Ribosome Biogenesis in the Yeast *Saccharomyces cerevisiae*

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ABSTRACT Ribosomes are highly conserved ribonucleoprotein nanomachines that translate information in the genome to create the proteome in all cells. In yeast these complex particles contain four RNAs (>5400 nucleotides) and 79 different proteins. During the past 25 years, studies in yeast have led the way to understanding how these molecules are assembled into ribosomes *in vivo*. Assembly begins with transcription of ribosomal RNA in the nucleolus, where the RNA then undergoes complex pathways of folding, coupled with nucleotide modification, removal of spacer sequences, and binding to ribosomal proteins. More than 200 assembly factors and 76 small nucleolar RNAs transiently associate with assembling ribosomes, to enable their accurate and efficient construction. Following export of preribosomes from the nucleus to the cytoplasm, they undergo final stages of maturation before entering the pool of functioning ribosomes. Elaborate mechanisms exist to monitor the formation of correct structural and functional neighborhoods within ribosomes and to destroy preribosomes that fail to assemble properly. Studies of yeast ribosome biogenesis provide useful models for ribosomopathies, diseases in humans that result from failure to properly assemble ribosomes.

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RIBOSOMES are the cellular machines that translate the genetic code in mRNA and catalyze protein synthesis in all organisms. These ancient, complex nanomachines consist of two ribonucleoprotein subunits. The small, 40S subunit (SSU) in yeast contains one ribosomal (r)RNA [18S, 1800 nucleotides (nt) long] and 33 different ribosomal proteins (r-proteins) while the large, 60S subunit (LSU) includes three rRNAs (5S, 121 nt; 5.8S, 158 nt; and 25S, 3396 nt) plus 46 r-proteins. The SSU serves as the decoding center to bring mRNA and aminoacylated transfer (t)RNAs together. The LSU is where the peptidyltransferase reaction occurs to catalyze peptide bond formation.

Studies of ribosome assembly began with experiments in the early 1970s to reconstitute bacterial ribosomal subunits *in vitro* from their RNA and protein components (Held *et al.* 1973; Nierhaus and Dohme 1974). Since then there has been significant progress to understand ribosome assembly. More detailed studies of bacterial ribosome assembly *in vitro* have been empowered by sophisticated biochemical and biophysical tools to assay binding of r-proteins to rRNA and pathways of rRNA folding. Development of assays for pre-rRNA processing and of genetic screens for ribosome assembly factors in yeast launched investigations of eukaryotic ribosome assembly *in vivo*. This work was complemented by methods to purify and characterize assembly intermediates. Here we review progress in the last 25 years to understand how ribosomes are constructed in yeast *in vivo*.

Ribosome assembly is a major undertaking for cells, requiring a significant fraction of the resources devoted to macromolecular synthesis and trafficking. All three RNA

polymerases participate. RNA polymerases I and III transcribe the rRNAs. The messenger (m)RNAs encoding r-proteins and ribosome assembly factors comprise at least 60% of the transcripts produced by RNA polymerase II and ultimately translated by ribosomes. More than one-half of the introns removed by the yeast splicing machinery are those present in r-protein pre-mRNAs. A major class of nuclear import and export cargo includes r-proteins and assembly factors entering the nucleus and preribosomes exiting the nucleus. Assembly of the ~5500 nt of RNA and 79 r-proteins into ribosomes requires 76 different small nucleolar RNAs and >200 different assembly factors. Not only is this construction project expensive, but also it must be completed many times and in a hurry! More than 2000 ribosomes are assembled each minute in a rapidly growing yeast cell (Warner 1999).

Ribosome construction also is subject to stringent inspection; elaborate mechanisms have evolved to monitor nascent ribosomes for correct assembly. This quality control is coupled to nuclear export of pre-rRNPs and remarkably includes testing "teenage" cytoplasmic ribosomes for proper assembly, immediately before the last steps of subunit construction.

Because production of ribosomes is so closely tied to the growth and proliferation of cells, dysregulation of ribosome assembly has profound consequences on the health of organisms. Complete loss-of-function mutations in most assembly factors and r-proteins are lethal in yeast and embryonic lethal in higher organisms. Perhaps more widespread is partial loss-of-function or haploinsufficiency of these ribosomal molecules, which in humans can present as a variety of maladies, including short stature, mental retardation, joint abnormalities, bone marrow failure, craniofacial dysmorphology, and predisposition to cancer. Pathways and participants in ribosome biogenesis in eukaryotes are proving to be largely conserved. Thus a complete understanding of ribosome assembly in yeast will enable more rapid understanding of the molecular basis of human disease caused by ribosomopathies.

Structure of Yeast Ribosomes

Cryoelectron microscope (cryo-EM) reconstructions and atomic resolution crystal structures of yeast and *Tetrahymena* ribosomes have significantly enhanced our ability to design and interpret experiments to understand assembly as well as function of eukaryotic ribosomes (Armache *et al.* 2010a,b; Ben-Shem *et al.* 2011; Klinge *et al.* 2011, 2012; Jenner *et al.* 2012; Melnikov *et al.* 2012; Rabl *et al.* 2012). The structures show that RNA is present in the core of each subunit, with r-proteins embedded on the surface and sometimes protruding into the rRNA core. The 18S rRNA sequences form four phylogenetically conserved secondary structural domains: the 5′, central, 3′ major, and 3′ minor domains (Figure 1A, left). These fold into tertiary structures (Figure 1B, left), which, together with r-proteins, form the body, shoulder, platform, head, and beak structures observed in SSUs (Figure 2A). The 5.8S and 25S rRNAs in the LSU fold into six conserved domains of secondary structure (I–VI), including base pairing between 5.8S and 25S rRNAs (Figure 1A, right). These six secondary domains then fold into tertiary structures found in mature 60S subunits (Figure 1B, right). Clearly discernible features of the LSU include the central protuberance (containing 5S rRNA), the L1 stalk, and the acidic stalk (Figure 2B). Comparison of these eukaryotic ribosome structures with those of bacterial and archaeal ribosomes reveals a universally conserved structural core (Melnikov *et al.* 2012) that contains the sites for ribosome functions: the peptidyltransferase center, the polypeptide exit tunnel, and the GTPase binding site in LSUs and the tRNA-binding sites (A, P, and E), the decoding center, and mRNA entry and exit sites in SSUs. Features specific to eukaryotes include rRNA expansion segments and long amino- or carboxy-terminal extensions of many r-proteins, located mostly on the solvent-exposed surface of the subunits.

Ribosomes Are Made in the Cell Nucleolus

Ribosomes are made in a non-membrane-bound subcompartment of the cell nucleus termed the nucleolus. The single *Saccharomyces cerevisiae* nucleolus is formed around the ~150 tandem repeats of the rDNA transcription unit found on chromosome XII (Figure 3) and is defined by the act of rRNA transcription (reviewed in more detail in Thiry and Lafontaine 2005). Its crescent shape is detectable via immunostaining of known nucleolar proteins (Hernandez-Verdun *et al.* 2010).

Transcription of Pre-rRNAs by RNA Polymerase I

RNA polymerase I is the specialized RNA polymerase that transcribes the pre-rRNA

Among the three RNA polymerases in eukaryotic cells, RNA polymerase I is the busiest. This enzyme transcribes the pre-rRNA that is processed to yield mature 18S, 5.8S, and 25S rRNAs. This represents 60% of the total cellular RNA transcription. In a single typical yeast cell generation, an astonishing 200,000 ribosomes are produced. To achieve this, RNA polymerase I maintains an elongation rate of 40–60 nt/sec (French *et al.* 2003; Kos and Tollervey 2010). The ribosome biogenesis machinery consisting of pre-rRNA processing and preribosome assembly factors must keep up this demanding rate of pre-rRNA synthesis.

Transcription of the 35S primary transcript by RNA polymerase I occurs in the cell nucleolus. The initial 6.6-kb pre-rRNA includes RNAs destined for both the SSU (18S) and the LSU (5.8S and 25S) (Figure 3). The 35S pre-rRNA also bears RNA sequences that do not become part of the mature ribosome and are processed away: the 5′ external transcribed spacer (5′ETS), internal transcribed spacers 1

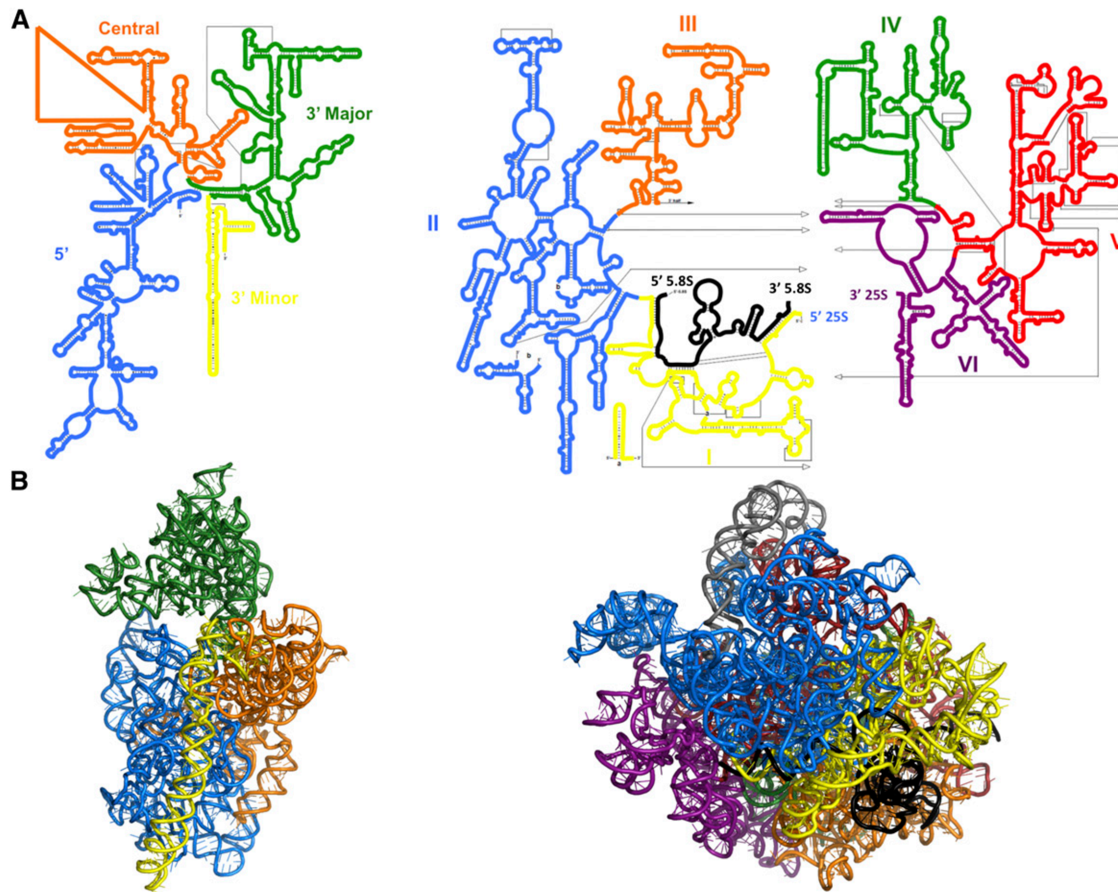

Figure 1 (A) Secondary structure of *S. cerevisiae* 18S, 25S, and 5.8S rRNAs. Left, the four domains of 18S rRNA secondary structure are indicated in different colors. Right, 25S rRNA contains six domains (I–VI) of secondary structure. The 5.8S rRNA (black) base pairs with domain I (adapted from www.rna.ccbb.utexas.edu). These secondary structures are phylogenetically conserved throughout all kingdoms, although eukaryotic rRNAs contain expansion segments not found in prokaryotic or archaeal rRNAs. (B) Tertiary structures of 18S rRNA (left) and 25S plus 5.8S rRNAs (right), from the crystal structure of yeast ribosomes (Ben-Shem *et al.* 2011).

and 2 (ITS1 and ITS2), and part of the noncoding transcribed spacer 3′ to the 25S coding sequence (3′ETS). The third RNA that is part of the LSU, the 5S rRNA, is transcribed by RNA polymerase III in the opposite direction.

The *S. cerevisiae* RNA polymerase I holoenzyme has 14 subunits and is 590 kDa in size (Table 1). Of the 14 subunits, all but Rpa34 and Rpa49 are shared or homologous with subunits in RNA polymerases II and III. A 12-Å cryo-EM structure of the complete RNA polymerase I and X-ray structures of subcomplexes reveal that the Rpa49/Rpa34 heterodimer retains features similar to those of TFIIF and TFIIE, further extending the similarities between RNA polymerase I and the other RNA polymerases (Kuhn *et al.* 2007; Geiger *et al.* 2010). Interestingly, pioneering mass spectrometry has revealed potential subcomplexes of RNA polymerase I that may serve as building blocks for its assembly (Lane *et al.* 2011).

Four general transcription factors or transcription factor complexes aid in the recruitment of RNA polymerase I to the promoter. They include UAS-binding upstream activity factor (UAF) (composed of Rrn5, Rrn9, Rrn10, and histones H3 and H4), TATA-binding protein (TBP), core factor (CF) (composed of Rrn6, Rrn7, and Rrn11 proteins and analogous to SL1 in mammals), and Rrn3 (TIF1A in mammals). Interestingly, only ~2% of RNA polymerase I is competent for transcription in yeast whole-cell extracts, and the initiation-competent RNA polymerase I is tightly associated with Rrn3 (Milkereit and Tschochner 1998). Furthermore, yeast Rrn7 and its human ortholog, TAF1B, play the role of TFIIB, functionally conserved among RNA polymerases, in preinitiation complex formation (Knutson and Hahn 2011; Naidu *et al.* 2011).

How is RNA polymerase I transcription regulated?

Classic experiments have demonstrated that ribosome biogenesis in yeast parallels growth rate (Kief and Warner 1981). The rRNA synthesis step in ribosome biogenesis is regulated by transcription initiation (French *et al.* 2003) and elongation (Zhang *et al.* 2010) and by the ratio of active to inactive rDNA repeats (Sandmeier *et al.* 2002). During growth of *S. cerevisiae* possessing an unperturbed rDNA locus, the number of active genes decreases from log to stationary phase, indicating that the proportion of active genes can be modulated between growth conditions (Dammann *et al.* 1993).

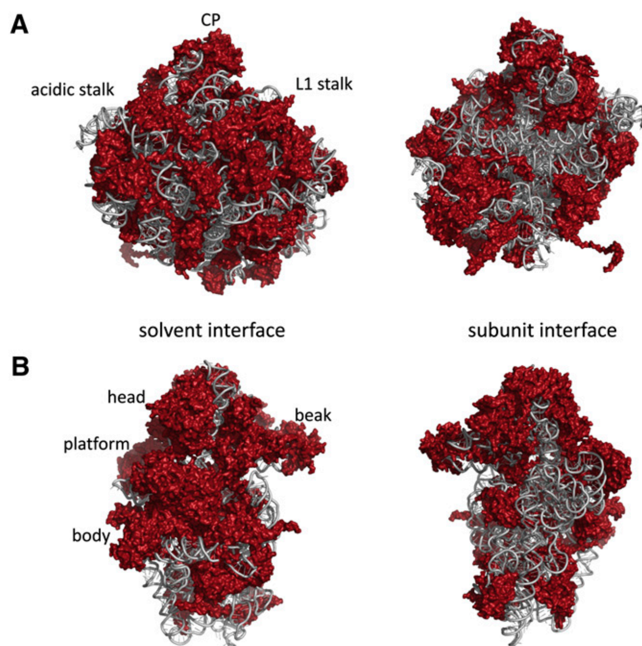

Figure 2 Crystal structure at 3.0-A resolution of *S. cerevisiae* 40S and 60S ribosomal subunits. (A) Views of the solvent-exposed surface (left) and subunit interface (right) of the 60S subunit. CP, central protuberance. (B) The solvent-exposed surface (left) and the subunit interface (right) of the 40S subunit. rRNA is represented in gray and r-proteins are in red. The crystal structure is adapted from Protein Data Bank files 3U5B, 3U5C, 3U5D, and 3U5E from Ben-Shem *et al.* (2011).

Measuring transcription by RNA polymerase I

There are several ways in which transcription of the rDNA can be assessed and quantified. Perhaps the most elegant is its direct visualization in chromatin spreads, using the electron microscope. First pioneered by Oscar Miller (Mcknight *et al.* 2012) using amphibian oocytes (Miller and Beatty 1969), the Beyer laboratory has recently used chromatin spreads to answer important questions about transcription and pre-rRNA processing in *S. cerevisiae* (French *et al.* 2003). Increasing lengths of rRNAs in the midst of its synthesis can be seen radiating from a strand of rDNA in chromatin spreads of the repeated units, with a dot of RNA polymerase I where each rRNA and rDNA strand meet (Figure 4). Counting the number of RNA polymerases in each transcription unit is one way of directly quantifying rRNA transcription. The Miller chromatin spreads are often referred to as "Christmas trees", with the rDNA representing the tree trunk and the rRNA, the branches, and the nascent RNPs (seen as knobs on the 5′ ends) representing the ornaments. A second quantitative method to assess rDNA transcription is nuclear run-on assays, where incorporation of ^{32}P[UTP] is measured after Sarkosyl is added to prevent reinitiation (Gallagher *et al.* 2004; Wery *et al.* 2009). A third method that is often used is the intensity of the 35S band, which is the primary transcript (Dez *et al.* 2007). In most cases, this does accurately echo what is seen with the other methods. However, this band results from the first pre-rRNA cleavage step at the 3′ end by Rnt1 (Kufel *et al.* 1999). Its intensity would thus result from the balance of rRNA synthesis, processing, and degradation. Since it is not the nascent transcript, but the product of the first processing step, there may be cases where its levels do not accurately reflect transcription.

Measuring active vs. inactive rDNA repeats

In a typical yeast cell, only about half of the 150 tandem repeats of the rDNA are being actively transcribed. The others are maintained in a transcriptionally inactive state. This ratio of active to inactive rDNA genes was discovered by their differential accessibility to the intercalator, psolaren (Toussaint *et al.* 2005). The actively transcribed rRNA genes are devoid of histones and are instead covered with the Hmo1 protein (analogous to UBF1 in mammals). As expected, RNA polymerase I associates with the slower-moving band but not the faster-moving one by chromatin endogenous cleavage (ChEC) (Merz *et al.* 2008). While biochemical analyses indicate that only about half of the repeats are active, single-transcript counting adds an interesting spin (Tan and van Oudenaarden 2010). These results suggest that an rDNA repeat cycles off every 1.6 sec, producing 190 RNA molecules. Therefore, the same set of rDNA repeats is not always off or always on. Within a single cell cycle, most of the repeats will thus have been active. Although only about half of them are used to synthesize rRNA at any one time, the large number of rDNA copies is maintained in yeast. Why? Recent experiments have shown that the "extra" (*i.e.*, untranscribed) copies function to protect yeast from mutagenic damage that would cause rDNA repeat instability (Ide *et al.* 2010).

Processing, Modification, and Folding of Pre-rRNA

Pre-rRNA processing is required to make ribosomes

The four transcribed spacers in pre-rRNA are removed by endonucleolytic and exonucleolytic processing reactions (Venema and Tollervey 1995) (Figure 5). Processing usually begins with cleavage by the the RNase III endonuclease Rnt1 to generate the 3′ end of the 35S primary pre-rRNA transcript (Kufel *et al.* 1999). Cleavages of the 35S pre-rRNA can occur first in the 5′ETS or in ITS1, at any one of these three sites, A_0, A_1, or A_2, as all of the related intermediates can be detected in growing yeast. The 5′ETS is removed in two steps, cleavage at site A_0 to generate 33S pre-rRNA and then cleavage at site A_1 to produce 32S pre-rRNA (Hughes and Ares 1991; Beltrame *et al.* 1994). Cleavage at the A_2 site in ITS1 generates the 20S and 27SA_2 pre-rRNAs, splitting the pathway of pre-rRNA processing and subunit maturation (Udem and Warner 1972). The separating cleavage in ITS1 can also be made by RNase mitochondrial RNA processing (MRP) at the A_3 site, producing 23S and 27SA_3 pre-rRNAs (Lygerou *et al.* 1996).

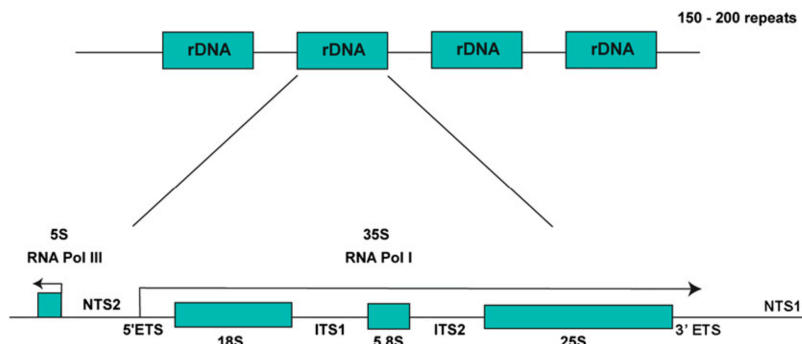

Figure 3 Organization of the rDNA locus in *S. cerevisiae*. The rDNA repeats (150–200) are located on chromosome 12. A single repeated unit is transcribed by RNA polymerase I (RNA pol I) to synthesize the 35S primary pre-rRNA transcript that will be processed to produce the mature 18S, 5.8S, and 25S rRNAs (arrow pointing right) and by RNA polymerase III (RNA pol III) to synthesize the 5S rRNA (arrow pointing left). NTS, nontranscribed spacer; ETS, external transcribed spacer; ITS, internal transcribed spacer.

The 20S pre-rRNA is packaged in 43S particles, and the $27SA_2$ pre-rRNA is found in 66S pre-rRNPs (Udem and Warner 1973; Trapman *et al.* 1975). The 43S pre-rRNPs are exported from the nucleolus through the nucleoplasm to the cytoplasm, where 20S pre-rRNA undergoes endonucleolytic cleavage at site D to remove the remaining ITS1 sequences, producing mature 18S rRNA (Fatica *et al.* 2003a). In contrast, maturation of 66S preribosomes is more complex and takes longer. Processing of the $27SA_2$ pre-rRNA continues in the nucleolus by two alternative pathways:

1. About 85–90% of $27SA_2$ pre-rRNA is shortened to the $27SA_3$ intermediate via endonucleolytic cleavage by the MRP RNAse at the A_3 site in ITS1 (Shuai and Warner 1991; Lindahl *et al.* 1992; Schmitt and Clayton 1993; Chu *et al.* 1994; Lygerou *et al.* 1996). The remaining ITS1 spacer sequences are removed from $27SA_3$ pre-rRNA by 5′–3′ exonucleases Rat1 and Rrp17, which halt at the $B1_S$ site to form the 5′ end of $27SB_S$ pre-rRNA (Henry *et al.* 1994; Oeffinger *et al.* 2009).
2. The other 10–15% of $27SA_2$ pre-rRNA is directly cleaved at the $B1_L$ site in $27SA_2$ pre-rRNA, by an unknown endonuclease, to generate $27SB_L$ pre-rRNA. The $27SB_S$ and $27SB_L$ pre-rRNAs undergo identical processing by endonucleolytic cleavage at the C_2 site in ITS2 to generate the 25.5S and $7S_S$ or $7S_L$ pre-rRNAs. The 5′ end of 25.5S pre-rRNA is trimmed by Rat1 to form mature 25S rRNA (Geerlings *et al.* 2000). The 3′ ends of 7S pre-rRNAs are processed in several steps to produce mature $5.8S_S$ and $5.8S_L$ rRNAs differing by 6 nt at their 5′ ends (Henry *et al.* 1994; Mitchell *et al.* 1996). This 5′ heterogeneity of 5.8S rRNA is conserved among eukaryotes, although its significance remains unclear. Apparently both forms are functional, since both are present in polyribosomes.

Cis-acting sequences in pre-rRNA necessary for many of these pre-rRNA processing steps have been identified by mutagenesis, using plasmid-based rDNA expression systems first developed in the Nomura laboratory (reviewed in Cole and LaRiviere 2008). Mutant rRNA expressed from a plasmid can be tagged with specific sequences to distinguish its fate from that of wild-type endogenous rRNA. The chromosomal rDNA can be deleted or its expression can be turned off, using a temperature-sensitive RNA polymerase I mutant, and plasmid-borne mutant rDNA expression can be driven by a RNA polymerase II promoter.

Although pre-rRNA processing intermediates are useful landmarks for the progression of subunit assembly, there is not necessarily an obligatory order for the entire processing pathway. While sometimes one processing step enables the next (Vos *et al.* 2004; Lamanna and Karbstein 2010), in other cases, blocking an "early" step does not prevent "later" steps from occurring (Torchet and Hermann-Le Denmat 2000). Thus, the apparent order of processing reactions can be dictated by the relative rate at which these sites are identified and used, rather than by simply completion of a previous step.

Pre-rRNA processing can occur cotranscriptionally

The widely held view for decades was that pre-rRNA modification and processing do not occur prior to the Rnt1 cleavage step that generates the 35S primary transcript (Venema and Tollervey 1999). Early experiments carried out in Jonathan Warner's laboratory on yeast spheroplasts (Udem and Warner 1972) observed synthesis of the 35S primary transcript prior to the commencement of pre-rRNA methylation and cleavage. The 35S pre-rRNA was readily detectable by metabolic labeling with labeled nucleosides and methyl-methionine and could be chased into the mature rRNAs. Similarly, Rudi Planta's laboratory (Trapman *et al.* 1975) detected a preribosome on sucrose gradients, 90S, which housed the 35S pre-rRNA (called 37S).

This view was challenged by two studies using very different methodologies. Careful analysis of yeast chromatin spreads in Ann Beyer's laboratory (Osheim *et al.* 2004) revealed cotranscriptional cleavage in the majority of nascent pre-rRNA transcripts. Surveying chromatin spreads of six different yeast strains in the electron microscope, the researchers visualized cotranscriptional cleavage in ITS1 in 40–80% of the nascent transcripts (Figure 4). This permitted the deduction that, indeed, "...co-transcriptional cleavage was the rule rather than the exception..." (Osheim *et al.* 2004, p. 948). David Tollervey's laboratory applied quantitative

Table 1 RNA polymerase I subunits

Gene name	Aliases	ORF	Essential?	Molecular weight (kDa)
RPA190	*RRN1*, A190	YOR341W	Yes	186
RPA135	*RPA2*, *RRN2*, *SRP3*, A135	YPR010C	Yes	136
RPC40	*RPC5*, AC40	YPR110C	Yes	38
RPC19	AC19	YNL113W	Yes	16
RPB5	ABC27	YBR154C	Yes	25
RPO26	*RPB6*, ABC23	YPR187W	Yes	18
RPB8	ABC14.5	YOR224C	Yes	17
RPB10	ABC10β	YOR210W	Yes	8
RPC10	*RPB12*, ABC10α	YHR143W-A	Yes	8
RPA12	*RRN4*, A12.2	YJR063W	No, ts	14
RPA43	A43	YOR340C	Yes	36
RPA14	A14	YDR156W	Yes	15
RPA49	A49	YNL248C	No, cs	47
RPA34	A34.5	YJL148W	No	27

ts: temperature-sensitive; cs: cold-sensitive

biochemical assays and mathematical modeling to the question of cotranscriptional cleavage (Kos and Tollervey 2010), arriving at the result that 70% of the transcripts were cotranscriptionally cleaved in their yeast strain. There is thus good agreement between the two studies that cotranscriptional cleavage occurs more frequently than not. However, cotranscriptional cleavage in ITS1 is not obligatory; yeast are able to and do make ribosomes both post- and cotranscriptionally.

Pre-rRNA modification can occur cotranscriptionally

The pre-rRNAs are heavily modified, largely by small nucleolar ribonucleoproteins (snoRNPs), which catalyze 2′-*O*-ribose methylation and pseudouridylation. These modifications can also occur cotranscriptionally on the nascent pre-rRNAs (Osheim *et al.* 2004; Kos and Tollervey 2010). snoRNPs are composed of a snoRNA and several protein components, among them the enzyme that catalyzes nucleotide modification. A single snoRNP can guide two RNA modification events at different sites.

The two major classes of snoRNPs that modify the pre-rRNA are named for their respective conserved snoRNA sequence motifs (Kiss *et al.* 2010; Watkins and Bohnsack 2012). The box H/ACA snoRNPs catalyze pseudouridylation at 44 sites on the rRNA, while the box C/D snoRNPs catalyze 2′-*O*-ribose methylation at 67 sites (Balakin *et al.* 1996; Kiss-Laszlo *et al.* 1996; Ganot *et al.* 1997; X. H. Liang *et al.* 2009; Watkins and Bohnsack 2012) (Figure 6). For each class of snoRNPs, catalysis is carried out by an enzyme stably associated with the snoRNA and is guided by a short snoRNA sequence. The modifications occur in sequences in the mature rRNAs that have functional importance in the ribosome (Decatur and Fournier 2002). While the lack of individual modifications or even groups of them has no effect on growth or ribosome biogenesis, depletion of the essential associated proteins disrupts ribosome biogenesis and cell growth (Schimmang *et al.* 1989; Tollervey *et al.* 1991, 1993; Gautier *et al.* 1997; Lafontaine and Tollervey 1999; Charette and Gray 2000). Additionally, misdirection of modification is deleterious (Liu *et al.* 2008). Modifications are important for optimal ribosome function, however, because ribosomes that lack them demonstrate reduced translation efficiency (Baxter-Roshek *et al.* 2007; Liang *et al.* 2007, 2009b; Jack *et al.* 2011).

The box C/D snoRNPs that carry out 2′-*O*-ribose methylation of the pre-rRNA in yeast are composed of a box C/D snoRNA, characterized by conserved box C, C′, D, and D′ motifs, and guide sequences of 10–21 nt that base pair to the RNA target (Figure 6). The guide sequences direct ribose methylation to the nucleotide base paired to the 5th nt upstream of the box D or D′ sequence (box D+5 rule). The four protein components of box C/D snoRNPs are fibrillarin/Nop1, Nop58, Nop56, and Snu13 (15.5 K in humans) (Table 2). Fibrillarin/Nop1 is the methyltransferase that catalyzes nucleotide modification, using S-adenosyl methionine as the methyl group donor (Singh *et al.* 2008), while Snu13 binds to the kink-turn in the box C/D snoRNA. Nop56 and Nop58 have extensive coiled-coil domains with which they heterodimerize. Although there are both single-particle electron microscopic and crystallographic structures of complete enzymatically active archaeal box C/D s(no)RNPs (Bleichert *et al.* 2009; Bleichert and Baserga 2010; Lin *et al.* 2011; Bower-Phipps *et al.* 2012), no structure yet exists for the yeast box C/D snoRNP.

Similarly, the box H/ACA snoRNPs that carry out pseudouridylation of the pre-rRNA in yeast are composed of a two-stem box H/ACA snoRNA, characterized by conserved H box or ACA motifs, and guide sequences of 4–8 nt (Figure 6) that base pair to the RNA target in the pseudouridylation pocket (reviewed in Kiss *et al.* 2010; Watkins and Bohnsack 2012). A single-box H/ACA snoRNA can target as many as two pseudouridylations. Box H/ACA snoRNPs consist of four protein components: Cbf5, Gar1,

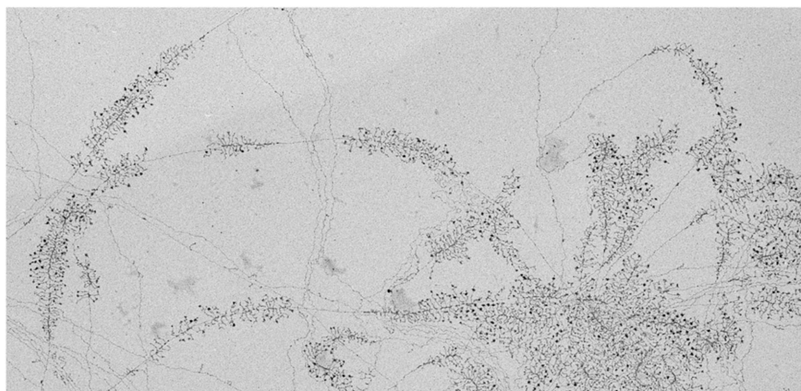

Figure 4 Yeast chromatin spreads of nucleolar contents analyzed by electron microscopy. Transcription of the repeated rDNA units can be visualized as "Christmas trees", where the trunk of the tree is the rDNA, the branches are the rRNA, and the ornaments are the knobs on the 5′ ends. The knob contains the SSU processome. Yeast chromatin spreads are courtesy of Sarah French and Ann Beyer.

Nop10, and Nhp2 (Table 2). Cbf5 (Dyskerin in humans) is the catalytic pseudouridine synthase. Atomic structures of active archaeal box H/ACA sRNPs are available (Li and Ye 2006; Wu and Feigon 2007; Duan *et al.* 2009; B. Liang *et al.* 2009; Hamma and Ferre-D'Amare 2010; Zhou *et al.* 2011), but no structure yet exists for the yeast box H/ACA snoRNP.

Coordination of folding and processing of pre-rRNA

Most of what we know about rRNA folding during ribosome assembly comes from studies of bacterial ribosomal subunit reconstitution *in vitro* (Nomura 1973; Nierhaus 1991; Woodson 2008, 2012; Sykes and Williamson 2009; Shajani *et al.* 2011). By itself, rRNA can rapidly fold into secondary structural domains (*cf*. Figure 1) and can then form tertiary structures resembling those in mature subunits (*cf*. Figure 2). RNAs often proceed down multiple alternative folding pathways, not all of which are productive. Binding of r-proteins is thought to stabilize productively folded rRNA conformers or function as chaperones to redirect misfolded rRNA (Semrad *et al.* 2004; Kovacs *et al.* 2009; Woodson 2012). Binding of r-proteins can alter RNA structure locally as well as distally to create binding sites for subsequently assembling r-proteins (Jagannathan and Culver 2004; Ramaswamy and Woodson 2009). In this way assembly *in vitro* may be driven by alternating and parallel series of rRNA folding and protein-binding steps.

Unlike the situation for *in vitro* reconstitution, pre-rRNA spacer sequences are present *in vivo*. It has been hypothesized that spacer sequences can form alternative conformers, by base pairing with sequences destined for mature rRNA, to prevent misfolding or to function as timers to prevent premature folding (van Nues *et al.* 1995b; Hughes 1996; Lamanna and Karbstein 2010; Dutca *et al.* 2011). Subsequent removal of these spacer sequences could then enable a functional sequence of subunit maturation steps. Two such examples have been described thus far. In early pre-40S particles, sequences in ITS1 3′ of the A_2 site are thought to base pair with the 3′ end of helix 44, to prevent formation of the top of helix 44 (Lamanna and Karbstein 2010). Cleavage of pre-18S rRNA at the A_2 site, followed by dissociation of the downstream ITS1 sequences, could enable a conformational switch to form a complete helix 44. This switch might allow proper positioning of the Nob1 endonuclease, to enable cleavage at the D site at later steps of subunit maturation. Thus, the order of two RNA cleavage reactions can be dictated by pre-rRNA folding. A similar scenario might occur during early stages of pre-60S subunit biogenesis (van Nues *et al.* 1995a). Sequences in the 3′ end of ITS1 are predicted to base pair with nucleotides in what will become the 5′ end of 5.8S rRNA. In mature ribosomes, these same sequences in 5.8S rRNA base pair with nucleotides in 25S rRNA to form helix 2 (Figure 7). Exonucleolytic removal of the 3′ end of ITS1 by Rat1 and Rrp17 during $27SA_3$ pre-rRNA processing may require unwinding this ITS1-5.8S rRNA helix, or alternatively might simply eliminate this helix. Either way, processing of $27SA_3$ pre-rRNA may be coupled to folding of the 5.8S/25S rRNA portion of domain I of rRNA.

Conformational switches can also be mediated by protein binding. Structural modeling and mutational analysis suggested that the ITS2 spacer can switch from a more flexible, open "ring" conformation to a stable, closed "hairpin" conformer during subunit biogenesis (Cote *et al.* 2002). DMS probing of ITS2 structure *in vivo* in wild-type cells and in mutants lacking Nop15 and Cic1 that bind ITS2 RNA showed that these proteins are important to prevent premature formation of the hairpin structure of ITS2 (Granneman *et al.* 2011). Thus, processing of spacer sequences *in vivo* is likely to be coupled to folding of the pre-rRNA as well as binding of proteins to the RNA.

Roles of Ribosomal Proteins in Assembly

Sixty-four of the 79 yeast r-proteins are essential for growth under laboratory conditions (Steffen *et al.* 2012), presumably because they are necessary for assembly and/or function of ribosomes. Nonessential r-proteins also may play important roles in ribosome biogenesis or function (Babiano *et al.* 2012; Baronas-Lowell and Warner 1990; Briones *et al.* 1998; DeLabre *et al.* 2002; Peisker *et al.* 2008; Remacha *et al.* 1995; Sachs and Davis 1990; Steffen *et al.* 2012; Yu and Warner 2001).

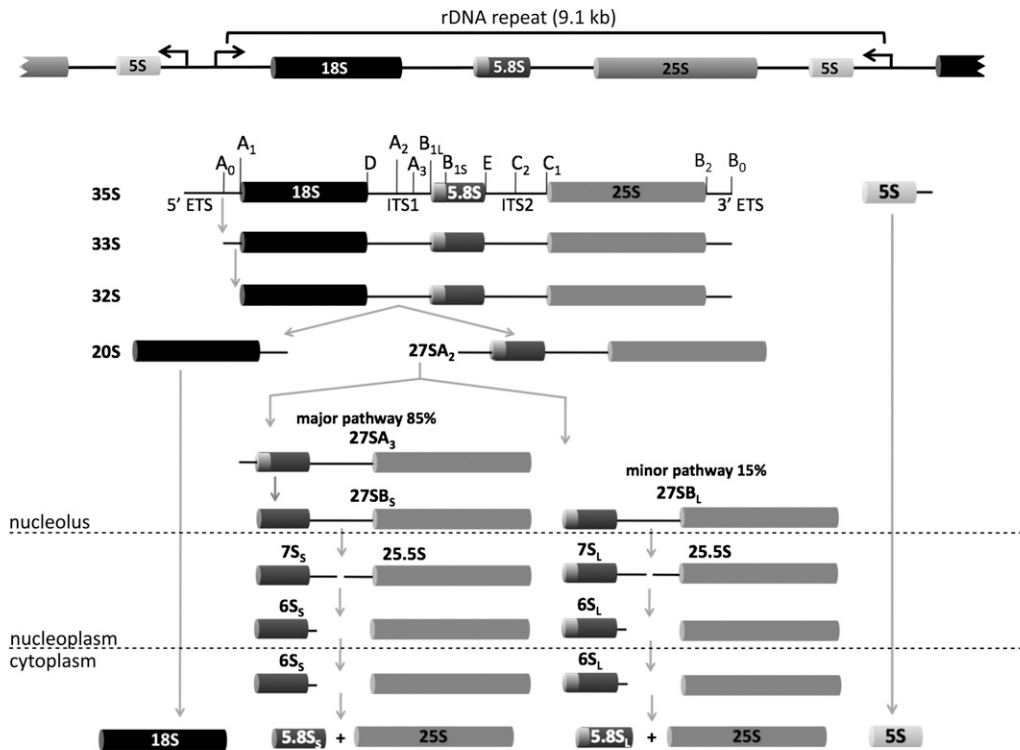

Figure 5 Pathway for processing of yeast pre-rRNA. The 35S pre-rRNA is transcribed by RNA polymerase I and contains sequences for 18S, 5.8S, and 25S rRNAs (black, dark gray, and light gray cylinders) flanked and separated by external and internal transcribed spacers (ETS and ITS, solid lines). Pre-5S rRNA (white cylinder) is transcribed by RNA polymerase III. Spacer sequences are removed from pre-rRNA by the indicated series of endonucleolytic and exonucleolytic processing steps, within assembling ribosomes. Each processing site is indicated. Processing begins in the nucleolus of the cell, but later steps occur in the nucleoplasm and cytoplasm. Note that in rapidly dividing cells, the majority of pre-rRNA undergoes cotranscriptional cleavage at the A_0, A_1, and A_2 sites before transcription is completed.

Fifty-nine of the 79 yeast r-proteins are encoded by two paralogous genes (Simoff *et al.* 2009). The amino acid sequences of 38 of these r-protein pairs differ from each other (usually by only 1-5 amino acids), suggesting the possibility of paralogue-specific functions. Consistent with this idea, deletion of one paralogue gene can result in a qualitatively different mutant phenotype than deletion of the other. These include defects in translation of localized *ASH1* mRNA (Komili *et al.* 2007), bud site selection (Ni and Snyder 2001), actin organization (Haarer *et al.* 2007), and sporulation (Enyenihi and Saunders 2003). Komili *et al.* (2007) proposed a ribosome code, in which physical heterogeneity of ribosomes could reflect functional heterogeneity, to regulate translation of different target mRNAs (reviewed in Gilbert 2011; Xue and Barna 2012). Thus, differential expression and assembly of r-protein paralogues under different conditions may provide additional flexibility for translational control.

All r-proteins directly interact with rRNA in mature ribosomes, although to varying extents. Some penetrate deeply into the rRNA core, and many have extensions that thread across the RNA surface (Armache *et al.* 2010a; Ben-Shem *et al.* 2011; Rabl *et al.* 2012). By affecting folding of both proximal and distal rRNA, r-proteins may help create binding sites for other proteins or trigger conformational switches in pre-rRNA necessary for removal of spacer sequences. Because most r-proteins are also exposed on the surface of the ribosome, they can serve as direct binding sites for assembly or translation factors.

The functions *in vivo* of r-proteins in yeast ribosome assembly were first examined by constructing strains conditional for their synthesis. Studies of individual yeast r-proteins (Moritz *et al.* 1990; Deshmukh *et al.* 1993; van Beekvelt *et al.* 2001; Jakovljevic *et al.* 2004, 2012; Leger-Silvestre *et al.* 2004; Martin-Marcos *et al.* 2007; Rosado *et al.* 2007b; Zhang *et al.* 2007; Robledo *et al.* 2008; Babiano and De La Cruz 2010; Neueder *et al.* 2010; Fernandez-Pevida *et al.* 2012; Gamalinda *et al.* 2013; Ohmayer *et al.* 2013) and, more recently, systematic examination of r-proteins (Ferreira-Cerca *et al.* 2005; Ferreira-Cerca *et al.* 2007; Poll *et al.* 2009; Jakovljevic *et al.* 2012; Gamalinda *et al.* 2013; Ohmayer *et al.* 2013) demonstrated that the absence of any one r-protein leads to a defect in a distinct pre-rRNA processing step. Interestingly, these phenotypes strongly correlate with the locations of the corresponding r-proteins within mature subunits. R-proteins located in the body of SSUs and bound closer to 5′ domains of 18S rRNA are important for early steps in pre-rRNA processing. In contrast, r-proteins important for later steps are predominantly located in the head of the SSU, bound to 3′ domains of 18S rRNA. Likewise, LSU r-proteins bound to domains I and II in the 5′ end of 25S/5.8S rRNA, in a band around the "equator" of the solvent-exposed surface of the 60S subunit, are necessary for early steps, processing of $27SA_2$ and $27SA_3$ pre-rRNA. R-proteins bound to domains I and III, located on the bottom of the subunit near the polypeptide exit tunnel, are necessary for a middle step of pre-rRNA processing, cleavage of 27SB pre-rRNA. Those r-proteins involved in late steps, processing of 7S pre-rRNA and nuclear export, are located near the central protuberance and on the subunit interface surface of LSUs.

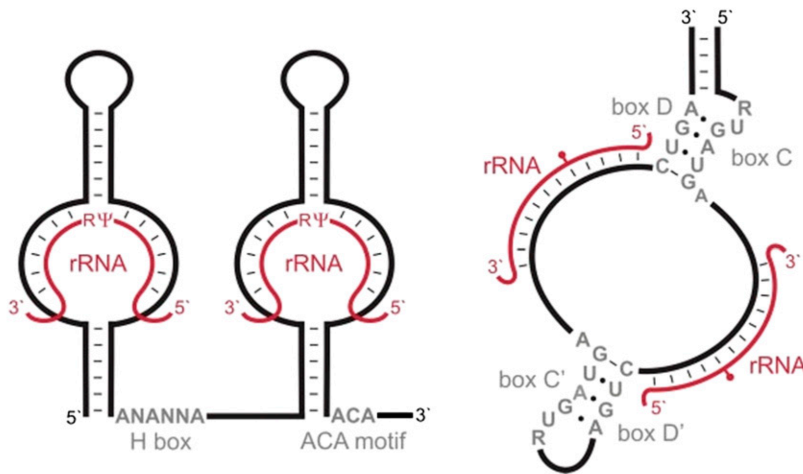

Figure 6 Box H/ACA and box C/D snoRNAs target pre-RNA modification via base pairing. A Box H/ACA snoRNA is shown on the left. A box C/D snoRNA is shown on the right. snoRNAs are indicated in black. Target rRNAs are indicated in red. This diagram is adapted from Figure 1 of Watkins and Bohnsack (2012).

Additional roles for r-proteins can be revealed by assaying effects of mutations more subtle than depletions. While depletion of S5 or S14 blocks early steps of pre-rRNA processing (Moritz *et al.* 1990; Jakovljevic *et al.* 2004; Ferreira-Cerca *et al.* 2007), alanine substitution mutations in three of the last four amino acids of S14 (Jakovljevic *et al.* 2004) or deletion of the last seven amino acids of S5 (Neueder *et al.* 2010) block a later step, cleavage at site D in 20S pre-rRNA. Thus S5 and S14 may direct proper folding of pre-rRNA necessary to form stable early assembly intermediates, but they also are important to create a proper local environment at the head/cleft interface of SSUs, near the 3′ end of 20S pre-rRNA, to enable a late step in pre-rRNA processing.

Ribosome Biogenesis Requires Many Ribosome Assembly Factors

In addition to r-proteins, ∼200 different assembly factors participate in the formation of ribosomes in yeast (Table 3 and Table 4). Most of these proteins are essential and conserved throughout eukaryotes, and all are present in assembling ribosomes. Yeast ribosome assembly factors were first discovered by classic or molecular genetic approaches (Fabian and Hopper 1987; Sachs and Davis 1990; Shuai and Warner 1991; Tollervey *et al.* 1991; Girard *et al.* 1992; Lee *et al.* 1992; Ripmaster *et al.* 1992; Jansen *et al.* 1993; Schmitt and Clayton 1993; Berges *et al.* 1994; Chu *et al.* 1994; Sun and Woolford 1994). Development of the tandem affinity purification method and development of mass spectrometric methods to identify proteins were important breakthroughs that enabled purification of preribosomes from yeast and led to identification of many more assembly factors (Bassler *et al.* 2001; Harnpicharnchai *et al.* 2001; Saveanu *et al.* 2001; Dragon *et al.* 2002; Fatica *et al.* 2002; Grandi *et al.* 2002). More than 70 proteins are reproducibly found in affinity-purified pre-40S ribosomal subunits and specifically function in their assembly (Table 3). Approximately 90 proteins can be found in 66S precursors to mature 60S subunits and play a role in assembly (Table 4). Among these proteins are endo- and exonucleases implicated in processing pre-rRNA, enzymes that modify RNA or proteins, RNA helicases/ATPases, AAA ATPases, GTPases, kinases and phosphatases, RNA-binding proteins, putative scaffolding proteins, and, interestingly, a few proteins that are highly homologous to r-proteins. Most of these factors have been assigned to function at a particular step in pre-rRNA processing, nuclear export, or subunit maturation, based primarily on the phenotypes observed upon their depletion.

Hierarchy of Assembly: Maturation from Complex to Simpler Preribosomal Particles

The original assembly maps that emerged from thermodynamic measurements of formation of stable bacterial r-protein–rRNA complexes *in vitro* suggested that assembly proceeds in a hierarchical, cooperative fashion (Held *et al.* 1973; Nomura 1973; Nierhaus and Dohme 1974). Primary rRNA-binding proteins are required to initiate assembly and enable the subsequent association of secondary binding proteins, which are necessary for assembly of tertiary r-proteins. More recent kinetic analyses of rRNA folding and r-protein binding to rRNA *in vitro*, as well as cryo-EM visualization of assembly intermediates, led to a more complex model for the assembly landscape *in vitro* (Woodson 2008, 2012; Sykes and Williamson 2009; Mulder *et al.* 2010; Shajani *et al.* 2011).

The hierarchy of assembly of r-proteins *in vivo* was first examined by pulse-labeling experiments in yeast, which demonstrated that many r-proteins associate with preribosomes at the earliest stages of subunit biogenesis (Kruiswijk *et al.* 1978). Consistent with this work, most r-proteins copurify with the earliest pre-rRNAs and assembly factors (Ferreira-Cerca *et al.* 2007; Babiano and De La Cruz 2010; Babiano *et al.* 2012; Jakovljevic *et al.* 2012; Gamalinda *et al.* 2013). However, the order of assembly of r-proteins relative to each other cannot be distinguished at any higher

Table 2 Protein components of yeast snoRNPs

Protein function	Box C/D snoRNP	Box H/ACA
Modification enzyme	Fibrillarin/Nop1	Cbf5
RNA binding	Snu13	Nhp2
	Nop56 (coiled coil)	Nop10
	Nop58 (coiled coil)	Gar1

resolution by existing methods. Nevertheless, there does appear to be a hierarchy of ever stronger association of r-proteins with preribosomes. Binding of r-proteins to pre-rRNA is strengthened as assembly proceeds; association with pre-rRNAs becomes less and less sensitive to high-salt washes, as subunits mature (Ferreira-Cerca *et al.* 2007). A few r-proteins, S10, S26, L10, L24, L29, L40, L42, P0, P1, and P2, do not join preribosomes until very late, in the cytoplasm (Saveanu *et al.* 2003; Ferreira-Cerca *et al.* 2005, 2007; Lo *et al.* 2010; Strunk *et al.* 2011). Delaying their assembly until this late stage may provide opportunities to more completely survey the structural and functional integrity of different domains. See *Cytoplasmic Maturation of Pre-40S and Pre-60S Subunits*.

As for r-proteins, the relative timing of association of assembly factors with preribosomes has been mapped by assaying which pre-rRNAs and proteins copurify with each epitope-tagged factor (Nissan *et al.* 2002; Schafer *et al.* 2003; Kressler *et al.* 2010). Most assembly factors are present in several consecutive assembly intermediates. Pre-40S and pre-60S subunits begin as relatively complex nucleolar particles containing many but not all of the assembly factors and r-proteins. As these initial preribosomes mature, they undergo remodeling, including release of some assembly factors, to form pre-rRNPs containing fewer constituents (Nissan *et al.* 2002; Schafer *et al.* 2003). This transition is more dramatic for pre-40S particles than for pre-60S ribosomes. The earliest precursor to 40S subunits contains the U3 snoRNA and >75 proteins (Figure 8 and Table 3) (Dragon *et al.* 2002; Grandi *et al.* 2002). Following cleavage at the A_2 site, the U3 snoRNA and a large number of the early-acting factors are jettisoned, and a few late-acting factors assemble onto pre-rRNPs in middle or late stages of subunit maturation (Panse and Johnson 2010; Strunk *et al.* 2011).

At least 45–50 assembly factors are present in early pre-60S ribosomes containing $27SA_2$ or $27SA_3$ pre-rRNA (Figure 8 and Table 4). Approximately one-third of these proteins are not detected in later preribosomes containing primarily 27SB pre-rRNA (Kressler *et al.* 2008). A small number of assembly factors join these midstage intermediates, to catalyze release of the early factors, enable processing of the ITS2 spacer from 27SB pre-rRNA, or facilitate nuclear export of the pre-rRNPs (Saveanu *et al.* 2001; Nissan *et al.* 2002; Lebreton *et al.* 2006a; Kressler *et al.* 2008; Ulbrich *et al.* 2009). Following export, 10–12 late-acting factors dock with cytoplasmic preribosomes, to facilitate the final steps in pre-rRNA processing, assembly of several late r-proteins, and removal of the last 6–8 other assembly factors (Lo *et al.* 2010; Bradatsch *et al.* 2012; Greber *et al.* 2012).

Nucleolar Pre-rRNA Cleavage Steps in Pre-40S Biogenesis

A large RNP, the SSU processome, is required for the nucleolar cleavage steps that mature the pre-18S rRNA

The 18S rRNA, destined for the mature SSU, is processed from the 35S pre-rRNA by a cleavage step in ITS1 that is usually cotranscriptional. This cleavage step separates the pre-18S rRNA from the pre-LSU rRNAs, 5.8S and 25S. The separating cleavage at A_2 as well as the other detectable cleavages in the pre-18S rRNA at sites A_0 and A_1 are dependent on the presence of the U3 snoRNA (originally referred to as snR17 in yeast) (Hughes and Ares 1991). While the U3 snoRNA belongs to the class of box C/D snoRNPs, it does not carry out rRNA methylation, but instead uses three short stretches of its 5′ 70 nt to base pair with three different sites in the 5′ETS to effect pre-rRNA cleavage (Beltrame and Tollervey 1992, 1995; Beltrame *et al.* 1994; Hughes 1996; Mereau *et al.* 1997; Sharma and Tollervey 1999; Borovjagin and Gerbi 2005; Dutca *et al.* 2011; Marmier-Gourrier *et al.* 2011). In general, the presence of the U3 snoRNA has been shown to be required for correct folding of the pre-rRNA (Dutca *et al.* 2011).

Base pairing between the U3 snoRNA and sequences at the start of the 18S rRNA has been proposed to prevent premature formation of the central pseudoknot (Hughes and Ares 1991). Additionally, U3 snoRNA sequences near the 3′ end (nucleotides 303–320) have recently been cross-linked to sequences in the 18S rRNA near the pseudoknot (Kudla *et al.* 2011). However, these nucleotides are part of a base-paired central stem that is required for function (Samarsky and Fournier 1998). Indeed, the stem as a structural component, and not the nucleotide sequence itself, is essential, making it difficult to reconcile a role for one of its strands in pre-rRNA base pairing.

The U3 snoRNA base pairs with the pre-rRNA to assemble a large ribonucleoprotein termed the SSU processome (Dragon *et al.* 2002). The SSU processome was originally purified from *S. cerevisiae*, using affinity methods, and contains ≥70 proteins (Table 3) that all co-immunoprecipitate the U3 snoRNA. What constitutes the SSU processome? It is likely a dynamic macromolecule that assembles around the nascent pre-SSU rRNA (23S portion of the 35S pre-rRNA) as it is being transcribed by RNA polymerase I (Dragon *et al.* 2002). In addition to the U3 snoRNP and other modification snoRNPs, numerous SSU ribosome biogenesis factors associate with the pre-SSU rRNA (Table 3). Many of the SSU processome protein components contain protein–protein interaction domains such as WD repeats and coiled-coil domains, although there are a few identifiable known RNA-binding domains. Some SSU r-proteins can also be found associated with it (Bernstein *et al.* 2004). However, assembly factors specific for LSU biogenesis are not found associated with the SSU processome in yeast. When depleted, almost all of the essential proteins cause defects in pre-rRNA cleavage at the A_0, A_1, and A_2 sites. Disruption or depletion of the nonessential proteins causes cold sensitivity, a well-known hallmark of defects in ribosome

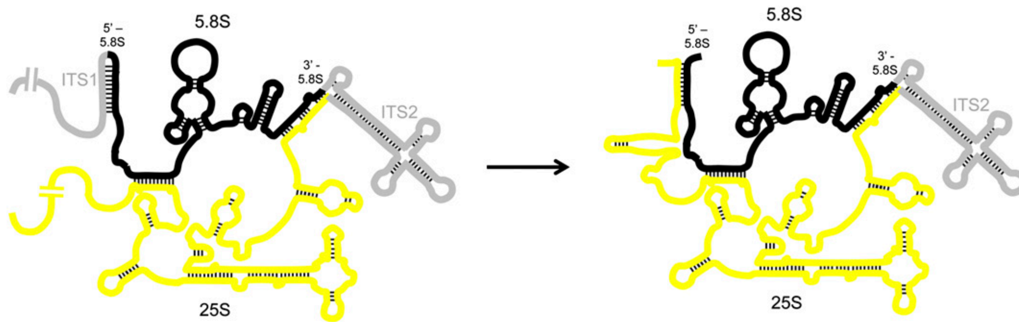

Figure 7 Conformational switch of pre-rRNA involving the removal of ITS1 spacer RNA. Left, sequences in the 3′ end of ITS1 spacer (gray) are predicted to base pair with nucleotides in what becomes the 5′ end of 5.8S rRNA (black). Right, in mature 60S subunits, these same sequences in 5.8S rRNA base pair with nucleotides in 25S rRNA to form helix 2, to which r-protein L17 binds. Removal of the 3′ end of ITS1 from $27SA_3$ pre-rRNA may be required to form a stable neighborhood including helix 2. Alternatively, remodeling of preribosomes to form a stable helix 2, including bound L17, may enable 5′ exonucleolytic processing of $27SA_3$ pre-rRNA.

assembly (Guthrie *et al.* 1969), and defects in cleavage at the A_1 and A_2 sites. Depletion experiments also indicate that the SSU processome corresponds to the large terminal knobs at the 5′ ends of most of the pre-rRNAs in Miller chromatin spreads, transforming the SSU processome into the "ornaments" on Miller's Christmas trees (Dragon *et al.* 2002).

Trapman and Planta (Trapman *et al.* 1975) predicted the occurrence of an entity like the SSU processome. They found that the percentage of protein (by mass) in yeast preribosomes decreased as they matured into ribosomes. This was in contrast to similar studies in bacteria where the percentage of protein increased upon ribosome maturation. This important observation suggested that there were additional ribosome biogenesis factors that facilitated preribosome assembly that did not become part of mature ribosomes, a so-called "...special class of proteins..." (Trapman *et al.* 1975, p. 103). However, the term "processome" itself was first coined sometime later, in 1993 (Fournier and Maxwell 1993).

At the same time, a macromolecule with similar composition was purified, characterized, and termed the "90S preribosome" (Grandi *et al.* 2002), after a term coined by Udem and Warner (1972) to describe a large preribosomal complex that processes the primary RNA polymerase I transcript, the 35S pre-rRNA. The 35S pre-rRNA contains the coding sequences for both LSU and SSU mature rRNAs; however, the newly purified 90S preribosome had no LSU biogenesis factors associated with it. As the 90S preribosome was named before the discovery that cotranscriptional cleavage in ITS1 was the norm (Osheim *et al.* 2004; Kos and Tollervey 2010), it is likely that it also represents a form of the SSU processome.

Even so, subsequent experiments uncovered the existence of what may be the 90S preribosome (Lebaron *et al.* 2005). Purification of RNPs associated with the jack-of-all-trades Prp43 DEAH Box protein revealed that it was associated with the 35S pre-rRNA, as well as the 27SA/B rRNAs (pre-LSU rRNAs) and the 20S rRNA (pre-SSU). Ribosome biogenesis factors required for both SSU and LSU biogenesis were thus found in association with it. One caveat, since Prp43 remains associated with the pre-SSU and LSU rRNAs after cleavage of the 35S primary transcript, is that it cannot definitively be said which of the copurifying proteins are specifically associated with the 35S pre-rRNA.

Subcomplexes are the functional units of the SSU processome

The SSU processome is assembled from protein and snoRNP subcomplexes in a hierarchical manner (Perez-Fernandez *et al.* 2007). Affinity purification of tagged SSU processome components present in the supernatant after prefractionation by high-speed centrifugation ($180,000 \times g \times 45$ min) followed by mass spectrometry revealed three subcomplexes: UtpA (Utp4, Utp8, Utp9, Utp10, Utp15, Utp17, and Pol5), UtpB [Pwp2 (aka Utp1), Utp6, Utp13, Utp12 (aka Dip2), Utp18, and Utp21], and UtpC (Utp22, Rrp7, Cka1, Cka2, Ckb1, and Ckb2) (Krogan *et al.* 2004). The U3 snoRNP, composed of a 333-nt-long snoRNA, and the fibrillarin (aka Nop1), Nop56, Nop58, Snu13, and Rrp9 proteins can also be detected as a subcomplex. There remain scores of SSU processome proteins not assigned to a subcomplex, suggesting that there may be additional subcomplexes yet to be discovered.

Most of the proteins that compose the UTP A subcomplex have been shown to have a dual function in pre-rRNA processing and optimal rDNA transcription (Gallagher *et al.* 2004). The transcription Utps (t-Utps) consist of Utp4, Utp5, Utp8, Utp9, Utp10, Utp15, and Utp17. The t-Utp subcomplex can be detected even in the absence of the SSU processome. Northern blot analysis revealed defects in pre-rRNA processing upon conditional t-Utp depletion. Similarly, direct counting of RNA polymerases in Miller chromatin spreads and nuclear run-on analysis of t-Utp-depleted yeast revealed reduced (but not absent) transcription. Note that there is overlap between the t-Utp and UTP A components (Krogan *et al.* 2004), and the terms are often used interchangeably. However, it is not yet known whether the two complexes represent different functional forms. The UTP A/t-Utp subcomplex is an early binder of the pre-rRNA and required for the recruitment of the other identified subcomplexes and for the recruitment of other SSU processome components (Perez-Fernandez *et al.* 2007). Immunoprecipitation experiments indicate that the first 135–138 nt of the

Table 3 Assembly factors that function in maturation of 40S ribosomal subunits in *Saccharomyces cerevisiae*

Name/alias	Human ortholog; accession no.	Subcomplex	Comments	Reference
Nop1/Lot3	Fibrillarin; NP_001427	Box C/D	2′-*O*-methyltransferase; GAR and fibrillarin domains	Lischwe *et al.* (1985); Ochs *et al.*, (1985) Tollervey *et al.* (1991)
Nop56/Ski1	NOP56; NP_006383	Box C/D	Nop5, NOSIC and Nop domains; RNAP I site and KKE/D repeats	Lafontaine and Tollervey (2000)
Nop58/Nop5	NOP58; NP_057018	Box C/D	Nop5, NOSIC, and Nop domains; CCs, RNAP I site, and KKE/D repeats	Wu *et al.* (1998)
Snu13	15.5K/NHP2-like protein 1; NP_001003796	Box C/D	rpL7Ae motif; binds to the K-turn motifs of U3 snoRNA; also part of the U4/U6-U5 tri-snRNP	Watkins *et al.* (2000)
Rrp9	U3-55K; NP_004695	U3 snoRNP	CCs and WD40 repeats; binds to the B/C motif of U3 snoRNA	Lukowiak *et al.* (2000); Venema *et al.* (2000)
Imp3	IMP3; NP_060755	Mpp10	rpS4/S9 RRM	Lee and Baserga (1999)
Imp4	IMP4; NP_219484	Mpp10	σ^{70}-like motif/Brix domain	Lee and Baserga (1999)
Mpp10	MPHOSPH10; NP_005782	Mpp10	CCs and glutamic acid-rich repeats; associated with the hinge region of U3 snoRNA	Baserga *et al.*, (1997); Dunbar *et al.* (1997)
t-Utp4	Cirhin; NP_116219	UtpA	WD40 repeats	Dragon *et al.* (2002); Gallagher *et al.* (2004); Krogan *et al.* (2004)
t-Utp5	WDR43; NP_055946	UtpA	Utp12 motif, WD40 and GAR repeats	Dragon *et al.* (2002); Gallagher *et al.* (2004)
t-Utp8		UtpA	No known motifs; also involved in nuclear tRNA export	Dragon *et al.* (2002); Gallagher *et al.* (2004); Strub *et al.* (2007)
t-Utp9		UtpA	WD40 repeats	Dragon *et al.* (2002); Gallagher *et al.* (2004); Krogan *et al.* (2004)
t-Utp10	BAP28/HEATR1; NP_060542	UtpA	t-Utp10 domain, HEAT/ARM-type fold and BAP28-like motif	Dragon *et al.* (2002); Gallagher *et al.* (2004); Krogan *et al.* (2004)
t-Utp15	UTP15; NP_115551	UtpA	WD40 repeats and Utp15 motif	Dragon *et al.* (2002); Gallagher *et al.* (2004); Krogan *et al.* (2004)
t-Utp17/Nan1	WD repeat domain 75; AAH40567	UtpA	WD40 repeats	Dragon *et al.* (2002); Gallagher *et al.* (2004); Krogan *et al.* (2004)
Pol5	Myb-binding protein 1A; AAF33021	UtpA	DNA polymerase φ; not required for DNA replication; required for rRNA transcription; ARM-type fold	Shimizu *et al.* (2002); Gallagher *et al.* (2004); Krogan *et al.* (2004)
Utp1/Pwp2	PWP2; NP_005040	UtpB	WD40 repeats and Utp12 motif	Dragon *et al.* (2002); Dosil and Bustelo (2004); Krogan *et al.* (2004); Champion *et al.* (2008)
Utp6	UTP6; NP_060898	UtpB	HAT motifs	Dragon *et al.* (2002); Dosil and Bustelo (2004); Krogan *et al.* (2004); Champion *et al.* (2008)
Utp12/Dip2	WDR3; NP_006775	UtpB	WD40 repeats and Utp12 domain	Dragon *et al.* (2002); Dosil and Bustelo (2004); Krogan *et al.* (2004); Champion *et al.* (2008)
Utp13	TBL3/transducin β-like 3; NP_006444	UtpB	WD40 repeats and Utp13 domain	Dragon *et al.* (2002); Dosil and Bustelo (2004); Krogan *et al.* (2004); Champion *et al.* (2008)
Utp18	UTP18/WDR50/CGI-48; NP_057085	UtpB	WD40 repeats	Bernstein *et al.* (2004); Dosil and Bustelo (2004); Krogan *et al.* (2004); Champion *et al.* (2008)

(continued)

Table 3, *continued*

Name/alias	Human ortholog; accession no.	Subcomplex	Comments	Reference
Utp21	WDR36/TA-WDRP; NP_644810	UtpB	WD40 repeats, Utp21 domain, and CCs	Samanta and Liang (2003); Bernstein *et al.* (2004); Dosil and Bustelo (2004); Krogan *et al.* (2004); Champion *et al.* (2008)
Rrp7	RRP7A; NP_056518	UtpC	No known motifs; also part of the CURI complex	Baudin-Baillieu *et al.* (1997); Krogan *et al.* (2004); Rudra *et al.* (2007)
Utp22	NOL6/NRAP; NP_075068	UtpC	NRAP domain; also part of the CURI complex	Peng *et al.* (2003); Samanta and Liang (2003); Bernstein *et al.* (2004); Krogan *et al.* (2004); Rudra *et al.* (2007)
Cka1	CSNK2A1; NP_808227	UtpC	α-catalytic subunit of CK2; also part of the CURI complex	Krogan *et al.* (2004); Rudra *et al.* (2007)
Cka2	CSNK2A1; NP_808227	UtpC	α′-catalytic subunit of CK2; also part of the CURI complex	Krogan *et al.* (2004); Rudra *et al.* (2007)
Ckb1	CSNK2B; NP_001311	UtpC	β-regulatory subunit of CK2; also part of the CURI complex	Krogan *et al.* (2004); Rudra *et al.* (2007)
Ckb2	CSNK2B; NP_001311	UtpC	β′-regulatory subunit of CK2; also part of the CURI complex	Krogan *et al.* (2004); Rudra *et al.* (2007)
Rrp36	C6orf153; NP_149103	UtpC?	No known motifs; contains DUF947	Gerus *et al.* (2010)
Utp2/Nop14	Nop14; NP_003694	Unclassified	Nop14-like domains and CCs; also involved in SSU nuclear export	Liu and Thiele (2001); Dragon *et al.* (2002); Milkereit *et al.* (2003)
Utp3/Sas10	UTP3; NP_065101	Unclassified	Utp3 domains, CCs, and a glutamic acid-rich region; disrupts silencing	Dragon *et al.* (2002)
Utp7/Kre31	WDR46/BING4; NP_005443	Unclassified	WD40 repeats and BING4CT; adenylate binding site	Dragon *et al.* (2002); Jwa *et al.* (2008)
Utp11	UTP11L/CGI-94; NP_057121	Unclassified	Utp11 domain and CCs	Dragon *et al.* (2002)
Utp14	UTP14A; NP_006640	Unclassified	Utp14 domain, CCs, and lysine-rich region; ATP/GTP binding site (P-loop)	Dragon *et al.* (2002)
Utp16/Bud21		Unclassified	U3 snoRNA-associated superfamily domain and CCs	Dragon *et al.* (2002)
Noc4/Utp19	NOC4L/MGC3162; NP_076983	Unclassified	Noc and CBF domains; also involved in SSU nuclear export	Milkereit *et al.* (2003); Bernstein *et al.* (2004)
Utp20	1A6/DRIM; NP_055318	Unclassified	ARM repeats, DRIM motif, and CCs	Samanta and Liang (2003); Bernstein *et al.* (2004)
Utp23	UTP23; NP_115710	Unclassified	Lysine-rich region; PINc nuclease domain not required for function	Bleichert *et al.* (2006)
Utp24/Fcf1	FCF1; NP_057046	Unclassified	PINc nuclease domain required for function	Bleichert *et al.* (2006)
Utp25	DEF/C1orf107; NP_055203	Unclassified	DEAD-box helicase-like motif; DUF1253 domain (digestive organ expansion factor)	Li *et al.* (2009); Charette and Baserga (2010); Goldfeder and Oliveira (2010)
Utp30	RSL1D1; NP_056474	Unclassified	rpL1 motif	Samanta and Liang (2003)
Bms1	BMS1; NP_055568	Unclassified	GTPase, stimulated by Rcl1; DUF663 and AARP2CN domains; lysine-rich region and CCs	Wegierski *et al.* (2001); Karbstein *et al.* (2005)
Dbp8	DDX49; NP_061943	Unclassified	DEAD-box RNA helicase; stimulated by Esf2; HELICc domain and CCs	Granneman *et al.* (2006)
Dhr1/Ecm16	DHX37; NP_116045	Unclassified	DEAH-box RNA helicase; HELICc, HA2, and OB Fold/DUF1605 domains	Colley *et al.* (2000); Granneman *et al.* (2006)
Dhr2	DEAH box polypeptide 8; AAH47327 (?)	Unclassified	DEAH-box RNA helicase; HELICc, HA2, and OB Fold/DUF1605 domains	Colley *et al.* (2000); Granneman *et al.* (2006)
Emg1/Nep1	EMG1/C2F; NP_006322	Unclassified	Member of α/β knot fold methyltransferase (SPOUT) superfamily; displays pseudouridine methyltransferase activity	Bernstein *et al.* (2004); Meyer *et al.* (2010); Wurm *et al.* (2010)
Krr1	KRR1/HRB2; NP_008974	Unclassified	KH domain and CCs	Bernstein *et al.* (2004)
Rcl1	RCL1; NP_005763	Unclassified	RNA-terminal phosphate cyclase-like protein; stimulates Bms1; no cyclase activity detected; also contains a RTC insert domain	Billy *et al.* (2000)
Nop19/Dhi1	BAF82397 (?)	Unclassified	DUF2702	

(continued)

Table 3, *continued*

Name/alias	Human ortholog; accession no.	Subcomplex	Comments	Reference
Rok1	DDX52; NP_008941	Unclassified	DEAD-box RNA helicase; HELICc domain	Granneman *et al.* (2006)
Rrp3	DDX47; NP_057439	Unclassified	DEAD-box RNA helicase; HELICc domain and CCs	Granneman *et al.* (2006)
Rrp5	PDCD11/NFBP; NP_055791	Unclassified	S1 RNA-binding motifs and HAT repeats; binds single-stranded tracts of U's; also participates in A3 cleavage in 5.8S processing	Venema and Tollervey (1996); Dragon *et al.* (2002)
Sof1	WDSOF1/DCAF13; NP_056235	Unclassified	WD40 repeats and Sof1 domain; similar to β-subunit of trimeric G-proteins	Jansen *et al.* (1993)
Dbp4/Hca4/ Ecm24	DDX10; NP_004389	Unknown	DEAD-box RNA helicase; HELICc motif and CCs	Liang *et al.* (1997)
Enp1/Meg1	bystin/BYSL; NP_004044	Unknown	bystin domain, glutamic acid-rich region, and CCs; also associates with U14	Chen *et al.* (2003); Bernstein *et al.* (2004)
Esf1	ESF1; NP_057733	Unknown	NUC153 domain, CCs, and lysine-rich region	Peng *et al.* (2004)
Esf2/Abt1	ABT1; NP_037507	Unknown	RRM and CCs; binds to RNA and stimulates ATPase activity of Dbp8	Hoang *et al.* (2005)
Fal1	DDX48/EIF4A3; NP_055555	Unknown	Member of the eIF4A subfamily of DEAD-box RNA helicases; HELICc domain	Kressler *et al.* (1997)
Fyv7		Unknown	rRNA processing domain, lysine-rich region, and CCs	Peng *et al.* (2003)
Gno1/Pxr1	PINX1; NP_060354	Unknown	Involved in snoRNA maturation and telomeres; G-patch, KKE/D repeats, and CCs	Guglielmi and Werner (2002)
Has1	DDX18; NP_006764	Unknown	DEAD-box RNA helicase; also required for LSU synthesis; HELICc domain	Emery *et al.* (2004)
Kre33	NAT10; NP_078938	Unknown	DUF1726, DUF699, and CCs	Grandi *et al.* (2002); Li *et al.* (2009)
Lcp5	Neuroguidin/NGDN; NP_001036100	Unknown	Utp3 domain and CCs	Wiederkehr *et al.* (1998)
Ltv1/Ykl2	LTV1; NP_116249	Unknown	Required for SSU nuclear export; component of the GSE complex; LTV domain and CCs	Loar *et al.* (2004)
Mrd1	RBM19; NP_057280	Unknown	RRMs and CCs	Jin *et al.* (2002)
Nop6		Unknown	RRM, lysine-rich region, and CCs, similar to hydrophilins	
Nop9	C14orf21; NP_777573	Unknown	Also required for SSU export; multiple pumilio-like RNA binding repeats	Thomson *et al.* (2007)
Nsr1/She5	Nucleolin/NCL; NP_005372	Unknown	RRMs and GAR domain; serine/glutamic acid-rich region and CCs	Kondo and Inouye (1992); Lee *et al.* (1992)
Pfa1/Sqs1	GPATCH2; NP_060510	Unknown	Stimulates the ATPase and helicase activities of Prp43p; R3H and G-patch domains	Lebaron *et al.* (2009)
Prp43/JA1	DHX15; NP_001349	Unknown	DEAH-box RNA helicase; also involved in mRNA splicing and LSU biogenesis; HELICc, HA2, and OB Fold/DUF1605 domains and CCs.	Combs *et al.* (2006)
Sen1/Cik3/ Nrd2	Senataxin/SETX/ALS4; NP_055861	Unknown	SF1/Upf1-like RNA helicase; also required for LSU synthesis and RNAP II termination; SEN1N domain, CCs, and lysine-rich region	Ursic *et al.* (1997)
Sgd1	NOM1; NP_612409	Unknown	MIF4G and MA3 domains and CCs	Li *et al.* (2009)
Slx9	DBF4-type zinc finger-containing protein 2; NP_065974	Unknown	No known domains	Bax *et al.* (2006)

pre-rRNA are sufficient for binding the t-Utps *in vivo* (J. E. G. Gallagher and S. J. Baserga, unpublished results).

Wery *et al.* (2009) have proposed that the t-Utp subcomplex is not required for transcription but for stabilization of the pre-rRNA. Transcription run-on analysis did not reveal defects in transcription upon t-Utp depletion. Furthermore, when the RNA surveillance proteins, Trf5 and Rrp6, were inactivated in a Utp5-depleted cell, pre-rRNA processing defects were rescued. Puzzlingly, the 18S rRNA that was detectably made during the rescue did not support growth. Likewise, it has been noted that no transcription defects occurred when Utp10 was depleted, although in this case transcription was assessed by examining levels of the primary transcript in a metabolic labeling (Dez *et al.* 2007) and not in a direct assay for transcription.

Conservation of a dual role in transcription and pre-rRNA processing for most of the t-Utps, however, has been observed in mammalian cells, using assays that measure transcription directly (Prieto and Mcstay 2007). Furthermore, siRNA-mediated knockdown of a metazoan-specific t-Utp, Nol11, also resulted in transcription defects in a transcription assay that uses a luciferase reporter gene (Freed *et al.* 2012). Accumulating evidence thus indicates that the function of the UTP A/t-Utp subcomplex in transcription and pre-rRNA processing is conserved from yeast to humans.

Table 4 **Assembly factors that function in maturation of 60S ribosomal subunits in *Saccharomyces cerevisiae***

Pre-rRNA processing step	Name/alias	Human ortholog; accession no.	Comments	Reference
A0, A1, A2, A3	Rrp5	PDCD11; NP_055791.1	S1-like RNA-binding domains	Venema and Tollervey (1996); Eppens *et al.* (1999); Vos *et al.* (2004)
	Nop4/Nop77	RBM28; NP_060547.2	4 RRMs	Sun and Woolford (1994)
	Dbp6	DDX51; NP_778236.2	DEAD-box RNA helicase	Kressler *et al.* (1998)
	Npa1/Urb1			Dez *et al.* (2004); Rosado and de la Cruz (2004)
	Npa2/Urb2			Rosado *et al.* (2007a)
	Nop8		Coiled coil and an RRM	Zanchin *et al.* (1999)
	Rsa3			De La Cruz *et al.* (2004)
	Dbp2	DDX5; NP_004387.1	DEAD-box RNA helicase	Bond *et al.* (2001)
	Noc1/Mak21	CEBPZ; NP_005751.2	CCAAT-binding factor	Milkereit *et al.* (2001)
	Noc2/Rix3	NOC2L; NP_056473.2	Coiled coils	Milkereit *et al.* (2001)
	Noc3	NOC3L; NP_071896.8	Coiled coils	Milkereit *et al.* (2001)
	Dbp3	DDX42; NP_031398.2	DEAD-box RNA helicase	Weaver *et al.* (1997)
	Dbp7	DDX31; NP_073616.6	DEAD-box RNA helicase	Daugeron and Linder (1998)
	Dbp9	DDX56; NP_061955.1	DEAD-box RNA helicase	Daugeron *et al.* (2001)
	Prp43/JA1	DHX15; NP_001349.2	DEAH-box RNA helicase; also involved in pre-mRNA splicing and SSU biogenesis	Combs *et al.* (2006); Leeds *et al.* (2006)
	Loc1		Also involved in *ASH1* mRNA localization	Urbinati *et al.* (2006)
	Mak5	DDX24; NP_065147.1	DEAD-box RNA helicase	Zagulski *et al.* (2003)
	Cbf5		Pseudouridine synthase catalytic subunit of box H/ACA snoRNPs	Lafontaine *et al.* (1998)
	Ssf1	PPAN; NP_064615.3	Brix domain	Fatica *et al.* (2002)
	Ssf2	EIF6; NP_064615.3	Brx1 domain	
	Rrp14	SURF6; NP_006744.2	Coiled coils	Oeffinger *et al.* (2007); Yamada *et al.* (2007)
	Rrp15	?????		De Marchis *et al.* (2005)
	Mak16	MAK16; NP_115898.2	HMG-like acidic region	Pellet and Tracy (2006)
	Nop16	NOP16; NP_057475.2		J. L. Woolford (unpublished results)
	Ebp2	EBNA1BP2; NP_001153408.1		Huber *et al.* (2000); Tsujii *et al.* (2000); Shimoji *et al.* (2012)
	Brx1	BRIX1; NP_060791.3	s70-like RNA-binding motif	Kaser *et al.* (2001); Shimoji *et al.* (2012)
	Rpf1	RPF1; NP_079341.2	s70-like RNA-binding motif	Wehner and Baserga (2002)
	Ytm1	WDR12; NP_060726.3	WD40 repeats	Miles *et al.* (2005); Sahasranaman *et al.* (2011)
	Erb1	BOP1; NP_056016.1	WD40 repeats	Pestov *et al.* (2001); Granneman *et al.* (2011); Sahasranaman *et al.* (2011)
	Nop7/Yph1	PES1; NP_055118.1	BRCT domain and coiled coils	Adams *et al.* (2002); Oeffinger *et al.* (2002); Granneman *et al.* (2011); Sahasranaman *et al.* (2011)
	Drs1	DDX27; NP_060365.7	DEAD-box RNA helicase	Merl *et al.* (2010); J. L. Woolford (unpublished results)
	Nop15	MKI67IP; NP_115766.3	RRM	Oeffinger and Tollervey (2003); Granneman *et al.* (2011); Sahasranaman *et al.* (2011)
	Cic1/Nsa3		Ribosomal protein L1 domain	Fatica *et al.* (2003b); Granneman *et al.* (2011); Sahasranaman *et al.* (2011)
	Rlp7	RL7L; NP_940888	Similar to r-protein L7	Dunbar *et al.* (2000); Sahasranaman *et al.* (2011)

(continued)

Table 4, *continued*

Pre-rRNA processing step	Name/alias	Human ortholog; accession no.	Comments	Reference
	Rrp1	RRP1B; NP_055871.1		Horsey *et al.* (2004); Sahasranaman *et al.* (2011)
	Nop12	RBM34; NP_055829.2	RRM	Wu *et al.* (2001); Granneman *et al.* (2011)
	Has1	DDX18; NP_006764.3	DEAD-box RNA helicase; also required for SSU biogensis	Dembowski *et al.* (2013)
	Rai1	DOM3Z; NP_005501.2	Binds to and stabilizes the exoribonuclease Rat1	Xue *et al.* (2000)
	Rat1/Xrn2	XRN2; NP_036387.2	Nuclear 5′–3′ single-stranded RNA exonuclease	Henry *et al.* (1994); Oeffinger *et al.* (2009)
	Xrn1	SEP1; NP_061874.3	Cytoplasmic 5′–3′ RNA exonuclease; also involved in mRNA decay	Henry *et al.* (1994); Oeffinger *et al.* (2009)
	Rrp17	NOL12; NP_077289	Nuclear 5′–3′ RNA exonuclease	Oeffinger *et al.* (2009)
C_2 cleavage	**Nip7**	NIP7; NP_057185.1	PUA domain	Zanchin *et al.* (1997); Talkish *et al.* (2012)
	Nop2/Yna1	NOP2; NP_006161.2	Putative RNA methyltransferase	Hong *et al.* (1997); Talkish *et al.* (2012)
	Spb4	DDX55; NP_065987.1	DEAD-box RNA helicase	De La Cruz *et al.* (1998); Garcia-Gomez *et al.* (2011)
	Rpf2	RPF2; NP_115570.1	Brix domain	Morita *et al.* (2002); Wehner and Baserga (2002); Zhang *et al.* (2007)
	Rrs1	RRS1; NP_055984.1		Tsuno *et al.* (2000); Zhang *et al.* (2007)
	Dbp10	DDX54; NP_001104792.1	DEAD-box RNA helicase	Burger *et al.* (2000)
	Mak11	PAK1IP1; NP_060376.2	WD40 repeats	Saveanu *et al.* (2007)
	Tif6 /Cdc95	EIF6; NP_852133.1	eIF6 domain	Basu *et al.* (2001); Senger *et al.* (2001)
	Rlp24	RSL24D1; NP_057388.1	TRASH domain and a coiled coil	Saveanu *et al.* (2003)
	Nog1	GTPBP4; NP_036473.2	GTPase	Kallstrom *et al.* (2003); Saveanu *et al.* (2003)
	Spb1	FTSJ3; NP_060117.3	Ado-Met dependent methyltransferase	Kressler *et al.* (1999)
	Nsa2	NSA2; NP_055701.1	Member of the ribosomal protein S8e superfamily	Lebreton *et al.* (2006a)
	Nog2/Nug2	GNL2; NP_037417.1	GTPase	Bassler *et al.* (2001); Saveanu *et al.* (2001)
	Nug1	GNL3L; NP_061940.1	GTPase	Bassler *et al.* (2001)
	Cgr1	CCDC86; NP_077003.1	Coiled coil	Moy *et al.* (2002)
27SB stability	Rix7	NVL; NP_002524.2	AAA-ATPase	Gadal *et al.* (2001a); Kressler *et al.* (2008)
	Nsa1	WDR74; NP_060563.2	Released from preribosomes by Rix7	Kressler *et al.* (2008)
7S processing and nuclear release of assembly factors	**Ipi3**	WDR18; NP_077005.2	WD40 repeats	Galani *et al.* (2004); Krogan *et al.* (2004)
	Ipi2/Rix1			Galani *et al.* (2004); Krogan *et al.* (2004)
	Ipi1			Galani *et al.* (2004); Krogan *et al.* (2004)
	Rea1/Mdn1	MDN1; NP_055426.1	Dynein-related AAA-type ATPase; Releases Ytm1 and Rsa4 from preribosomes	Galani *et al.* (2004); Ulbrich *et al.* (2009); Bassler *et al.* (2010)
	Rsa4	NLE1; NP_060566.2	WD40 repeats	De La Cruz *et al.* (2005)
	Mtr4/Dob1	SKIV2L2; NP_056175.3	DEAD-box RNA helicase	De La Cruz *et al.* (1998)
Nuclear export	Nop53	GLTSCR2; NP_056525.2	Coiled coil	Granato *et al.* (2005); Granato *et al.* (2008)
	Sda1	SDAD1; NP_060585.2	Also required for actin cytoskeleton organization	Dez *et al.* (2006)

(continued)

Table 4, *continued*

Pre-rRNA processing step	Name/alias	Human ortholog; accession no.	Comments	Reference
	Rrp12	RRP12; NP_055994.2	HEAT repeats	Oeffinger *et al.* (2004)
	Arx1	PA2G4; NP_006182.2	Coiled coil	Lebreton *et al.* (2006b); Bradatsch *et al.* (2007, 2012)
	Alb1		Nuclear shuttling factor	Lebreton *et al.* (2006b); Bradatsch *et al.* (2007, 2012)
	Nmd3/Src5	NMD3; NP_057022.2	Coiled coil	Hedges *et al.* (2005); West *et al.* (2005)
	Mex67	TAP; NP_006353.2	Also involved in nuclear RNA export	Yao *et al.* (2007, 2008)
	Mtr2		Also involved in nuclear RNA export	Yao *et al.* (2007, 2008)
	Bud20		Zinc-finger domain	Bassler *et al.* (2012)
6S processing	Ngl2		Ccr4-like Rnase	Faber *et al.* (2002); Thomson and Tollervey (2010)
	Rex1/Rnh70	REX1; NP_065746.3	3′–5′ exoribonuclease	Van Hoof *et al.* (2000)
	Rex2		3′–5′ exoribonuclease	Van Hoof *et al.* (2000)
Cytoplasmic maturation	Drg1/Afg2	SPATA5; NP_660208.2	AAA-ATPase; releases Nog1 and Rlp24	Pertschy *et al.* (2007)
	Rei1	ZNF622; NP_219482.1	Zinc-finger domains; release of Arx1 and Alb1	Lebreton *et al.* (2006b); Parnell and Bass (2009)
	Reh1		Zinc-finger domains	Lebreton *et al.* (2006b); Parnell and Bass (2009)
	Jjj1	DNAJC21; NP_919259.3	Contains a zinc-finger domain and a DNA J domain; release of Arx1 and Alb1	Demoinet *et al.* (2007); Meyer *et al.* (2007, 2009); Greber *et al.* (2012)
	Ssa1	HSP70; NP_005337.2	ATPase; member of HSP70 family; releases Arx1 and Alb1	Meyer *et al.* (2007)
	Ssa2	HSC70; NP_006588.1	ATPase; member of HSP70 family; releases Arx1 and Alb1	Meyer *et al.* (2007)
	Yvh1	DUSP12; NP_009171.1	Dual-specificity phosphatase	Kemmler *et al.* (2009); Lo *et al.* (2009)
	Mrt4	MRT04; NP_057267.2	Similar to the r-protein P0	Kemmler *et al.* (2009); Rodriguez-Mateos *et al.* (2009)
	Sdo1	SBDS; NP_057122.2	Responsible for Shwachman–Bodain–Diamond syndrome; release of Tif6	Menne *et al.* (2007)
	Efl1/Ria1	EFTUD1; NP_078856.4	Cytoplasmic GTPase; releases Tif6	Senger *et al.* (2001)
	Lsg1/Kre35	LSG1; NP_078856.4	Cytoplasmic GTPase; releases Nmd3	Kallstrom *et al.* (2003); Hedges *et al.* (2005)
	Sqt1	AAMP; NP_001078.2	WD40 repeats	West *et al.* (2005)
Unknown	Puf6	KIAA0020; NP_055693.4	Pumilio family RNA-binding domain	
	Fpr4		Peptidyl-prolyl *cis-trans* isomerase	
	Fpr3		Peptidyl-prolyl *cis-trans* isomerase	

Consecutive assembly factors in boldface type represent subcomplexes.

Five snoRNPs participate in pre-18S rRNA cleavage

In addition to RNase MRP and the U3 snoRNP, there are 3 snoRNPs that play a role in pre-18S processing: U14, snR30, and snR10 (reviewed in Kiss *et al.* 2010; Watkins and Bohnsack 2012). U14 is a box C/D snoRNA while snR30 and snR10 are box H/ACA snoRNAs (Table 5). The U14 and snR30 snoRNAs are encoded by essential genes whereas snR10 is not (Table 5). The U14 and snR10 snoRNPs carry out dual roles in both pre-rRNA processing and rRNA modification (Liang and Fournier 1995; Morrissey and Tollervey 1997; Liang *et al.* 2010). In contrast to these two snoRNPs but like the U3 box C/D snoRNP, snR30 base pairs to the pre-rRNA to effect pre-18S rRNA processing but has no known RNA modification target (Atzorn *et al.* 2004; Fayet-Lebaron *et al.* 2009; Lemay *et al.* 2011). RNA affinity purification of snR30 revealed that the PIN-like domain protein Utp23 and Kri1 associate specifically with different subsets of snR30 snoRNPs (Hoareau-Aveilla *et al.* 2012). Furthermore, depletion of Utp23 resulted in accumulation of snR30 RNA in large preribosomal complexes, suggesting that Utp23 is

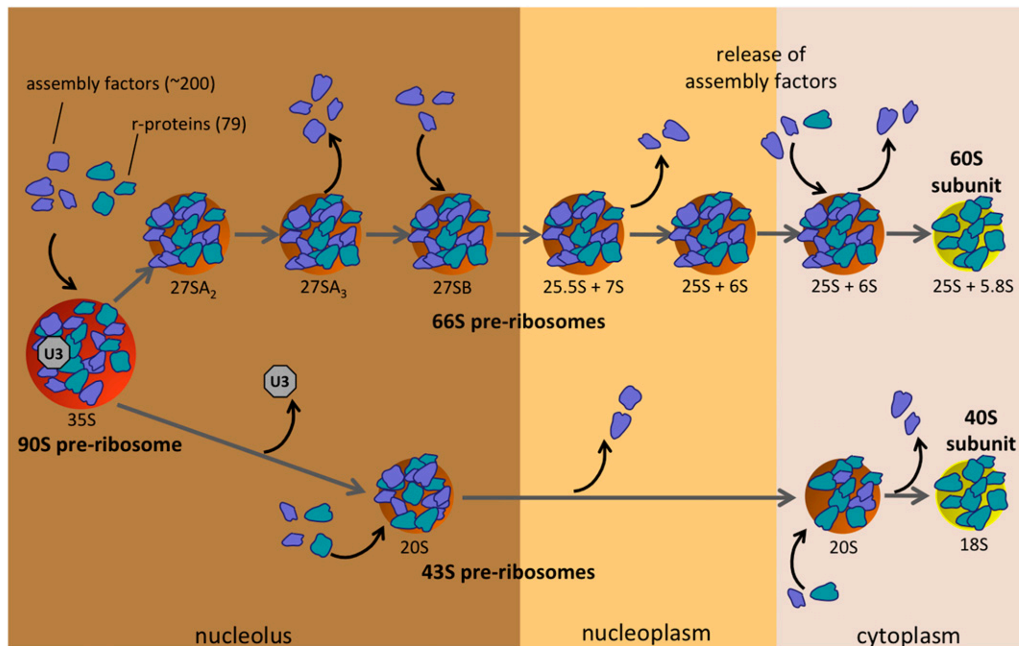

Figure 8 Pathway for maturation of preribosomes to form 40S and 60S ribosomal subunits. Sequential assembly intermediates are shown, distinguished by the pre-rRNA processing intermediates contained within them. Most r-proteins (light blue) and many assembly factors (dark blue) associate with the early nucleolar/nuclear precursor particles. Some assembly factors join preribosomes in middle steps of assembly or even during late steps in the cytoplasm. Release of assembly factors from preribosomes occurs at early, middle, or late stages of subunit maturation.

required for release of snR30 from assembling preribosomes. The snR30 RNA has been shown to be required for assembly of the SSU processome (Lemay *et al.* 2011; Hoareau-Aveilla *et al.* 2012).

While many ribosome assembly factors and snoRNPs are required for pre-rRNA cleavage in nucleolar SSU biogenesis, identification of the *bona fide* cleavage enzymes for 18S rRNA biogenesis has been more difficult. Utp24 has been proposed as a candidate cleavage enzyme for the A_1 and A_2 sites (Bleichert *et al.* 2006). Bioinformatic analysis indicated the presence of a PINc domain in Utp24, a domain associated with nuclease activity. Mutation of residues in the predicted active site in Utp24 caused reduced cell growth and defects in cleavage at the A_1 and A_2 sites, consistent with a role as the cleavage endonuclease for these steps in nucleolar SSU biogenesis. The cyclase-like protein, Rcl1, has also been proposed to be an endonuclease that cleaves at the A_2 site (Billy *et al.* 2000; Horn *et al.* 2011) as recombinant Rcl1 cleaves an rRNA fragment containing the A_2 site *in vitro*. However, extensive mutagenesis in the active site predicted by the crystal structure of the *Kluyveromyces lactis* Rcl1 revealed relative insensitivity to mutation (Tanaka *et al.* 2011), although a triple mutation affects growth *in vivo* and A_2 cleavage *in vitro* (Horn *et al.* 2011).

RNA helicases in nucleolar ribosome biogenesis

There are 19 RNA helicases involved in ribosome biogenesis in *S. cerevisiae*. All but one, Mtr4, are SF2 family helicases characterized by DEAD- or DEAH-box protein motifs (referred to as DExD/H-box RNA helicases; Table 6) (Fairman-Williams *et al.* 2010). Seven are required for SSU biogenesis, 10 participate in LSU biogenesis, and 2 are required for both SSU and LSU biogenesis (Table 6) (reviewed in Martin *et al.* 2012; Rodriguez-Galan *et al.* 2013). All are encoded by essential genes except for Dbp2, Dbp3, and Dbp7. Prp43 is unique among these helicases as it also is required for pre-mRNA splicing. This helicase therefore holds an important role in RNA metabolism in both the nucleolus and the nucleus.

The DExD/H motif is defined by a conserved helicase core consisting of 13 protein motifs (Q, I, Ia, Ib, Ic, II, III, IV, Iva, V, Va, Vb, and VI) that participate in ATP binding and hydrolysis and RNA binding and unwinding or RNA–protein complex rearrangement (Rodriguez-Galan *et al.* 2013). Structural studies indicate that motifs Q-III and IV–VI each fold into a Recombinase A-like (RecA-like) domain. Yeast Dbp8, Prp43, Dbp3, Dbp4, Rok1, and Rrp3 expressed and purified from *Escherichia coli* all have RNA-dependent ATPase activity (Granneman *et al.* 2006; Garcia and Uhlenbeck 2008; Lebaron *et al.* 2009). Of note is that the ATPase activity of Dbp8 and of Prp43 has been shown to be stimulated by interaction with dedicated nucleolar protein cofactors. The ATPase activity of Dbp8 is stimulated by binding to Esf2, a protein that bears the RRM RNA-binding motif (Granneman *et al.* 2006). Similarly, the ATPase activity and RNA duplex unwinding activity of Prp43 are stimulated by binding to the nucleolar G-patch protein, Pfa1/Sqs1 (Lebaron *et al.* 2009). Dbp3, Dbp4, Rok1, and Rrp3 have also been shown to unwind RNA duplexes (Garcia *et al.* 2012). While the Prp43-Pfa1 combination is capable of unwinding RNA duplexes of 21 bp, the Dbp3, Dbp4, Rok1, and Rrp3 helicases are capable of unwinding only 10-bp RNA duplexes. When challenged with an RNA duplex of just 3 additional base pairs, they fail to dissociate the strands. As it may be that these RNA helicases too require cofactors for maximal activity, it will be interesting to test them together when they are identified.

A posited function for the RNA helicases is in unwinding the 76 snoRNAs base paired to the pre-rRNA in the pre-SSU

Table 5 Yeast snoRNAs with roles in pre-rRNA processing

snoRNA	Class	Essential?	Function
U3	Box C/D	Yes	Processing
U14	Box C/D	Yes	Processing and 2′-*O*-methylation
snR30	Box H/ACA	Yes	Processing
snR10	Box H/ACA	No	Processing and pseudouridylation
RNase MRP	Unique	Yes but not for processing	Processing

and LSU ribosomal complexes. Consistent with such a function, depletion of Dbp4, Has1, Rok1, and Prp43 impaired release of assorted snoRNAs from large pre-SSU ribosomal complexes (Kos and Tollervey 2005; Liang and Fournier 2006; Bohnsack *et al.* 2008, 2009). In general, an intact helicase active site was required for snoRNA release. However, these RNA helicases were not unique in this requirement as depletion of nonhelicase proteins Utp23, Esf1, and Esf2 (Hoang *et al.* 2005; Liang and Fournier 2006; Hoareau-Aveilla *et al.* 2012) yielded similar impaired snoRNA release. Indeed, both Utp23 and the helicase Rok1 caused impaired release of snR30. The likeliest scenario reconciling these findings is that the impaired snoRNA release observed upon depletion of these four helicases reflects impaired remodeling or rearrangement of the large pre-SSU ribosomal complexes as they mature to ribosomes. The helicases may thus be required for RNA–protein remodeling of the SSU processome or for RNA–RNA dissociation of the pre-18S rRNA itself. Consistent with this, depletion of five essential RNA helicases required for SSU biogenesis had no effect on snoRNA release from pre-40S RNPs, perhaps because their enzymatic role cannot be detected by this assay.

An exciting innovation has been the development of an *in vivo* protein–RNA cross-linking method that has enabled mapping of helicase interactions with the pre-rRNA. This method, called UV cross-linking and analysis of cDNAs (CRAC) in yeast, relies on UV cross-linking of a His6-TEV–Protein-A-tagged protein followed by double affinity purification and sequencing of cDNAs representing the associated RNA fragments (Bohnsack *et al.* 2009, 2012; Granneman *et al.* 2009). While Rok1 was not detectably cross-linked, Prp43 was cross-linked to pre-rRNA and to the U6 spliceosomal snRNA, reflecting its dual roles in rRNA biogenesis and pre-mRNA splicing. The major cross-linking site in the pre-18S rRNA was to helix 44, placing it near the site D cleavage site that is processed by Nob1 in the cytoplasm to make the mature 18S 3′ end and suggesting that it may play a role in the necessary structural rearrangements.

Nucleolar and Nucleoplasmic Steps Required to Generate 60S Ribosomal Subunits

Maturation of 66S preribosomes to generate mature 60S subunits includes the following steps: construction of stable early assembly intermediates containing $27SA_2$ pre-rRNA, cleavage of $27SA_2$ pre-rRNA at the A_3 site and removal of ITS1 to form 27SB pre-rRNA, release of early-acting assembly factors, cleavage at the C_2 site in ITS2, removal of ITS2 from 25.5 and 7S pre-rRNAs, nuclear export of pre-60S particles, and cytoplasmic maturation of subunits.

Construction of stable early assembly intermediates

The roles of assembly factors present in the earliest preribosomes (Fatica *et al.* 2002; Dez *et al.* 2004), but that exit before 27SB pre-rRNA processing is completed (Table 4), are the least well defined among LSU assembly factors. Depletion of these factors results in a severe decrease in the steady-state levels of 27S and 7S pre-rRNAs. Pulse-chase experiments confirm that 27S pre-rRNAs are rapidly turned over in the absence of these proteins. Among these early factors are six putative RNA-dependent RNA helicases (Dbp2, Dbp3, Dbp6, Dbp7, Dbp9, and Prp43), whose likely function is to rearrange RNA or RNP structure. Thus, during these initial stages of maturation, preribosomes may undergo multiple complex rearrangements to form productive, stable intermediates, while failure to do so may result in particularly unstable particles.

Seven of these early factors, Dbp6, Dbp7, Dbp9, Npa1, Npa2, Nop8, and Rsa3, exhibit multiple genetic interactions with each other and with r-protein L3 (De La Cruz *et al.* 2004) and thus may function together. Npa1, Npa2, Nop8, Rsa3, and Dbp6 form a discrete subcomplex, which might function as a scaffold to mediate topological rearrangements by the RNA helicases and organize assembly of early pre-60S ribosomes (Rosado *et al.* 2007a).

We still lack a fundamental understanding of the earliest stages of 60S subunit assembly. How is nascent pre-rRNA initially folded into secondary structures and then compacted into tertiary structures, together with r-proteins and early assembly factors? Do these early steps of pre-60S subunit assembly occur by identical steps when pre-rRNA processing occurs cotranscriptionally *vs.* post-transcriptionally? What triggers cleavage of the A_3 site by the MRP endonuclease? How is this cleavage coupled to processing of 3′ETS (Allmang and Tollervey 1998)? What dictates the frequency of the minor, alternative ITS1 processing pathway to directly cleave the B_L site, and what molecules catalyze this cleavage?

Steps in assembly required for processing of $27SA_3$ pre-rRNA

Removal of ITS1 from $27SA_3$ pre-rRNA to form 27SB pre-rRNA requires the 12 "A3 factors" Ebp2, Brx1, Pwp1, Nop12, Nop7, Ytm1, Erb1, Rlp7, Nop15, Cic1/Nsa3, Drs1, and Has1 (Figure 9 and Table 4 and references therein). Upon depletion of each of these proteins, levels of $27SA_3$ pre-rRNA increase and amounts of 27SB pre-rRNA decrease significantly.

Table 6 RNA helicases involved in ribosome biogenesis

	LSU	Both SSU and LSU
Dbp4, Dbp8, Dhr1, Dhr2, Fal1, Rok1, Rrp3	Dbp2[a], Dbp3[a], Dbp6, Dbp7[a], Dbp9, Dbp10, Drs1, Mak5, Mtr4, Sbp4	Has1, Prp43

[a] Not essential for growth in yeast.

Ebp2, Brx1, and Nop12 copurify together in a subcomplex along with Pwp1 and r-proteins L8 and L15 (Zhang *et al.* 2004). Consistent with this finding, Nop12, L8, and L15 are located near each other in preribosomes, adjacent to the proximal stem formed by base pairing between the 3′ end of 5.8S rRNA and the 5′ end of 25S rRNA (Ben-Shem *et al.* 2011; Granneman *et al.* 2011; Klinge *et al.* 2011). Several observations indicate that the other 8 A3 factors also may be located adjacent to each other in the same neighborhood of preribosomes, near the proximal stem: (1) Erb1, Ytm1, and Nop7 form a protein subcomplex, via direct interactions of Nop7 and Ytm1 with Erb1 (Harnpicharnchai *et al.* 2001; Miles *et al.* 2005; Tang *et al.* 2008); (2) Erb1 and Nop7 cross-link to RNA near each other in 25S rRNA (Granneman *et al.* 2011; J. Dembowski and R. Babiano, personal communication); (3) Nop15, Rlp7, and Cic1 cross-link to the ITS2 spacer RNA (Granneman *et al.* 2011), whose 5′ and 3′ ends are located near the Erb1 and Nop7 binding sites and the proximal stem; (4) Drs1 associates with the Nop7 subcomplex under certain conditions (Merl *et al.* 2010); and (5) Has1 interacts strongly with Nop15 in a two-hybrid assay (Dembowski *et al.* 2013).

Assembly of these A3 factors into preribosomes is hierarchical (Figure 9). Ebp2 and L8 are each required for assembly of Nop7, Erb1, Ytm1, Nop15, Rlp7, Cic1, and Has1 (Jakovljevic *et al.* 2012; Shimoji *et al.* 2012). Nop7, Erb1, Ytm1, Rlp7, Nop15, and Cic1 are mutually interdependent for assembly and are necessary for association of Drs1 and Has1, but not the Ebp2 complex, with pre-rRNPs (Sahasranaman *et al.* 2011). Upon depletion of Drs1 or Has1, all of the other A3 factors assemble efficiently, placing these two DEAD-box proteins at the end of the assembly hierarchy (Dembowski *et al.* 2013; J. Talkish, personal communication).

Several observations indicate that these A3 factors might not directly participate in processing of 27SA$_3$ pre-rRNA, but instead may play structural roles in subunit biogenesis, to produce stable assembly intermediates that can undergo proper processing of 27SA$_3$ pre-rRNAs:

1. Except for the DEAD-box RNA helicases Drs1 and Has1, the A3 factors are not predicted to have enzymatic functions. Most of the A3 factors likely function by binding to RNA. However, their binding sites apparently are distant from the 5′ end of 27SA$_3$ pre-rRNA; they bind near the proximal stem of pre-rRNA (Granneman *et al.* 2011).
2. The effect on pre-rRNAs in the A3 mutants is not due to failure to recruit processing nucleases to preribosomes; these enzymes are present in pre-rRNPs in the A3 mutants (Sahasranaman *et al.* 2011).
3. Consistent with a structural role for the A3 factors, pre-rRNAs undergo rapid turnover in their absence, more so than when later steps in processing and assembly are blocked (Dunbar *et al.* 2000; Pestov *et al.* 2001; Adams *et al.* 2002; Gadal *et al.* 2002; Oeffinger *et al.* 2002; Fatica *et al.* 2003b; Oeffinger and Tollervey 2003; Miles *et al.* 2005).

Assaying effects of depleting A3 factors on the assembly of r-proteins provided important clues to the roles of A3 factors in construction of LSUs. In the absence of A3 factors, assembly of r-proteins L17, L26, L35, and L37 is greatly diminished (Sahasranaman *et al.* 2011; Jakovljevic *et al.* 2012). These four r-proteins bind to the helices formed by base pairing of 5.8S and 25S rRNA (Ben-Shem *et al.* 2011; Klinge *et al.* 2011). Thus this stage of subunit construction, during which ITS1 sequences are removed from 27SA$_3$ pre-rRNA, also might include stabilizing base pairing of the 5′ end of 25S rRNA with the 5′ and 3′ ends of 5.8S rRNA, as well as association of r-proteins with these rRNA domains. The absence of A3 factors and these r-proteins in nascent particles exposes a considerable portion of domains I, II, and/or III of 25S/5.8S rRNA, potentially explaining the rapid turnover of pre-rRNAs in A3 mutants.

Processing of 27SA$_3$ pre-rRNA to generate the 5′ end of 5.8S rRNA (the Bs site) provides a prototype to think about two important unresolved questions in ribosome biogenesis: How are the precise ends of mature rRNAs generated? What features in preribosomes precisely halt exonucleases that generate these ends? In mature ribosomes, r-protein L17 binds to the 5′ end of 5.8S rRNA (Ben-Shem *et al.* 2011; Klinge *et al.* 2011) and thus might function as a roadblock during assembly to prevent further exonucleolytic processing past the B$_S$ site. Consistent with this idea, when L17 is depleted, but adjacent r-proteins L26, L35, and L37 are present, a 5′-truncated form of 5.8S rRNA is produced, differing in length equivalent to the footprint of L17 (Sahasranaman *et al.* 2011). In the absence of the A3 factors, the nucleases Rat1 and Xrn1 are present in preribosomes, but L17 and the three nearby r-proteins are not (Sahasranaman *et al.* 2011). This suggests an efficient mechanism for quality control of processing: Exonucleases that normally serve as processing enzymes could readily convert to turnover machines when processing goes awry. However, such a mechanism might be used for quality control of LSU biogenesis, but not for SSUs. The 5′ and 3′ ends of 25S and 5.8S rRNAs are generated by exonucleases, while both ends of 18S rRNA result from endonucleolytic cleavage.

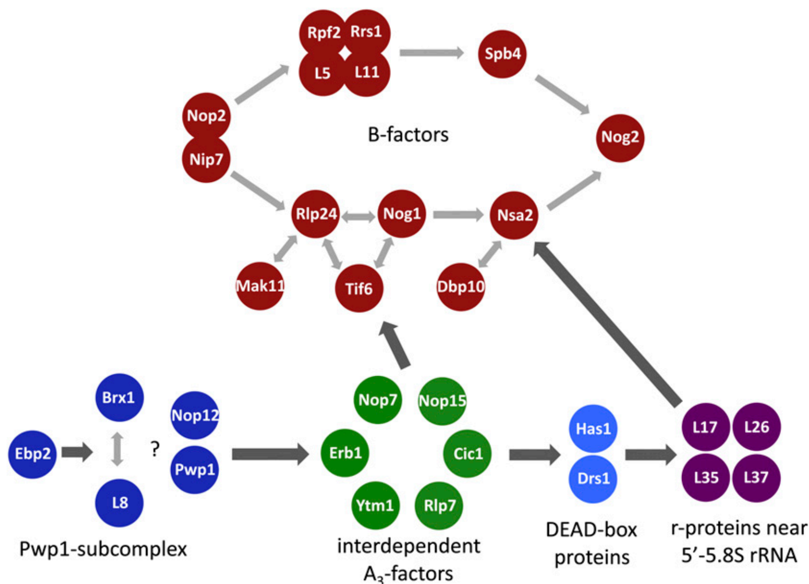

Figure 9 Hierarchical pathway for stable association with preribosomes of assembly factors and some r-proteins necessary for early and middle steps of 60S subunit assembly. Proteins that form the Pwp1 subcomplex (blue) function together to process $27SA_2$ and $27SA_3$ pre-rRNAs and are required for stable association of A3 factors (green). The A3 factors are interdependent for their association with preribosomes and are necessary for $27SA_3$ pre-rRNA processing. The presence of the A3 factors is required for assembly of the DEAD-box proteins Drs1 and Has1 (light blue). In turn, these two factors are necessary for the stable association with preribosomes of r-proteins L17, L26, L35, and L37 (purple), which bind to helices formed between 5.8S and 25S rRNAs. Both the A3 factors and r-proteins bound to the 5′ end of 5.8S rRNA are required for the stable association with preribosomes of a subset of factors required for 27SB pre-rRNA processing (red). The B factors assemble into preribosomes in two parallel pathways that converge to recruit the GTPase Nog2.

Processing of 27SB pre-rRNA

ITS2 is the last spacer removed during assembly of 60S subunits (Figure 5). This occurs in multiple steps, as preribosomes exit the nucleolus and transit from the nucleoplasm to the cytoplasm. First, the C_2 site in 27SB pre-rRNA is cleaved to produce 25.5S and 7S pre-rRNAs, then 5′–3′ exonucleolytic trimming of 25.5S pre-rRNA generates 25S rRNA, and then 3′–5′ exonucleolytic processing of 7S pre-rRNA produces 5.8S rRNA.

Fourteen assembly factors ("B factors") are required for cleavage at the C_2 site (Table 4 and Figure 9). Upon depletion of each of 11 of these factors, 27SB pre-rRNAs accumulate and little or no downstream 25.5S or 7S pre-rRNAs are made. Although depletion of Drs1 or Has1 blocks $27SA_3$ pre-rRNA processing, missense mutations that inactivate Drs1 or Has1 but still allow their assembly into preribosomes block the next step, 27SB pre-rRNA processing (Adams *et al.* 2002; Dembowski *et al.* 2013). Nog2 plays a role in both C_2 cleavage and processing of 7S pre-rRNA (Saveanu *et al.* 2001). B factors include five potential RNA-binding proteins (Nip7, Rpf2, Nsa2, Rlp24, and Tif6), four RNA helicases (Spb4, Drs1, Has1, and Dbp10), two GTPases (Nog1 and Nog2), at least one potential scaffolding protein (Mak11), and one putative methyltransferase (Nop2), but no known nucleases.

Most A3 and B factors assemble into early preribosomal particles containing $27SA_2$ pre-rRNA (Saveanu *et al.* 2003, 2007; Zhang *et al.* 2007; Garcia-Gomez *et al.* 2011; Talkish *et al.* 2012). Nsa2 and Nog2 assemble later, just before the ITS2 spacer is removed (Saveanu *et al.* 2001; Lebreton *et al.* 2006a). B factors assemble sequentially with preribosomes, via two mostly independent pathways that converge on Nog2 (Figure 9) (Talkish *et al.* 2012). All of the B factors are necessary to recruit Nog2, and a subset (Nop2/Nip7, Dbp10, Tif6, Rlp24, and Nog1) recruits Nsa2. This convergent pathway might enable quality control to ensure that the irreversible processing of 27SB pre-rRNA cannot occur until the subunits have undergone proper maturation. Assembly could then be powered forward by a single step.

Association of B factors with preribosomes is simplified and facilitated by formation of several subcomplexes. Rpf2 and Rrs1 form a subcomplex with r-proteins L5 and L11 plus 5S rRNA, which assemble into preribosomes in a concerted fashion to form the central protuberance (Zhang *et al.* 2007). Nip7 and Nop2 form a heterodimer (Talkish *et al.* 2012); while Rlp24, Nog1, and Mak11 exhibit significant genetic and physical interactions (Saveanu *et al.* 2003, 2007); and Nog1 is linked genetically and physically to Nsa2 (Lebreton *et al.* 2006a).

The 27SB pre-rRNA containing ITS2 is the longest-lived pre-rRNA processing intermediate, perhaps reflecting complex remodeling of pre-rRNPs after removal of ITS1 and before ITS2 can be removed. ITS2 may facilitate base pairing between 5.8S rRNA and sequences at the 5′ end of 25S rRNA. Removal of ITS2 could be coupled with release of early factors and exit of preribosomes from the nucleolus into the cytoplasm. Interestingly, none of the known B factors binds to ITS2. Is the "B phenotype", analogous to the A3 phenotype, due to inability to create particles sufficiently stable and long-lived that they can undergo cleavage at the C_2 site, rather than a direct result of blocking pre-rRNA processing?

Nucleolar release of assembly factors from preribosomes

Just as the docking and presence of assembly factors on preribosomes drive subunit biogenesis, release of such proteins from pre-rRNPs may also trigger changes essential for maturation of subunits. Mechanisms of assembly factor removal have been studied in detail for a few factors that function during intermediate steps of assembly and for

factors released from late cytoplasmic particles (see *Cytoplasmic Maturation of Pre-40S and Pre-60S Subunits*).

During the transition from early preribosomes to intermediate pre-rRNPs, the AAA ATPases Rix7 and Rea1 catalyze removal of Nsa1 and Ytm1 plus Rsa4, respectively (Kressler *et al.* 2008; Ulbrich *et al.* 2009; Bassler *et al.* 2010). Amino-terminal extensions of these ATPases are thought to direct them to their protein substrates, and ATP-dependent conformational switches are thought to disrupt pre-rRNP architecture, to release target factors from preribosomes. Cryo-EM reconstructions of Rix1-purified preribosomes revealed that the AAA domains of Rea1 bind near the central protuberance of preribosomes, via connections with the Rix1/Ipi1/Ipi3 subcomplex, while the C-terminal, conserved metal ion-dependent adhesion site (MIDAS) domain located at the end of the hinged "tail" structure of Rea1 may contact the pre-rRNP at different points (Ulbrich *et al.* 2009). The Rea1 MIDAS domain binds to the MIDAS interacting domain (MIDO) motifs in Ytm1 or Rsa4. Mutations within these MIDO domains that disrupt these interactions prevent removal of Rsa4 or Ytm1 from preribosomes by Rea1 and cause a dominant negative block in subunit biogenesis (Bassler *et al.* 2010).

All of the assembly factors eventually must dissociate from pre-rRNPs, but only a modest number of NTPases are present on preribosomes. Do Rix7 and Rea1 catalyze the removal of any other of the factors that dissociate from pre-rRNPs when Nsa1 and Ytm1 are released? How many different factors are removed in one operation or by one enzyme? Exactly when and how are the early factors that dissociate before or upon formation of 27SB pre-rRNA removed from preribosomes? Are these early factor release steps coupled to removal of the ITS2 spacer? Do any of the DEAD-box ATPases catalyze release of assembly factors? How does release of assembly factors power progression of particle maturation?

Processing of the 3′ end of 7S pre-rRNA to form mature 5.8S rRNA

Following endonucleolytic cleavage of ITS2 at the C_2 site, ITS2 spacer sequences are removed from the resulting 7S and 25.5S pre-rRNAs. The 5′–3′ exonucleases Rat1 and Rrp17 rapidly process 25.5S pre-rRNA to 25S rRNA (Geerlings *et al.* 2000). Trimming of 7S pre-rRNA to $5.8S_S$ or $5.8S_L$ rRNAs requires at least four steps and a number of different nucleases and occurs sequentially in the nucleus and then the cytoplasm (Henry *et al.*1994; Thomson and Tollervey 2005, 2010). The core exosome, which includes 3′–5′ exonucleases as well as endonucleases, removes ~110 nt, starting from the C_2 site, to produce 5.8S extended at its 3′ end by ~30 nt (Mitchell *et al.* 1996; Allmang *et al.* 1999). Rrp6, the nuclear-specific component of the exosome, removes another ~22–25 nt, to form the 6S pre-rRNA (5.8S rRNA 3′ extended by 5–8 nt) (Briggs *et al.* 1998). Following export of preribosomes to the cytoplasm, the exonucleases Rex1 and Rex2 continue to shorten the 6S pre-rRNA to a form containing ~5 nt of ITS2 sequences at its 5′ end (Van Hoof *et al.* 2000), and then Ngl2 removes the remaining few nucleotides of ITS2 to create mature 5.8S rRNA (Faber *et al.* 2002; Thomson and Tollervey 2010).

Seven late-acting, nuclear assembly factors also are required for processing of 7S pre-rRNA to 5.8S rRNA. These include the AAA ATPase Rea1 and the Rix1, Ipi1, and Ipi3 subcomplex (Galani *et al.* 2004). Removal of Ytm1, Rsa4, or other assembly factors from preribosomes by Rea1 might be necessary to provide access for nucleases or to properly organize the RNP environment for processing of the 3′ end of 7S pre-rRNA.

Why is 3′-end processing of 5.8S rRNA so complex, requiring handover from several enzymes in several compartments? Exactly how is processing coordinated at each end of ITS2 to generate the 3′ end of 5.8S rRNA and the 5′ end of 25S rRNA? Is this coupled with the earlier removal of ITS1 sequences to produce the 5′ end of 5.8S rRNA? Is export of preribosomes to the cytoplasm coupled with late steps of pre-rRNA processing and, if so, how?

Nuclear Export of Pre-40S and Pre-60S Particles

Pre-40S and pre-60S subunits must be rapidly exported from the nucleus, to meet the cell's high demand for protein synthesis capacity. In fast-growing yeast cells, one preribosome is exported through each nuclear pore every 2–3 sec (Warner 1999). Several challenges must be overcome to enable this efficient export: (1) The hydrophilic surface of preribosomes must navigate though the hydrophobic mesh of FG repeat-containing nucleoporins inside the nuclear pore and (2) export of incompletely or improperly assembled preribosomes must be avoided.

GFP fusions to r-proteins L11, L25, S2, and S3 were used as reporters to find that components of the general export machinery, including the nuclear export receptor Crm1, the Ran GTPase Gsp1, and several nucleoporins, are necessary for export of both nascent ribosomal subunits (Hurt *et al.* 1999; Moy and Silver 1999; Stage-Zimmermann *et al.* 2000).

Ltv1 and Pno1/Dim2 have been proposed to function as adaptors for nuclear export of pre-40S ribosomes (Seiser *et al.* 2006; Vanrobays *et al.* 2008). Both contain NES sequences that could bind Crm1. Depletion of each protein or deletion of the NES in either protein prevents nuclear export of these particles. However, this export defect in *ltv1* mutants may be indirect, due to inhibition of maturation of pre-40S ribosomes, resulting in sequestration of export factors with abortive cytoplasmic pre-40S assembly intermediates (Fassio *et al.* 2010).

At least five proteins (Arx1, Bud20, Ecm1, Mex67, and Nmd3) function together to enable nuclear export of pre-60S subunits (Ho *et al.* 2000; Gadal *et al.* 2001b; Bradatsch *et al.* 2007; Yao *et al.* 2007, 2010; Hung *et al.* 2008; Bassler *et al.* 2012).

Nmd3, the Mex67-Mtr2 complex, and Arx1 bind to different domains of pre-60S subunits (Figure 10). Nmd3 functions

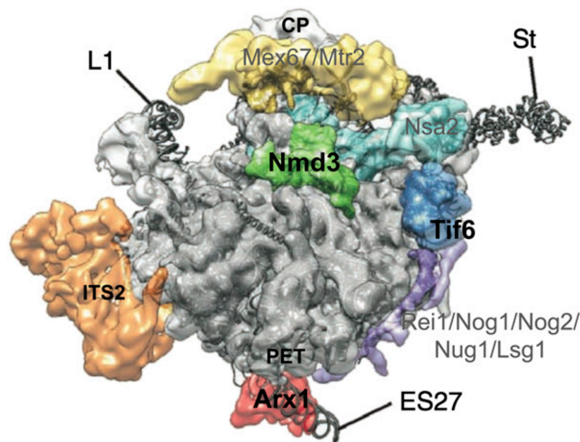

Figure 10 Locations on late pre-60S ribosomes of export factors Mex67/Mtr2, Nmd3, and Arx1 and assembly factors Tif6 and Rei1. Cryo-EM reconstructions of particles purified using TAP-tagged Arx1 were modeled onto the crystal structure of the mature 60S ribosomal subunit. This model is adapted from Figure 2 of Bradatsch *et al.* (2012).

as an export adaptor, by bridging preribosomes with the export receptor Crm1 (Ho *et al.* 2000; Gadal *et al.* 2001b). Nmd3 contacts the subunit interface of pre-60S subunits near helices 38 and 95 and r-protein L10 and contains an NES that presumably binds to Crm1 (Sengupta *et al.* 2010). Deletion of this NES creates a dominant negative effect on subunit export; the truncated Nmd3 can still bind preribosomes but cannot link up with Crm1 (Ho *et al.* 2000).

The Mex67/Mtr2 heterodimer and Arx1 directly link preribosomes with the FG repeat-containing nucleoporins lining the nuclear pore (Yao *et al.* 2007, 2008). Yeast-specific loops of Mex67 bind to 66S preribosomes *in vivo* and to 5S rRNA *in vitro* and are required for pre-60S export. Thus, binding of Mex67/Mtr2 to nucleoporins and to 66S particles may direct them to and through the nuclear pore.

The amino acid sequence of Arx1 is very similar to that of methionyl aminopeptidase (MetAP), which binds to the polypeptide exit tunnel of mature 60S subunits and removes N-terminal methionines from nascent polypeptides (Bradatsch *et al.* 2007). Cryo-EM reconstructions and crystal structures showed that Arx1 binds to r-proteins L25, L26, and L35, adjacent to the polypeptide exit tunnel (Bradatsch *et al.* 2012; Greber *et al.* 2012) (Figure 10).

Assembly factors Rrp12 and Sda1, which contain HEAT-repeat motifs found in export receptors, are required for export of both precursor particles (Oeffinger *et al.* 2004; Dez *et al.* 2007). Rrp12 binds to rRNA *in vitro* as well as the Ran GTPase (Gsp1) and FG-repeat nucleoporins and might coat the hydrophilic surface of preribosomes and effect transit of the subunits through the nucleoporin meshwork inside nuclear pores.

Depletion of many r-proteins prevents nuclear export of preribosomes. These defects might reflect direct roles in export, *e.g.*, recruiting export adaptors such as Arx1. Alternatively, these export defects may be indirect effects of either blocking assembly, causing turnover of preribosomes before export can occur, or failure to recycle export factors back to the nucleus because they are trapped on abortive intermediates in the cytoplasm. At present, S15 is the only candidate for an r-protein more directly involved in nuclear export of preribosomes. Upon depletion of S15, intact 43S particles containing 20S pre-rRNA and an apparently complete set of assembly factor and r-proteins accumulate in the nucleus (Leger-Silvestre *et al.* 2004).

During late nuclear steps in assembly and export to the cytoplasm, preribosomes may undergo "structural proofreading" to monitor whether they are correctly assembled. Successful binding of Nmd3 to the subunit interface (Sengupta *et al.* 2010), Arx1 to the surface surrounding the polypeptide tunnel exit (Bradatsch *et al.* 2012; Greber *et al.* 2012), and Mex67/Mtr2 to 5S rRNA at the central protuberance (Yao *et al.* 2007) likely depends on formation of the proper local RNP architecture of these three separate domains in preribosomes (Figure 10). Thus, coupling binding of these factors to export could provide a means to segregate only properly assembled subunits to the cytoplasm.

Cytoplasmic Maturation of Pre-40S and Pre-60S Subunits

Nascent 40S and 60S subunits entering the cytoplasm utilize complex pathways to complete their maturation to functional subunits (Zemp and Kutay 2007; Panse and Johnson 2010). Several steps must be completed and challenges overcome during this last stage of ribosome assembly: (1) final steps of processing of pre-rRNA, assembly of the last r-proteins, and release and recycling of several assembly factors; (2) preventing premature association of newly exported, but inactive subunits with the translational machinery; and (3) interrogating functional domains for their correct assembly.

Newly exported pre-rRNPs contain only a few remaining assembly factors and likely bear close resemblance to mature subunits. Thus, important clues to the function of these late-acting factors and for mechanisms of their removal are provided by cryo-EM reconstructions of particles bound to the late factors, using the crystal structures of mature subunits as guides (Figure 10 and Figure 12).

Release of the last assembly factors from pre-60S subunits and incorporation of the last r-proteins

Newly exported pre-60S subunits still contain several assembly factors and lack r-proteins L10, L24, L29, L40, L42, P0, P1, and P2. Initial insights into the late steps of maturation of these particles were provided by the discovery that the NTPases Drg1, Efl1, and Lsg1 are present on cytoplasmic pre-60S subunits (Becam *et al.* 2001; Senger *et al.* 2001; Kallstrom *et al.* 2003; Hedges *et al.* 2005; West *et al.* 2005; Demoinet *et al.* 2007; Meyer *et al.* 2007; Pertschy *et al.* 2007). At first it was puzzling to discover that mutations in or depletions of these cytoplasmic NTPases blocked earlier, nuclear steps in subunit biogenesis and nuclear

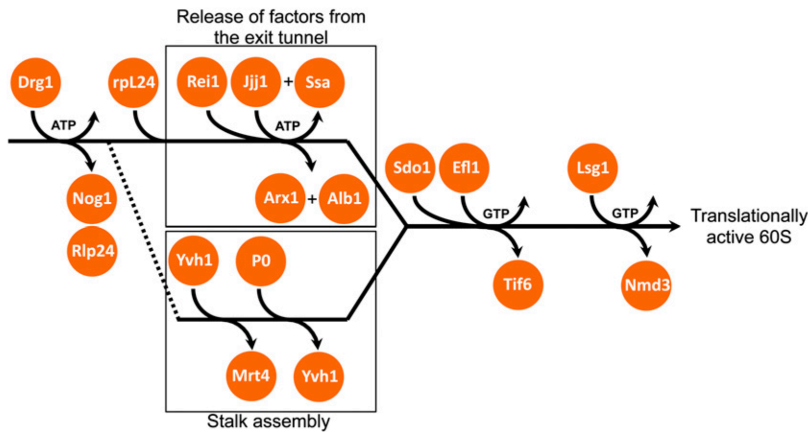

Figure 11 Pathway for late steps of maturation of pre-60S subunits in the cytoplasm. Assembly factors Nog1, Rlp24, Arx1, Alb1, Mrt4, Tif6, and Nmd3 are sequentially released from 66S preribosomes in the cytoplasm by the ATPase Drg1; and Ssa/Ssb by the GTPases Efl1 and Lsg1; and the phosphatase Yvh1 with the aid of Jjj1, Rei1, and Sdo1. This pathway also enables late assembly of r-proteins L24 and P0, replacing their homologues Rlp24 and Mrt4, respectively. This pathway is adapted from Figure 7 of Lo *et al.* (2010).

export. These paradoxic phenotypes were explained by the failure of the NTPases to release and recycle back to the nucleus shuttling assembly factors that are necessary for nuclear steps in subunit construction. Target proteins of each cytoplasmic release factor were identified by suppressor mutations that weaken binding of the target protein to preribosomes, just enough that they bypass the requirement for that release factor, yet allow the shuttling protein to bind preribosomes sufficiently well to function in nuclear subunit biogenesis (Senger *et al.* 2001; Menne *et al.* 2007; Lo *et al.* 2010).

Sequential recruitment and activation of release factors, followed by release of target proteins, triggers activation of the next release factor and release of its corresponding substrate (Figure 11). The pathway proceeds in an ordered, stepwise fashion, as follows: Rlp24 binds to the AAA ATPase Drg1 (Pertschy *et al.* 2007; Lo *et al.* 2010) and stimulates its ATPase activity, which then catalyzes exchange of Rlp24 with its paralogous r-protein L24 (Kappel *et al.* 2012). Interaction of Drg1 with nucleoporin Nup116 is necessary for Rlp24 release, suggesting coupling between nuclear export of nascent particles and initiation of cytoplasmic maturation steps. Interaction of L24 with Rei1 then enables docking of Rei1 to preribosomes. Rei1, together with the Hsp70 ATPase Ssa1/Ssa2, and its cofactor Jjj1, release the export adaptor Arx1 and its partner Alb1 from the polypeptide exit tunnel (Lebreton *et al.* 2006b; Demoinet *et al.* 2007; Meyer *et al.* 2007, 2009; Hung *et al.* 2008).

In a parallel pathway, r-protein L12 recruits Yvh1, which catalyzes replacement of Mrt4 with its paralogue, r-protein P0 (Kemmler *et al.* 2009; Lo *et al.* 2009). This enables assembly of the stalk structure (r-proteins P0, P1, and P2) that recruits GTPases to mature 60S subunits during protein synthesis. Assembly of the stalk then enables recruitment and activation of the GTPase Efl1, which, together with Sdo1, catalyzes displacement of Tif6 (Becam *et al.* 2001; Senger *et al.* 2001; Menne *et al.* 2007). This step is coupled to the final step of maturation, release of the export adaptor Nmd3 by the GTPase Lsg1 (Kallstrom *et al.* 2003; Hedges *et al.* 2005; West *et al.* 2005).

Preventing premature translation initiation

Pre-40S particles exported to the cytoplasm contain seven assembly factors and lack r-proteins S10 and S26 (Strunk *et al.* 2011). These factors are well positioned to shield pre-40S subunits from premature association with translation initiation factors or with 60S ribosomal subunits. Cryo-EM reconstructions and RNA–protein cross-linking showed that assembly factors Tsr1, Rio2, and Dim1 are located on the subunit interface of pre-40S particles and overlap binding sites of translation initiation factors eIF1, eIF1A, and eIF2- tRNAi Met, respectively (Figure 12, left). The endonuclease Nob1 and its regulator Pno1/Dim2 function in cytoplasmic 43S particles to cleave 20S pre-rRNA to form 18S rRNA. The binding sites for these two proteins overlap that for eIF3 on the platform of 40S subunits (Figure 12, center). Enp1 and Ltv1 may prevent premature rearrangements that enable recruitment of mRNA during translation initiation, by perturbing proper interactions of r-protein S3 to enable opening of the mRNA channel (Figure 12, right). Because the binding site for S10 overlaps with that for Ltv1/Enp1, and the S26 binding site lies near that for Dim2/Pno1, cytoplasmic release of Ltv1 and Pno1/Dim2 may be coupled to late assembly of r-proteins S10 and S26.

Pre-60S subunits are prevented from participating prematurely in translation, by blocking their association with 40S subunits. Both Nmd3 and Tif6 bind to the subunit-joining interface of 60S particles (Figure 10) and prevent binding of the LSU to SSUs (Valenzuela *et al.* 1982; Gartmann *et al.* 2010: Sengupta *et al.* 2010). Thus, release of Tif6 and Nmd3 during the last cytoplasmic stages of subunit maturation could license nascent subunits to successfully engage with each other to catalyze translation (Becam *et al.* 2001; Senger *et al.* 2001; Hedges *et al.* 2005; West *et al.* 2005; Menne *et al.* 2007). Likewise, Arx1 may prevent premature association of factors that facilitate maturation of nascent polypeptides emerging from the ribosome.

Test driving pre-40S or pre-60S subunits in a translation-like process

Depletion or mutations of r-proteins S0, S14, S20, or S31 or assembly factors Fap7, Rio1, or Nob1 block late steps of

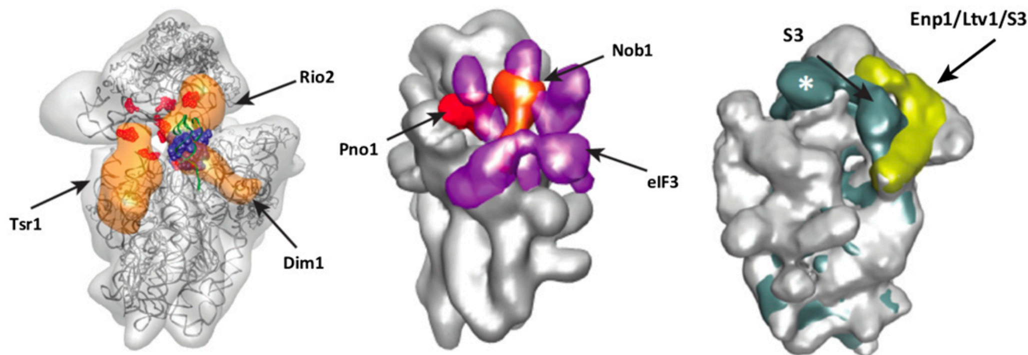

Figure 12 Assembly factors prevent premature association of translation initiation factors with pre-40S ribosomes. Left, Tsr1, Rio2, and Dim1 are located on the subunit interface of pre-40S ribosomes, overlapping with the binding sites of translation initiation factors eIF1 (blue) and eIF1A (binding site in red) and the P-site tRNA (green). Center, Nob1 and Pno1/Dim2 bound to pre-40S ribosomes prevent association of eIF3 (purple). Right, The presence of Ltv1 and Enp1 on the solvent side of pre-40S particles prevents proper interaction of r-protein S3 with helix 16, thus blocking opening of the mRNA entry channel. This diagram is adapted from Figure 1 of Karbstein (2013).

pre-40S subunit maturation, but curiously also result in accumulation of cytoplasmic 20S pre-rRNA that cosediments on sucrose gradients with 80S ribosomes and polysomes (Ford *et al.* 1999; Jakovljevic *et al.* 2004; Granneman *et al.* 2005; Soudet *et al.* 2010). Subsequent investigations of Fap7 as well as the GTPase eiF5B/Fun12 led to the discovery of a "translation-like" cycle in which pre-40S particles are monitored for their proper maturation into functional subunits (Figure 12) (Granneman *et al.* 2005; Lebaron *et al.* 2012; Strunk *et al.* 2012). The 80S-like complexes that accumulate in the absence of Fap7 contain pre-40S particles and mature 60S subunits. The apparent absence of mRNA and eIF2 tRNAi Met from these complexes indicated that they are not *bona fide* intermediates in translation initiation. Rather, the pre-40S particles in these "vacant couples" may be monitored for their proper assembly. The GTPase eIF5B that promotes 60S subunit joining during translation initiation also enables joining of pre-40S particles to 60S subunits and activates Nob1-mediated processing of 20S pre-rRNA to 18S rRNA. The ABC-like ATPase Rli1 and Dom34, which normally dissociate subunits after translation termination, also disassemble the "80S-like" complexes after other assembly factors have been removed. Thus, formation and disassembly of this 80S-like complex provide tests for assembly of important functional domains in pre-40S subunits: the subunit interface that binds to 60S subunits and the domains required for binding and activation of translation factors eIF5B, Rli1, and Dom34.

Late, cytoplasmic steps in maturation of pre-60S subunits are thought to include "functional proofreading", to test the proper assembly of the tRNA P site near the catalytic center of the ribosome and assembly of the GTPase activating site (Bussiere *et al.* 2012). The flexible loop subdomain of r-protein L10 lies adjacent to the P-site tRNA in translating ribosomes. This L10 loop may detect proper assembly of the catalytic center and transmit a molecular signal to activate Efl1 and release Tif6. Docking of Efl1 to the stalk and subsequent activation of Efl1 by both the stalk and the "P-site loop" of L10, followed by release of Tif6, could couple proofreading of the P site and the GTPase activating site with the licensing of nascent 60S subunits for binding to 40S subunits, to engage in protein synthesis.

Surveillance and Turnover of Misassembled Preribosomes

Because ribosome assembly involves multiple complex functions, mistakes are likely to occur, which could significantly affect cells. For example, the rapid production of large numbers of ribosomes requires efficient recycling of assembly factors. If abortive assembly intermediates were to accumulate, these factors might be sequestered by them and could not be recycled. Or misassembly could create malfunctioning ribosomal subunits whose presence could interfere with remaining functional subunits. Even single missense mutations in r-proteins or rRNA can alter the structure and dynamics of otherwise intact ribosomal subunits, leading to decreased fidelity of protein synthesis (Dinman 2009). Thus, mechanisms for proofreading assembly of preribosomes and for destruction of misassembled preribosomes are of crucial importance. The existence of such mechanisms has long been inferred from phenotypes of assembly mutants. Blocking biogenesis of subunits at almost any step does not result in accumulation of excessive amounts of pre-rRNA intermediates; instead, these pre-rRNAs are turned over.

The first breakthrough in discovering mechanisms for surveillance and destruction of misassembled ribosomes was finding that the exosome complex of exonucleases functions not only in processing of pre-rRNAs, but also in turnover of aberrant pre-rRNA processing intermediates (Allmang *et al.* 2000; Dez *et al.* 2006; Wery *et al.* 2009). The discovery that pre-rRNAs in the *rrp6* mutants are adenylated at their 3′ ends provided a potential clue for mechanisms of turnover (Fang *et al.* 2004; Kuai *et al.* 2004). Addition of approximately four to six A residues is performed by nuclear TRAMP complexes, containing either poly(A) polymerase Trf4 or Trf5, plus the RNA helicase Mtr4, and RNA-binding protein Air1 or Air2 (LaCava *et al.* 2005; Dez *et al.* 2006; Houseley and Tollervey 2006). Failure to add adenosines to pre-rRNA in *trf4* or

trf5 mutants results in stabilization of the pre-rRNAs, while inactivating both the TRAMP complex and the exosome results in even greater stabilization. Small amounts of oligo(A)-containing pre-rRNAs and rRNAs are also found in wild-type cells, suggesting that the surveillance machinery detects mistakes in ribosome biogenesis even under normal growth conditions (Kuai *et al.* 2004).

How do the TRAMP complex and the exosome function together to turn over pre-rRNA? The Mtr4 helicase may help unwind or disassemble structured pre-rRNAs or pre-rRNPs. Adenylation of pre-rRNA may create a better substrate for binding and 3′ degradation by the exosome. The TRAMP complex exhibits distributive addition of adenosines *in vitro*, suggesting a model in which frequent dissociation of the complex from its RNA substrate could increase access of the exonucleases (LaCava *et al.* 2005; Houseley and Tollervey 2006). Trf4, Trf5, Air1, and Air2 chromatin immunoprecipitate (ChIP) with rDNA. This requires ongoing pre-rRNA synthesis, indicating that nucleolar surveillance can initiate cotranscriptionally (Wery *et al.* 2009). The 3′-adenylated rRNA and GFP-tagged TRAMP or exosome components are enriched in similar discrete subnucleolar structures, called "Nobodies" (Kadowaki *et al.* 1994; Dez *et al.* 2006). Thus turnover of aberrantly assembled ribosomes might occur in a destruction complex.

It is not at all clear how improperly assembled preribosomes are recognized. Because turnover can occur when any one of the mutiple assembly steps is blocked, a common substrate is not immediately evident. The turnover machinery, or molecules that recruit it, might always be present in preribosomes and could function when proper pre-rRNPs do not form fast enough. Houseley and Tollervey have hypothesized a kinetic proofreading mechanism, based on Hopfield's ideas (Hopfield 1974; Houseley and Tollervey 2009) and similar to that proposed for turnover when spliceosome assembly or pre-mRNA splicing fails to occur properly (Burgess and Guthrie 1993; Staley and Guthrie 1998). At multiple different points in the assembly pathway, pre-rRNPs might contain an NTPase, driving an irreversible reaction to form a high-energy intermediate, which can either be converted to the proper downstream assembly complex or undergo disassembly and destruction. Producing such an intermediate could introduce a time delay to allow cells to discriminate properly assembled complexes from improperly formed RNPs that might dissociate more rapidly. The existence of 19 DEAD-box ATPases necessary for ribosome assembly in yeast suggests that multiple consecutive proofreading modules might function to correct mistakes in assembly.

Yeast as a Disease Model

S. cerevisiae has been an important model cell for the study of ribosomopathies, the human diseases that affect ribosome biogenesis. The detailed dissection of ribosome biogenesis in yeast, the genetic tools available, the conservation of many of the participating proteins, and the relative cost-effectiveness of working with yeast have all contributed to its use for dissecting molecular pathogenesis. Studies in yeast have revealed important aspects of the underlying pathophysiology of two human genetic diseases of ribosome biogenesis: Diamond–Blackfan anemia (DBA) and Shwachman–Diamond syndrome (SDS).

DBA is a rare human genetic disease that causes bone marrow failure usually in the first year of life (Ball 2011; Ebert and Lipton 2011; Ellis and Gleizes 2011; Horos and von Lindern 2012). The bone marrow failure is primarily of the erythroid lineage, causing a severe anemia. Over 50–60% of the occurrences of DBA can be traced to mutation of an r-protein gene encoding a protein from either subunit. Haploinsufficiency of the affected r-protein results in autosomal dominant genetic transmission. There is an increased risk of cancer over time (Vlachos *et al.* 2012).

Over 60 mutations in Rps19 are found in 25% of the patients with DBA (Leger-Silvestre *et al.* 2005; Gregory *et al.* 2007; Ellis and Gleizes 2011). These include mutations that result in premature stop codons as well as missense mutations. Studies in yeast have shown that the Rps19 protein is essential for cell growth. Deletion of one of the two copies of the gene results in reduced growth, consistent with haploinsufficiency as part of the disease pathophysiology. Rps19 is required to process the precursors that make the 18S rRNA and for SSU assembly (Leger-Silvestre *et al.* 2005). Some rps19 missense mutations that cause DBA impart reduced growth and defects in ribosome biogenesis in yeast (Leger-Silvestre *et al.* 2005; Gregory *et al.* 2007). Many of the missense mutations cluster near each other on the *Pyrococcus abyssi* S19 structure (Gregory *et al.* 2007). In general, mutations that cause DBA result in defects in the nucleolar steps of ribosome biogenesis (Moore *et al.* 2010).

Like DBA, SDS is also a rare human genetic disease of congenital bone marrow failure (Burroughs *et al.* 2009). However, SDS often presents with other symptomatology such as exocrine pancreatic insufficiency, skeletal abnormalities, and neutropenia. Mutations in the Shwachman–Diamond–Bodian gene (SBDS in humans; SDO1 in yeast) result in autosomal recessive inheritance. Patients are predisposed to myelodysplastic syndrome and acute myelogenous leukemia.

Yeast genetics revealed important clues about the pathophysiology of SDS. Deletion of SDO1 in yeast results in a severe growth defect (10 hr doubling time) (Menne *et al.* 2007). A genetic screen for spontaneous extragenic suppressors revealed mutations in the TIF6 gene (eIF6 in humans). Consistent with a role in 60S biogenesis, *sdo1Δ* cells demonstrate underaccumulation of 60S subunits in the cytoplasm. The proposed disease mechanism, based on these yeast studies, was that mutation or deletion of SDO1 resulted in defective Tif6 recycling from the cytoplasm to the nucleolus, leading to aberrant translational activation in the cytoplasm. Further studies in mice corroborated and extended these observations from yeast and permitted the

conclusion that SBDS is indeed a ribosomopathy (Finch *et al.* 2011). Unlike DBA, SBDS is primarily caused by defects in cytoplasmic steps in ribosome biogenesis (Moore *et al.* 2010).

Yeast has also been used as a model system to test the effect of the Rpl10 R98S mutation identified in exome sequencing of T-cell acute lymphoblastic leukemias. Results indicated that the mutation affected cell growth and caused defective ribosome biogenesis (De Keersmaecker *et al.* 2013).

While yeast has been clearly valuable for the study of some particular ribosomopathies, at least in one case it has not proved to be a good model system. North American Indian childhood cirrhosis is caused by an R565W mutation in hUTP4/Cirhin (Chagnon *et al.* 2002). When an analogous mutation was made in the yeast Utp4 protein, no effect was observed on growth or ribosome biogenesis (Freed *et al.* 2010). However, introducing the mutation in the human hUTP4/Cirhin protein abrogated interaction with the ribosome biogenesis factor NOL11 in the yeast two-hybrid system (Freed *et al.* 2012), giving the first hints at a potential pathophysiology. It remains to be seen whether the NAIC missense mutation confers defective ribosome biogenesis in human cells.

Perspectives

Several classes of questions need to be addressed to better understand ribosome assembly, including those already posed in this review.

Linking assembly with transcription of rRNA

Only in the last few years has it become evident that in fast-growing yeast, ribosome assembly occurs cotranscriptionally. How is transcription coupled with assembly and with the state of the cell? What triggers the switch from cotranscriptional assembly to post-transcriptional assembly when cell growth rates decrease? Do assembly and pre-rRNA processing occur differently cotranscriptionally *vs.* post-transcriptionally?

Folding of pre-rRNA

A major unanswered question and perhaps the greatest technical challenge to understand ribosome assembly is to understand how pre-rRNA folds during assembly *in vivo*. How does pre-rRNA transition from secondary to proper tertiary structure? There are hints that the 5′ and 3′ ends of the pre-rRNA communicate with each other during early steps. Is this indeed the case, and how does it work? How flexible is the folding pathway? Are there multiple, different productive folding pathways? How are kinetically trapped, unproductive conformers avoided or resolved? How does binding of r-proteins and assembly factors affect pre-rRNA folding and how does pre-rRNA folding dictate binding of these proteins?

What is the role of transcribed spacers? Do they function as internal RNA chaperones? Are they binding sites for eukaryotic-specific assembly factors? Do conformational switches involving spacers enable their removal or does removal trigger the switches? Do decisions for the irreversible removal of spacers serve as checkpoints in assembly?

Protein and RNA interaction networks in assembly

Based on their amino acid sequence, most of the ribosome assembly factors (and all of the r-proteins) are predicted to lack enzymatic activity. Thus, they are more likely to function to establish stable and productive preribosome structures, through specific, although transient interactions with each other and with pre-rRNA. Locating each assembly factor (and r-protein) within preribosomes provides valuable clues to their function. The CRAC protocol and cryo-EM have begun to provide such information. Yet we have probably discovered only a small fraction of the network of protein–protein and protein–RNA interactions in assembling ribosomes (other than those predicted for r-proteins, based on structures of the mature particles). How are these interactions established during assembly, how do they change during assembly, and how do they drive assembly forward? While the CRAC method has opened possibilities to map certain direct interactions of assembly factors with pre-rRNA, additional approaches would be useful to map other contact points. Higher-resolution cryo-EM promises to add to this picture as well.

Likewise, we need improved tools to identify protein–protein interactions. Thus far they have largely been identified by two-hybrid assays or by discovery of stable assembly subcomplexes. These interactions need to be mapped in more detail and in the context of preribosomes. More sophisticated use of cross-linkers coupled with the development of mass spectrometric tools to identify cross-linking sites might provide powerful solutions to this problem (Stengel *et al.* 2012).

Roles of assembly factors with enzymatic activities

Assembly factors include 19 different DEAD/DEAH-box ATPases, 3 AAA ATPases, and 6 different GTPases, as well as several kinases and a phosphatase. Do the NTPases serve as remodelers, timers, or switches during ribosome assembly? Do NTP-independent as well as NTP-dependent functions of NTPases enable formation of proper RNP architecture preribosomes? Do these proteins serve as timers, by preventing certain steps in assembly, until successful formation of a specific preribosome structure activates NTP hydrolysis to trigger release of the factor?

Surveillance and turnover of misassembled ribosomes

An important challenge facing us is to discover how misassembled preribosomes are recognized and marked for destruction. Are unprotected ends of RNAs primary targets? Do post-translational modifications occur in response to misassembly and trigger turnover of preribosomal proteins? How are mistakes in assembly and subsequent turnover of misassembled preribosomes communicated to the pathways that regulate growth and proliferation of cells?

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