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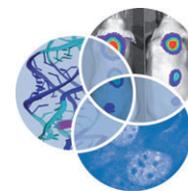
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An overview of pre-ribosomal RNA processing in eukaryotes

Anthony K. Henras, Célia Plisson-Chastang, Marie-Françoise O'Donohue, Anirban Chakraborty[†] and Pierre-Emmanuel Gleizes*

Ribosomal RNAs are the most abundant and universal noncoding RNAs in living organisms. In eukaryotes, three of the four ribosomal RNAs forming the 40S and 60S subunits are borne by a long polycistronic pre-ribosomal RNA. A complex sequence of processing steps is required to gradually release the mature RNAs from this precursor, concomitant with the assembly of the 79 ribosomal proteins. A large set of *trans*-acting factors chaperone this process, including small nucleolar ribonucleoproteins. While yeast has been the gold standard for studying the molecular basis of this process, recent technical advances have allowed to further define the mechanisms of ribosome biogenesis in animals and plants. This renewed interest for a long-lasting question has been fueled by the association of several genetic diseases with mutations in genes encoding both ribosomal proteins and ribosome biogenesis factors, and by the perspective of new anticancer treatments targeting the mechanisms of ribosome synthesis. A consensus scheme of pre-ribosomal RNA maturation is emerging from studies in various kinds of eukaryotic organisms. However, major differences between mammalian and yeast pre-ribosomal RNA processing have recently come to light. © 2014 The Authors. *WIREs RNA* published by John Wiley & Sons, Ltd.

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INTRODUCTION

The eukaryotic ribosome consists of two subunits formed by the intricate association of 79 ribosomal proteins (RPs) with 4 distinct ribosomal RNAs (rRNAs).^{1,2} The small subunit (40S) comprises the 18S rRNA assembled to 33 RPs (RPSs), whereas the large subunit (60S) contains the 5S, 5.8S, and 25S/28S rRNAs associated with 46 RPs (RPLs). The production of this huge molecular machine consumes the major part of the cellular energy³ and occupies vast

nuclear domains before its final maturation in the cytoplasm.⁴ Indeed, ribosome biogenesis is an oriented process starting in the nucleolus where three of the rRNAs (18S, 5.8S, and 25S/28S rRNAs) are produced as a long primary transcript originating from the head-to-tail tandem repeats of rDNA (~150 copies per haploid genome in yeast *Saccharomyces cerevisiae* and 300–400 in a diploid human cell), which are transcribed by RNA polymerase I (Pol I). In baker's yeast, the rDNA repeat also encodes the 5S rRNA, which is transcribed in the reverse direction. In human cells, the rDNA clusters, called nucleolar organizer regions (NORs), are localized on the short arms of the five acrocentric chromosomes (HSA13–15, HSA21, and HSA22),^{5,6} while the precursor to the 5S rRNA is synthesized from multiple genes located in close proximity to the nucleolus.⁷

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Within the eukaryotic primary rRNA transcript, the mature 18S, 5.8S, and 25S/28S rRNAs are separated by the internal transcribed spacers 1 (ITS1) and 2 (ITS2) and flanked by the 5' and 3' external transcribed spacers (5'-ETS and 3'-ETS). The nascent primary transcripts associate co-transcriptionally with some RPs, numerous pre-ribosomal factors (PRFs), and small nucleolar ribonucleoprotein particles (snoRNPs) to form a series of large RNPs,⁸ in which pre-rRNA folding and modification take place together with RP assembly. Along this process, the transcribed spacers are sequentially eliminated through a complex series of endonucleolytic and exonucleolytic cleavages. The production pathways of the two ribosomal subunits diverge after cleavage in the ITS1. The maturing pre-60S particles form the so-called granular component of the nucleolus, seen by electron microscopy (EM),⁴ whereas pre-40S particles are more rapidly exported to the cytoplasm. Proteomic studies have established a highly dynamic pattern in the protein composition of these pre-ribosomes, with gradual association of RPs and association/dissociation of PRFs, either as individual factors or as multiprotein modules.^{9,10} The major part of this process occurs in the nucleolus, but additional maturation events take place in the nucleoplasm. Pre-ribosomes are then actively exported to the cytoplasm, where they undergo final processing to form the mature ribosomal subunits. Here, we provide an overview of the mechanisms underlying pre-rRNA conversion into mature 18S, 5.8S, and 25/28S rRNAs, as it emerges from the study of unicellular and multicellular eukaryotes. Synthesis and maturation of the 5S rRNA have been reviewed elsewhere.¹¹

DIFFERENT PATHWAYS FOR PRE-rRNA PROCESSING

Maturation of the nascent pre-rRNA begins with the early stages of the production of the 18S rRNA, namely endonucleolytic processing of the 5'-ETS and cleavage within the ITS1 (Figures 1 and 2). In yeast cells, the 18S rRNA is exclusively generated by a series of endonucleolytic cleavages within the 5'-ETS and ITS1 sequences (Figure 1), whereas a combination of endonucleolytic and exonucleolytic processing steps in the ITS1 is involved in mammalian cells (Figure 2). Following cleavage of the ITS1 in the early precursors, the 5' end of the 5.8S rRNA is tailored through two alternative processing pathways that yield a long and a short form. Elimination of the ITS2 is initiated by endonucleolytic cleavages allowing 3'→5' and 5'→3' exonucleases to respectively generate the mature 3'

end of the 5.8S rRNA and the mature 5' end of the 25S/28S rRNA.

The timing of 5'-ETS removal relative to ITS1 cleavage in the primary transcript varies among organisms or cell lines and may also differ from one cell type to another in multicellular organisms.^{15,24–26} In human cells, early cleavage of the 5'-ETS yields the 41S pre-rRNA, whereas the presence of the 30S pre-rRNA indicates that ITS1 cleavage at site 2 precedes 5'-ETS cleavage (Figure 2). Similarly, ITS1 cleavage at site E prior to the cleavage at site 2 results in the formation of the 36S pre-rRNA, which is commonly observed in mouse cell lines. This kinetic variability, long acknowledged in the case of animals and plants, may also apply to *S. cerevisiae*, albeit to a lesser extent. While 5'-ETS is cleaved first in the major pathway in yeast, the 23S pre-rRNA, produced by ITS1 direct cleavage at site A₃ prior to 5'-ETS, is present at low levels in wild-type yeast cells.^{27,28} The 22S (A₀–A₃) and 21S (A₁–A₃) pre-rRNAs are also detected. These species accumulate in ribosome biogenesis mutant strains, where they are targeted for degradation. They are therefore usually considered as dead-end intermediates. However, these RNAs are strongly co-immunoprecipitated with several components of the processing machinery in wild-type cells.^{29,30} In addition, the 21S pre-rRNA accumulates in yeast strains bearing mutations in *RRP5* or *PFA1*, in which A₂ cleavage is strongly inhibited.^{31,32} These mutant strains display a growth rate indistinguishable from wild-type cells, suggesting that the 21S intermediate can be productively converted to the mature 18S rRNA in yeast. In higher eukaryotes, this kinetic variability in the early cleavages is often interpreted as the existence of different processing pathways. It is tempting to speculate that it represents a potential source of structural and functional heterogeneity among ribosomes, an idea that requires further experimental support. These alternative pathways may also contribute to the robustness of the maturation process and to its regulation. It is of note that pre-rRNA maturation also includes nonuniversal processing steps in some organisms (discussed in Ref 26).

These differences in cleavage kinetics may also be linked to the timing of pre-rRNA processing relative to transcription. While detection of the full-length rRNA precursors (35S pre-rRNA in yeast and 47S pre-rRNA in mammals) clearly points to the occurrence of post-transcriptional processing, co-transcriptional cleavage of the pre-rRNA also takes place in yeast, as evidenced from the visualization of rDNA transcription units by EM after chromatin spreading. The so-called Christmas trees display nascent transcripts of variable lengths, proportional to

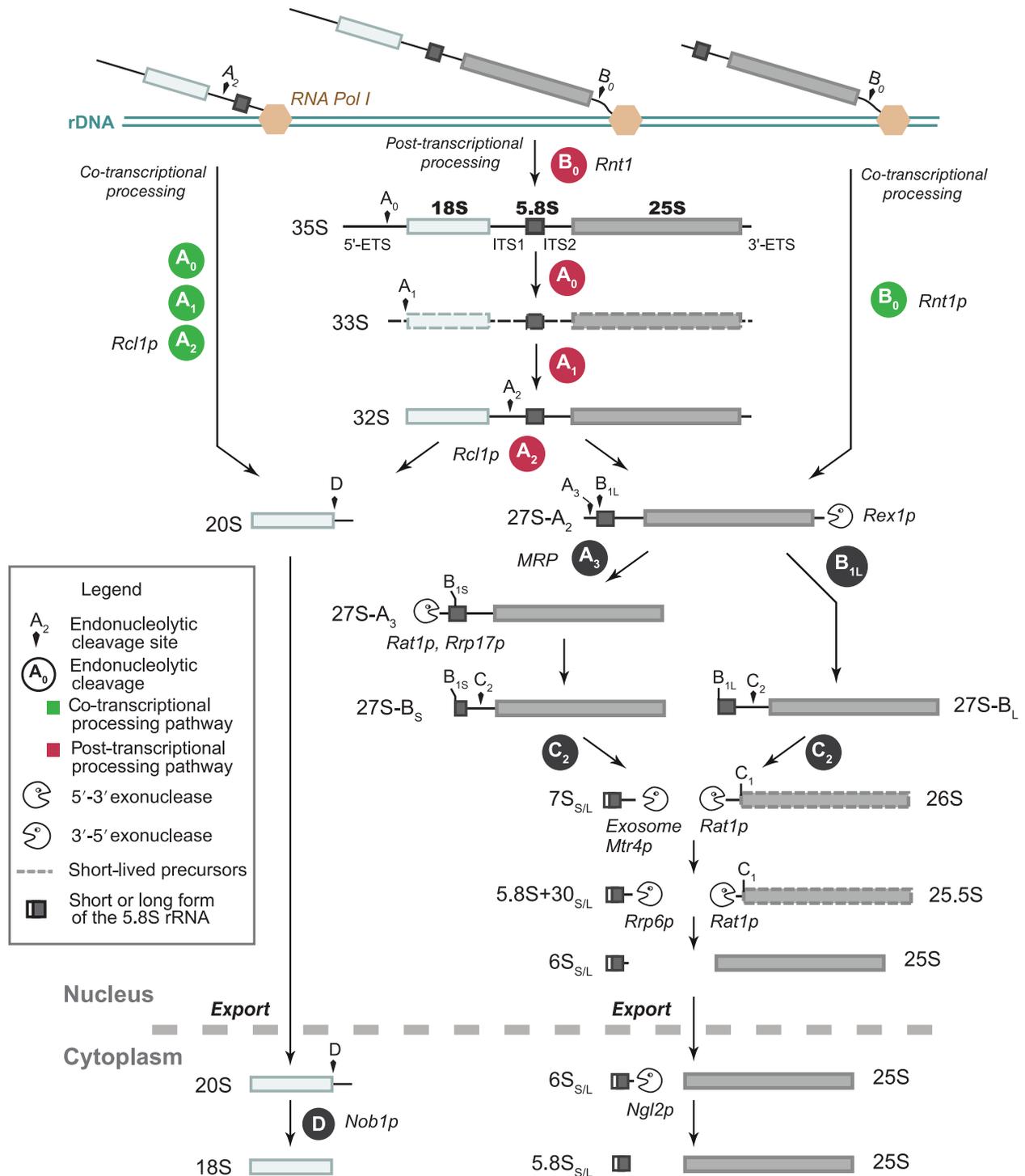


FIGURE 1 | Pre-ribosomal RNA (rRNA) processing in yeast *Saccharomyces cerevisiae*. The majority of the nascent transcripts are cleaved co-transcriptionally at sites A_0 , A_1 , and A_2 , yielding the 20S and 27S- A_2 pre-rRNAs (green).¹² Alternatively, the full-length 35S pre-rRNA is processed post-transcriptionally (red). After elimination of the 5'-ETS and cleavage at site A_2 , maturation of the 18S rRNA 3' end from the 20S pre-rRNA requires a single endonucleolytic cleavage step by Nob1p, which takes place in the cytoplasm after nuclear export of the pre-40S particle. Maturation of the large subunit follows two pathways, which yield two versions of the 5.8S rRNA 5' end. The major pathway produces a short form by endonucleolytic cleavage of the 27S- A_2 pre-rRNA at site A_3 by RNase MRP and subsequent exonucleolytic processing of the 27S- A_3 pre-rRNA by Rat1p and Rrp17p.¹³ Alternatively, the 27S- A_2 pre-rRNA is cleaved at site B_{1L} , yielding a long form of the 5.8S rRNA. Maturation of the 5.8S rRNA 3' end is completed in the cytoplasm by exonuclease Ng12p.¹⁴

26S precursors (Figure 2), both of which accumulate upon impairment of ribosome biogenesis.^{39,40} These cleavages are generated by endoribonucleases, as the spacer fragments corresponding to the intervening sequences were detected in yeast^{41–43} as in mouse cells.⁴⁴ At present, the enzymes responsible for the 5'-ETS endonucleolytic cleavages remain unknown. However, a large number of factors are required for processing of the 5'-ETS including the U3, snR30/U17, and U14 snoRNAs, the SSU processome,^{35–37} and about half of the RPSs^{40,45} (see below). Sites A0 and 1 are thought to be located close to one another in the 5'-ETS structure, and are always found near binding sites of the U3 snoRNA.^{46,47} The U3 snoRNA plays a central role in 5'-ETS processing by hybridizing with distant segments of the pre-rRNA and chaperoning RNA folding,^{26,48–51} which was shown to orientate the order of the early cleavages in *Xenopus laevis*.^{26,52} According to secondary structure predictions made for the human and murine 5'-ETS,^{53,54} sites A0 and 1 flank the stem region of a very large structural domain, the size of which is bigger than that of the 18S rRNA. In yeast, degradation of the 5'-ETS fragments is mostly ensured by the exosome,^{42,55} whereas it primarily requires the cooperative action of the 5'→3' exoRNase XRN2 and the exosome in mammalian cells.^{16,44,56}

An additional very early cleavage site in the 5'-ETS, called A' or O1, was described in animals and plants, but the frequency of this cleavage varies among species and cell types.^{26,57–59} This site is characterized by an evolutionarily conserved motif (ECM) of 11 nucleotides, which allows recruitment of the U3 snoRNP and of the abundant phosphoprotein nucleolin. Efficient cleavage at A' was shown to depend on the U3, U14, E1, and E3 snoRNPs,^{60,61} but recent data indicate that the sole UTP-A complex, recruited upstream of U3 snoRNA, is strictly necessary in human cells.⁵⁶ Surprisingly, this endonucleolytic cleavage requires the 5'→3' exonuclease XRN2.^{56,62} This cleavage appears optional for production of mature rRNAs because further processing of the 5'-ETS at sites A0 and 1 is not precluded by defects in A' cleavage.^{56,63} Conversely, affecting cleavage at sites A0 and 1 by depleting RPs does not hamper A' cleavage.⁴⁰ This cleavage could facilitate access of the processing machinery for the subsequent maturation steps.

Processing of the ITS1

Recent studies have highlighted major differences in the formation of the 3' end of the 18S rRNA between yeast and mammals. While this process is accomplished through two endonucleolytic cleavages (sites A₂ and D) in yeast (Figure 1), it requires the combined action of both endonucleases and

exonucleases in human cells (Figure 2). As in yeast, two endonucleolytic sites have been identified within the human ITS1 so far: site 2 and site E. Usually, cleavage at site 2 occurs first. In human cells, available data suggest that it is located ~900 nucleotides downstream of the 18S 3' end.⁵⁹ Cleavage at site E occurs after nucleotides C5605 and C5608, i.e., 78 and 81 nucleotides after the 18S–ITS1 junction, resulting in the formation of 18S-E pre-rRNA (Figure 3).¹⁶ Unlike in yeast, the pre-rRNAs generated by cleavage in the ITS1 are then trimmed by 3'→5' exonucleolytic activities.^{16,17,64,65} Removal of around 250 nucleotides at the 3' end of the 21S pre-rRNA produces the 21S-C intermediate. This exonucleolytic step stops at the boundary of a highly conserved domain of ITS1 in mammals (domain C in Figure 3). Similarly, the 3' end of the 18S-E precursor is gradually shortened in the nucleus and the cytoplasm.¹⁶ The identity of these exonucleases and their role need to be clarified. The exosome has been proposed to ensure the trimming of the 21S pre-rRNA.^{16,17,65} It was suggested that processing of the 21S RNA by the exosome represents a major pathway for generating the 18S-E pre-rRNA,⁶⁵ but rapid amplification of cDNA 3' ends (3'-RACE) analysis of the 18S-E pre-rRNA does not support this hypothesis.¹⁶ Other 3'→5' exonucleases are likely to be involved in the processing of the 18S-E pre-rRNA.¹⁶ After nuclear export, the PIN domain-containing protein NOB1 likely cleaves the 18S-E pre-rRNA at site 3, which yields the mature 18S rRNA.^{16,65} Exonucleolytic processing of the 18S-E pre-rRNA in the cytoplasm could facilitate access of NOB1 to site 3. Alternatively, 3'→5' trimming could proceed up to the 18S rRNA 3' end and constitutes an alternative maturation pathway. Interestingly, the 18S-E pre-rRNA is oligouridylated in the cytoplasm, which might stimulate the recruitment of an exonuclease.¹⁶ In yeast, the pre-40S particles containing the 20S pre-rRNA are exported to the cytoplasm, where endonucleolytic cleavage by Nob1p forms the mature 18S rRNA.^{67,68} Recently, it was shown that the final maturation stages of the 40S subunits in yeast involve a 'translation-like' cycle, where the cytoplasmic pre-40S particles associate with mature 60S subunits for a proofreading test prior to processing by Nob1p.^{67,69}

In mammalian cells, the efficiency of cleavage at site 2 is rather insensitive to defects in cleavage at sites A0, 1, and E. It depends on a number of factors including PES1, BOP1, NOL12, and RPs of the large subunit (RPL15 and RPL26).^{16,65,66} Absence of any of these proteins slows down cleavage at site 2 relative to 5'-ETS processing and affects processing by XRN2, leading to accumulation of 41S and 36S

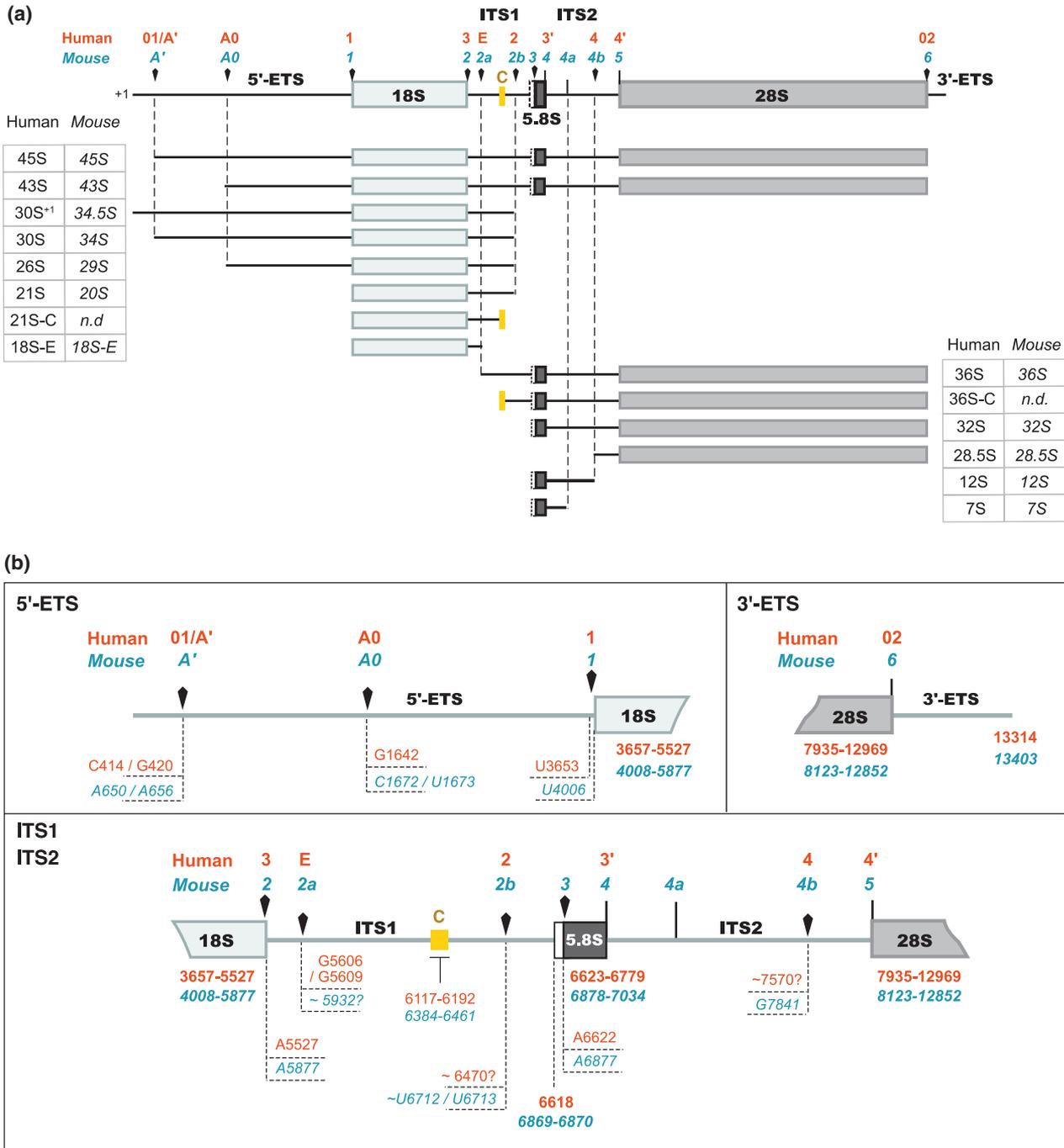


FIGURE 3 | The different pre-rRNAs detected in human and murine cells and their nomenclature.^{15,16,24,39,40,44,47,59,66} (a) Definition of the major pre-ribosomal RNAs (rRNAs). Arrowheads indicate the endonucleolytic cleavage sites. The yellow box corresponds to a highly conserved domain in ITS1 among mammals. Its boundaries define the 5' end of the 36S-C and the 3' end of the 21S-C, suggesting that exonucleolytic trimming of these extremities is stopped by a secondary structure and/or a protein complex anchored on this region.¹⁶ The 36S-C and the 30S⁺¹/34.5S species are examples of precursors that are only detected upon perturbation of ribosome biogenesis. (b) Map of the processing sites in the human (orange) and murine (blue) pre-rRNAs. Arrowheads indicate the endonucleolytic cleavage sites. The nucleotides correspond to the residue located 5' to the cleavage site. The extremities of the 18S, 5.8S, and 28S rRNAs are indicated in bold letters. The mapping of these sites is partly discussed in the text and was extensively reviewed by Mullineux and Lafontaine.⁵⁹ Human site E was determined by primer extension and 3'-RACE.¹⁶ Localization of site 4b in mouse was mapped by primer extension.⁴⁴ The human sites equivalent to sites 3 and 4a in mouse have not been formally designated. Details on site 4a are given in the text. The numbering of the nucleotides refers to GenBank sequences U13369.1 (human rDNA) and BK000964.3 (mouse rDNA).

pre-rRNAs (Figure 2). Interestingly, the orthologs of PES1, BOP1, and NOL12 in *S. cerevisiae* (Nop7p, Erb1p, and Rrp17p) are part of the 'A₃ factors', which are required for 5'→3' exonucleolytic tailoring of the 5' extremity of the 5.8S_S rRNA by Rat1p after cleavage at site A₃ (see below). These observations draw a strong parallel between site 2 in human and site A₃ in yeast. In contrast, cleavage at site E in human requires prior cleavage at sites A0 and 1 and is blocked upon knockdown of RPS18, RPS19, or Bystin/ENP1,^{40,64} similar to what has been observed in yeast for site A₂.⁷⁰ Therefore, processing sites E and 2 appear to be functionally related to sites A₂ and A₃ in yeast, respectively. In yeast, Rcl1p, a protein belonging to the RNA cyclase family, has been proposed to be the enzyme responsible for cleavage at site A₂.⁷¹ RCL1, the human ortholog of Rcl1p, is required for cleaving the ITS1 at site E.⁶⁵ Cleavage at site A₃ in yeast is catalyzed by the RNase MRP, a RNP composed of a structural RNA associated with 10 essential proteins.⁷² However, knockdown of MRP components was found to have no effect on ITS1 cleavage in human cells.⁶⁵ Nevertheless, these strong functional analogies allow envisioning a unified nomenclature of the endonucleolytic cleavage sites in eukaryotes.

MATURATION OF THE LARGE SUBUNIT rRNAs

Maturation of the 5' end of the 5.8S rRNA is coordinated with formation of the 3' end of the 25S/28S rRNA. In yeast, mutation of the 3'-ETS impairs endonucleolytic cleavages both at sites A₃ and B_{1L}, indicating that these processing events are somehow coupled.⁷³ A similar phenotype was observed upon loss-of-function of the U8 snoRNA in *X. laevis*.⁷⁴ In yeast, the 3'-ETS is cleaved by endonuclease Rnt1p at site B₀, which may participate in transcription termination⁷⁵ (Figure 1). This leaves a ~20-nucleotide extension that is removed by the 3'→5' exonuclease Rex1p in the 27S-A precursors.⁷⁶

Two alternative processing pathways produce 5.8S rRNAs with different 5' ends. In yeast, the 5' end of the major form (5.8S_S for 5.8S short) results from 5'→3' exonucleolytic trimming of the 27S-A₃ pre-rRNA down to site B_{1S} by two exoribonucleases: Rat1p, the activity of which is stimulated by its co-factor Rai1p, and Rrp17p.^{13,77–81} Rat1p is recruited within the particles containing the 27SA₂ intermediate, prior to A₃ cleavage.⁸² It requires a series of factors referred to as the 'Nop7p-subcomplex'⁸³ or 'A₃ cluster',⁸² which bind to the 3' end of the 5.8S rRNA, the ITS2, and the 5' end of the 25S rRNA, and coordinate the processing events

occurring at the 5' and 3' ends of the 5.8S rRNA.⁸² The 5' end of the minor form (5.8S_L for 5.8S long) is produced through a direct cleavage at site B_{1L} of the 27SA₂ RNA, and probably also the 27SA₃, by a yet unidentified endonucleolytic activity.⁸⁴ Endonucleolytic processing of the ITS2 at site C₂ is initiated only after maturation of both the 5' end of the 5.8S rRNA and 3' end of the 25S rRNA.⁸⁵ Cleavage at C₂ yields the 7S pre-rRNA, which corresponds to the mature 5.8S rRNA with a ~140-nucleotide extension at its 3' end. Production of the mature 3' end of the 5.8S rRNA from this 7S precursor involves a surprisingly complex succession of exonucleases (Figure 1). First, the nuclear exosome initiates the 3'→5' processing of the 7S intermediates from site C₂.^{85,86} This process is assisted by the RNA helicase Dob1p/Mtr4p, which probably functions in unwinding the secondary structures or displacing the bound proteins that may otherwise hamper progression of the exosome.^{42,85,87} Progression of the exosome is stopped ~30 nucleotides from the 5.8S rRNA 3' end because of steric hindrance, resulting in the 5.8S + 30 intermediates. The Rrp6p exonuclease in turn further degrades the ITS2 sequence and leaves approximately eight nucleotides, generating the 6S intermediates.^{85,88} A role of exonucleases Rex1p, Rex2p, and Rex3p in this process was also proposed,^{89,90} but may rather correspond to a quality control pathway.¹⁴ Finally, the Mg²⁺-dependent exonuclease Ngl2p trims the 3' end of the 6S pre-rRNA in the cytoplasm, producing the mature 3' end of the 5.8S rRNA.^{14,84} Interestingly, the 5'→3' exonuclease Rat1p⁹⁰ and its co-factor Rai1p⁸¹ are also required for the 3'-end processing of the 5.8S rRNA. These factors may coordinate the 5'- and 3'-end processing of this rRNA. The downstream intermediate of C₂ cleavage, the 26S RNA, corresponds to the 25S rRNA with a 5' extension. Removal of this segment is performed by exonuclease Rat1p.^{79,91} The Las1p protein is required for the processing events occurring at both ends of the ITS2 sequence.⁹⁰

This processing scheme, first described for yeast cells, appears to be conserved across evolution, as shown by recent studies in human cells or in *Arabidopsis thaliana*.^{17,92} Formation of the 3' end of the 28S rRNA is a very early event that may participate in transcription termination in mammalian cells.⁷⁵ 60S particles in animals and plants also contain two forms of 5.8S rRNA, suggesting molecular mechanisms similar to those in yeast. In mouse and human cells, XRN2 (yeast Rat1p) processes the downstream products generated at sites 2 and E¹⁶ and is required for tailoring the 5' end of the 32S RNA.⁴⁴ The 5.8Ss rRNA is lost upon depletion

of BOP1 (yeast Erb1p) in mouse cells.⁶⁵ However, unlike Rat1p depletion, XRN2 knockdown does not strongly affect the ratio of 5.8S_L to 5.8S_S rRNAs.^{65,90} Endonucleolytic cleavage of ITS2 (sites 4 in human and 4b in mouse, Figure 3) in the 32S rRNA gives rise to the 12S and 28.5S species, which are precursors to the mature 5.8S and the 28S rRNAs, respectively. The 5' end of the 28.5S rRNA is then trimmed by XRN2 to form the 28S rRNA.⁴⁴ Processing of 5.8S rRNA 3' end from the 12S precursor is a multistep mechanism, as in yeast. Mammalian cells display a shorter precursor, the 7S pre-rRNA.^{93,94} This species was proposed to result from a second endonucleolytic cleavage within ITS2 (site 4a in mouse, Figure 3).^{21,22} However, intermediate processing fragments between the 12S and 7S pre-rRNAs accumulate upon depletion of the exosome components, including its catalytic subunits RRP6 and DIS3.^{16–18} In addition, the nucleolar exonuclease ISG20-L2 was proposed to take part in this process.¹⁹ Efficient maturation of the 7S pre-rRNA requires several subunits of the exosome¹⁷ and of the exosome-associated factors MTR4, MPP6, and CD1.^{17,18} Depletion of RRP6 results in very strong accumulation of a smaller intermediate, coined 5.8S+40, very similar to the 5.8S+30 species in yeast.¹⁷ Finally, a 5.8S rRNA precursor extended of a few nucleotides in 3' (termed 6S in Figure 2) undergoes final maturation by the 3'→5' exoribonuclease ERI1, as reported in mouse cells.²⁰ Noticeably, this role of ERI1 is conserved in *Schizosaccharomyces pombe* and *Caenorhabditis elegans*.⁹⁵ Studies in yeast¹⁴ and in *X. laevis* oocytes²³ have shown that the very last step of the 5.8S rRNA 3'-end maturation takes place in the cytoplasm. Consistently, ERI1 is a cytoplasmic protein in *S. pombe* and in *C. elegans*. ERI1 is not essential for cell viability in *S. pombe* and in *C. elegans*, indicating that 60S particles containing 6S rRNA are competent for translation.⁸⁴ Mice devoid of ERI1 also accumulate functional 60S particles containing 5.8S rRNA with an extended 3' end, but display embryonic growth defects with less than 10% survival at weaning and die within 2 days postpartum.²⁰ Overall, these studies indicate that the maturation scheme of the large subunit rRNAs in mammals resembles that in yeast, but further studies are necessary to precisely define the relative contribution of the various exoribonucleases and the occurrence of secondary endonucleolytic cleavages.

ROLE OF THE RIBOSOMAL PROTEINS

Exhaustive analyses in yeast and mammalian cells have shown that RPs are required for structural assembly, pre-rRNA processing, and nuclear export

of the pre-ribosomal particles.^{40,45,96–100} For example, only a few RPs are dispensable for production of the mature 18S rRNA, namely Rps12p and Rps25p in yeast,⁴⁵ and RPS25 in human.^{40,97} Similar to RPS12 (human) and Rps12p (yeast), human RPL26, unlike its yeast counterpart Rpl26p,¹⁰¹ is essential for 60S subunit production in human cells,¹⁰² pointing toward the evolution of RPs' functions.

The precise functions of RPs in pre-rRNA processing and ribosome biogenesis remain elusive. X-ray and cryo-EM structures of mature ribosomes have shown that most RPs share common structural features, in particular globular domains interspersed by poorly structured loops that bind to rRNA or other RPs.¹⁰³ Studies in prokaryotes and eukaryotes indicate that the interaction network between proteins and RNA, at first somewhat loose and dynamic, gradually tightens during subunit assembly, and eventually forms stable ribosomal subunits.^{96,99,104,105} It is generally thought that during subunit assembly, RPs act as chaperones assisting the proper folding of the nascent pre-rRNAs. Indeed, *in vitro* assembly of bacterial ribosomes showed that RPs are required in a hierarchical order.¹⁰⁶ A recent study on the *Escherichia coli* 30S small ribosomal subunit directly demonstrated the guiding role of Rps4 on the correct folding of 16S rRNA helices h3 and h18, allowing in turn the folding of h1 as well as subsequent recruitment of other proteins on the 16S rRNA.¹⁰⁷

Depletion of eukaryotic RPs results in blockades at different pre-rRNA processing steps. In human cells, initiation of cleavage at sites A0, 1, and E (but not cleavage at site A') strictly depends on assembly of half of the small subunit RPs (RPSs).⁴⁰ This first set of RPSs makes contacts with the 5' part of the 18S domain (Figure 4). They include the orthologs of the primary and secondary binders that were identified in *in vitro* assembly of the prokaryotic small subunit, and were indeed found to associate early with pre-ribosomal particles in mammalian cells.¹⁰⁹ The second set of RPSs is not required to initiate removal of the 5'-ETS, but for efficient processing of the ITS1 at sites 1, E, or 3 as well as nuclear export.⁴⁰ Those proteins are incorporated later in pre-ribosomes and structure the 3' region of the 18S rRNA (Figure 4). Similar results were also found in yeast.^{45,96} Thus, the 40S structure is sequentially assembled: first, the 'body' domain is formed, which contains the 5' end and central domains of 18S rRNA and primary and secondary binding RPs, followed by the 'head' domain, which is composed of the major 3'-end domain of 18S rRNA and secondary/tertiary binding RPs (Figure 4). This *in vivo* two-stepped, 5'→3' oriented assembly of the

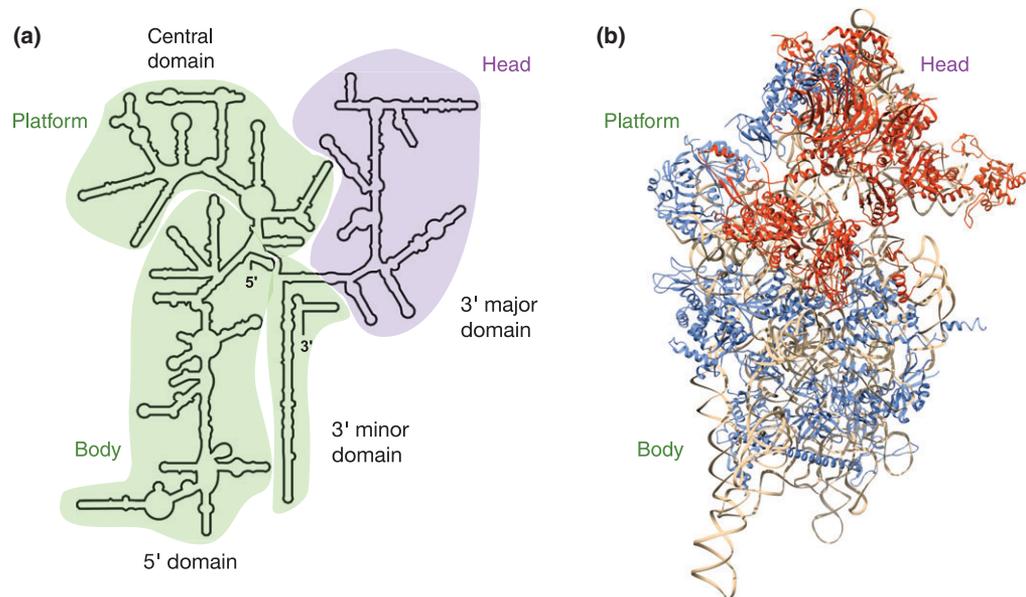


FIGURE 4 | Role of the ribosomal proteins in formation of the 40S subunit. (a) Secondary structure of the human 18S rRNA (<http://apollo.chemistry.gatech.edu/RibosomeGallery/>). Four topological domains can be distinguished, which form distinct features in the 3D structure: the body and the platform outlined in green, and the head outlined in purple. (b) Two functional groups in RPS proteins. A first class of RPSs in human cells is strictly required for initiating cleavage at sites A0, 1, and E. These ‘initiation RPSs’ or i-RPSs (blue) are associated with the 5’ part of the RNA (body, platform, and back of the head). The ‘progression RPSs’ or p-RPSs (orange) are subsequently required for efficient processing at sites 1, E, or 3; they assemble on or around the head,⁴⁰ consistent with a delay in the formation of this structure with respect to the body and the platform. Consistent data were found in yeast.^{45,96} The figure shows the position of these proteins in the structure of the human 40S subunit solved by cryo-electron microscopy (PDB 3J3A).¹⁰⁸ The 18S rRNA appears in beige.

eukaryotic 40S was also observed in prokaryotes in *in vitro* kinetic studies.^{104,110,111} Such a strong interdependency between formation of large-scale structural domains and pre-rRNA processing was also recently demonstrated for the large subunit in yeast.¹⁰⁰ Joining of RPLs and folding of rRNA are tightly connected and induce a hierarchical assembly of the structural domains in the 60S subunit. Binding of early-acting RPLs to domains corresponding to the 5’ part of the 25S and 5.8S rRNAs stabilizes a pre-ribosomal structure that facilitates A₂ and A₃ cleavages. These early-acting RPLs form a clamp-like region cupping the convex solvent side of the future 60S subunit, which favors formation of the 27S-B pre-rRNA. Subsequently, stable assembly of middle-acting RPLs allows cleavage of the 27S-B pre-rRNA at the C₂ site in ITS2. Binding of middle- and late-acting RPLs to the 3’ region of the mature 25S rRNA sequence is coupled to 7S pre-rRNA processing. Finally, the 5S RNP complex (a structural module comprising the 5S rRNA, Rpl5p, and Rpl11p and recruited in early pre-60S particles) must undergo a semicircular movement to adopt its final position and form the central protuberance of the 60S subunit.¹¹² This step requires correct folding and processing of the 6S precursor to mature 5.8S rRNA, chaperoned by late-acting RPLs.

FUNCTIONS OF THE *trans*-ACTING FACTORS

The maturation of pre-ribosomes involves a wealth of *trans*-acting proteins and snoRNPs that are required at specific stages. The snoRNPs (~80 in yeast and 200 in human) belong to two families characterized by specific motifs in their RNA component, the C/D or H/ACA boxes.^{113,114} As described earlier, a subset of snoRNPs, including U3, snR30/U17, U14, and U8, function as RNA chaperones assisting early cleavages of the pre-rRNA. However, most box C/D and box H/ACA RNPs catalyze ribose methylation and uridine isomerization into pseudouridine, respectively, at specific positions of the mature rRNA sequences within the pre-rRNA. Each snoRNA base-pairs with the pre-rRNA at the vicinity of a nucleotide to be modified, thereby guiding the enzymatic modification ensured by one of the core proteins of the snoRNPs: the methyltransferase Nop1p/fibrillarlin in box C/D snoRNPs and the pseudouridine synthase Cbf5p/Nap57p/Dyskerin in H/ACA snoRNPs. The modified nucleotides are mostly found in functionally important regions of the ribosome and are necessary for efficient and accurate translation.^{114,115} Indeed, knockdown of a single snoRNA is sufficient to alter development of zebrafish embryos.¹¹⁶ In addition, a

few nucleotides are methylated on the base moiety by methyltransferases Dim1p,¹¹⁷ Bud23p,¹¹⁸ Rrp8p,¹¹⁹ Bmt2p,¹²⁰ Nop2p, and Rcm1p.¹²¹ While several of these methyltransferases are essential for viability in yeast, their enzymatic activity is not always required, suggesting a facultative role of these modifications in ribosome activity.^{118,122} In the yeast decoding region, a hypermodified uridine residue combines pseudouridylation with base methylation and addition of a 3-amino-3-carboxypropyl group. Absence of these individual modifications was found to delay formation of the 18S rRNA.¹¹⁵

Many of the PRFs belong to recognizable protein families such as AAA ATPases, GTPases, RNA helicases, or kinases.¹²³ The precise molecular function of the vast majority of these factors remains ill defined. RNA helicases involved in ribosome biogenesis are expected to modulate RNA–RNA interactions within the pre-rRNAs or between the pre-rRNAs and snoRNAs. The yeast helicases Dbp4p,¹²⁴ Rok1p,¹²⁵ Has1p,¹²⁶ and Prp43p¹²⁷ appear to function in the unwinding of snoRNA/pre-rRNA base-pairings, while the release of U8 in mouse requires DDX51.¹²⁸ The Mtr4p/Dob1p helicase unwinds pre-rRNA secondary structures, thereby facilitating processing or degradation by the exosome.^{42,87} Helicases may also function as RNPs and displace RNA-bound proteins at specific stages of the maturation process. Several AAA-ATPases were shown in yeast to function in the release of *trans*-acting factors at specific stages of the maturation pathway. The best characterized example in yeast is Rea1p, a component of pre-60S particles. Rea1p mechanically extracts protein modules from the particles at different stages of the maturation pathway in the nucleus.^{129,130} Down the line in the pre-60S pathway, another AAA-ATPase, Drg1p, is required to initiate the release of PRFs in the cytoplasm.¹³¹ Regarding GTPases, Fun12p promotes joining of the late pre-40S particles to mature 60S subunits in the ‘translation-like’ cycle proposed to proofread these future 40S subunits in yeast cells.⁶⁹ Recently, the GTPase Nog2p/Nug2p has been suggested to control recruitment of Crm1p-adaptor Nmd3p and regulate nuclear export of pre-60S particles to the cytoplasm.¹³² As another example, the GTPase Efl1p functions in the release of Tif6p from cytoplasmic pre-60S particles, which allows neo-synthesized 60S particles to engage in translation.^{133,134} Kinases such as Rio2p or Hrr25p in yeast, R1OK2 or CK1 α /CK1 ϵ in human cells, are required for structural remodeling of the pre-40S particles and release of PRFs in the cytoplasm.^{135–138} Many PRFs with no predicted enzymatic activity contain putative RNA-binding domains (e.g., GAR, RRM, KH, BRX, S1, dsRBD,

and Zinc finger domains) and/or protein–protein interaction domains (WD40, HEAT, TPR, and HAT). Some of these factors function as protein modules that form before they associate with pre-ribosomes, such as the subcomplexes of the SSU processome: UTP-A,^{28,139} UTP-B,^{139–141} UTP-C,¹³⁹ Mpp10p-Imp3p-Imp4p,¹⁴² Bms1p-Rcl1p,^{143–145} or the pre-60S particle modules Rrp5p-Noc1p-Noc2p,¹⁴⁶ Noc4p-Nop14p,²⁹ and Ytm1p-Nop7p-Erb1p.^{83,147} Finally, an important function of PRFs is also to shield the pre-ribosomal particles from premature interactions with the translation machinery.⁶⁹

NUCLEOLAR SURVEILLANCE PATHWAYS

Accuracy of pre-RNA processing appears to involve constant monitoring by quality control mechanisms. In the yeast nucleus, improperly processed pre-rRNAs are rapidly targeted by a degradation mechanism known as the nucleolar surveillance pathway.^{30,148–150} These pre-rRNAs are tagged for degradation through the addition of a short tail consisting of four to five adenosines at their 3' end by two related complexes, TRAMP4 and TRAMP5. As their names indicate, these complexes contain either the Trf4p or Trf5p homologous proteins belonging to the β -nucleotidyl transferase family, the Air1p/Air2p zinc knuckle RNA-binding proteins and the Mtr4p/Dob1p RNA helicase of the DEXD/H family. Trf4p and Trf5p both display a distributive polyadenylation activity *in vitro*^{151,152} and are directly responsible for the oligoadenylation of the targeted substrates within the TRAMP complexes. The Air2p protein is an important structural component as it mediates binding of the TRAMP complexes to RNA.¹⁵³ The Mtr4p/Dob1p helicase appears to bind to oligo-A tails at the 3' end of the targeted substrates and tightly control their length.⁸⁷ The 3'→5' degradation of the oligoadenylated pre-rRNAs is mainly carried out by Rrp6p, although the core exosome might also be involved.¹⁵² Mtr4p has been suggested to mediate recruitment of the exosome to the adenylated substrates,¹⁵² possibly via its so-called KOW domain.^{153,154} Although the pre-rRNAs targeted by the nucleolar surveillance pathway are mostly degraded from the 3' end, some are also attacked from their 5' end by the Rat1p exonuclease.⁸¹

The substrate specificities of the TRAMP4/5 complexes remain unclear at present, but experimental evidence points to partially overlapping functions for these complexes.^{152,155} Deletion of *TRF5* strongly reduces the accumulation of oligoadenylated pre-rRNAs observed in the absence of Rrp6p^{148,152}

and partially restores the accumulation of faulty pre-rRNAs degraded upon loss-of-function of PRFs.^{30,148,149} Thus, TRAMP5 mainly functions in oligoadenylation of the pre-rRNAs targeted by the nucleolar surveillance pathway. Deletion of *TRF4* results in similar phenotypes, although less pronounced, suggesting a redundant function. Interestingly, inactivation of the nucleolar surveillance pathway restores not only the accumulation of the targeted pre-rRNAs^{30,148,150,152} but also the production of the mature rRNAs in some cases.¹⁴⁸ Therefore, in addition to ill-processed pre-rRNAs, the nucleolar surveillance mechanism may also target pre-rRNAs whose processing is only kinetically delayed.

How the nucleolar surveillance pathway in yeast recognizes the defective pre-rRNAs remains unclear. Based on ChIP experiments, it has been proposed that the TRAMP complexes monitor pre-rRNA processing co-transcriptionally.¹⁴⁸ Recruitment of the nucleolar surveillance machinery might be mediated by the Nrd1p–Nab3p–Sen1p complex, which interacts with the Spt4p–Spt5p RNA Pol I elongation complex.¹⁵⁶ Upon alteration of pre-rRNA processing, the TRAMP and exosome complexes transiently accumulate with pre-rRNAs into a subnucleolar focus called the No-body, which is probably the site for elimination of the targeted pre-rRNAs.¹⁵⁰

Similar mechanisms appear to exist in mammalian cells as well. In mouse cells treated with low concentrations of actinomycin D, aberrant pre-rRNAs resulting from abortive elongation by RNA Pol I are polyadenylated by PAPD5, a mammalian ortholog of Trf4p/Trf5p, and are subsequently degraded by the exosome.¹⁵⁷ Unlike Trf4p and Trf5p, which require Air1p/Air2p for binding to RNA, PAPD5 possesses its own RNA-binding domain. However, decay of aberrant pre-rRNAs in mouse also strongly depends on 5'→3' exonucleolytic processing by XRN2/Rat1.⁴⁴ As mentioned above, XRN2 is required for degrading the excised 5'-ETS fragments, as well as for processing the 5' end of the 36S, 32S, and 28S RNAs.^{16,44} Therefore, processing by XRN2 follows all the intermediate endonucleolytic cleavages in pre-rRNA processing, namely A', A0 in the 5'-ETS, E and 2 in the ITS1, and 4 in the ITS2. Similarly, the 'upstream' products of these cleavages are trimmed by processive 3'→5' exoRNases such as the exosome, ERI1, or ISG20-L2.^{16,17,65} Each endonucleolytic cleavage could thus be envisioned as a quality control step where exoribonucleases would either process or degrade the cleavage products, depending on their conformity for the subsequent maturation step.

CONCLUSION

This overview of pre-rRNA processing shows that despite a deep understanding of these mechanisms, basic elements are still missing in the blueprint of this giant Meccano, like the exact position of some cleavage sites in metazoans or the identity of several endonucleases. But new tools are now available to investigate these questions. For example, high-throughput RNA sequencing can be used to explore pre-rRNA processing and modification on a large scale and to locate maturation factors in the pre-ribosome structure by cross-linking techniques.^{158–160} Genome-wide siRNA screens^{17,161} and nucleolus proteomic^{9,10,162} analyses have provided large sets of functional data and revealed potential maturation factors. Three-dimensional EM has delivered the first structures of pre-ribosomes and will be instrumental to apprehend the structural remodeling of the ribosomal precursors.^{111,112,130,135,163–166} Recent findings in mammalian cells have revealed significant divergence of the mechanisms of pre-rRNA processing in evolution and sequencing of whole genomes should allow tackling questions such as the co-evolution of rRNAs and their processing machineries. Part of the increased complexity of the ribosome and its synthesis in evolution might be ascribed to additional connections with other cellular processes and to adaptive regulatory responses. In that respect, the discovery of ribosomopathies, a growing set of genetic pathologies or syndromes associated with mutations in genes encoding ribosome biogenesis factors, has shed new light on the link between ribosome biogenesis and cell fate.¹⁶⁷ Disorders in pre-rRNA processing, nucleolar organization, and ribosomal subunit accumulation have been detected in cells from patients suffering ribosomopathies, including Diamond–Blackfan anemia,^{102,168,169} Schwachman–Diamond syndrome,¹⁷⁰ aplasia-cutis congenita,¹⁷¹ or trichothiodystrophy.¹⁷² Dysregulated ribosome activity is also a common feature in many malignancies. Changes in the number and size of nucleoli have been used as prognostic marker (AgNOR analysis) for aggressive cancers for many years.¹⁷³ Although an elevated ribosome activity might simply correlate with increased protein synthesis in cancer cells, recent studies rather suggest that increased RNA Pol I-mediated rDNA transcription is a critical step in tumorigenic transformation,¹⁷⁴ and new RNA Pol I inhibitors are currently used in clinical trial.¹⁷⁵ Modulation of pre-rRNA processing or nucleotide modification (pseudouridylation, ribose or base methylation) could contribute to the making of different ribosomes, under normal or pathological conditions. Hence, the ribose methylation level in

rRNAs was recently shown to vary in cancer cells¹⁷⁶ or upon p53 loss-of-function.¹⁷⁷ Changes in the quality or the quantity of ribosomes may directly alter translational regulation of major regulators of cell growth and differentiation.^{176–178} Furthermore, recent data support the hypothesis along which ribosomes may display structural and functional heterogeneities in different tissues.¹⁷⁹ The tissue-specific

phenotypes observed in ribosomopathies call for a deeper understanding of the intricate relationship between ribosome biogenesis and cell regulatory mechanisms. These questions will require further extending the study of pre-rRNA processing from unicellular organisms or isolated cells to multicellular genetic models.

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