of ribosomopathies.

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