REVIEW

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Noncoding RNAs in eukaryotic ribosome biogenesis and function

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The ribosome, central to protein synthesis in all cells, is a complex multicomponent assembly with rRNA at its functional core. During the process of ribosome biogenesis, diverse noncoding RNAs participate in controlling the quantity and quality of this rRNA. In this Review, I discuss the multiple roles assumed by noncoding RNAs during the different steps of ribosome biogenesis and how they contribute to the generation of ribosome heterogeneity, which affects normal and pathophysiological processes.

Ribosomes are ribonucleoprotein (RNP) nanomachines that convert the genetic information encoded in mRNAs into proteins. At the ribosome's functional core is rRNA, a ribozyme that catalyzes the critical steps of decoding and amino acid polymerization[1]. The human ribosome contains four rRNAs and 80 ribosomal proteins (RPs; 79 in yeast) organized into two subunits, each carrying out specialized functions in translation (**Box 1**).

Ribosome biogenesis is a highly orchestrated process involving hundreds of molecular components and assembly factors (AFs). In eukaryotes, the process starts with precursor (pre)-rRNA synthesis in the nucleolus, where the synthesized pre-rRNA is modified, folded and processed. These steps are catalyzed with the aid of small nucleolar RNAs (snoRNAs), which are active as part of small nucleolar ribonucleoprotein particles (snoRNPs). All ribosomal components are then assembled and transported to the cytoplasm, and there are quality-control steps throughout[2].

Understanding ribosome biogenesis is essential because ribosomes are indispensable to all life forms. Indeed, ribosome assembly dysfunction leads to 'ribosome diseases', or ribosomopathies. These severe human diseases result from mutations in RPs or ribosome-assembly factors, and they are characterized by hematological defects, skeletal problems and increased cancer susceptibility[3,4]. An in-depth understanding of ribosome biogenesis will also allow the exploration of the functional significance of ribosome diversity.

For many years, it was assumed that all ribosomes within a cell are identical and that all cells in an organism express only one type of ribosome. This simplistic view has been called into question[5,6]. In fact, the notion of a heterogeneous population of ribosomes that might be functionally specialized was introduced more than 20 years ago[7]. Heterogeneous ribosomes differ in rRNA or ribosomal-protein composition as a result of stage-specific expression of rDNA genes, cell-specific activation of cryptic or alternative pre-rRNA–processing pathways, differential modifications of rRNA or RPs, assembly of alternative forms of RPs or a variation in ribosomal-protein copy number per ribosome.

This Review provides a current overview of the roles of various noncoding RNAs (ncRNAs) in eukaryotic ribosome biogenesis in budding yeast and human cells. Because ribosome biogenesis requires snoRNPs, current knowledge on snoRNP assembly is also reviewed, and parallels with ribosomal assembly are highlighted. Finally, this Review discusses how ncRNAs regulate ribosome synthesis and function under normal and pathophysiological conditions.

The ribosome-assembly machinery

There are six important steps in ribosome biogenesis (**Fig. 1**): (i) synthesis of components (rRNAs, RPs, AFs and snoRNAs); (ii) processing of pre-rRNAs (cleavage); (iii) covalent modification of pre-RNAs, RPs and AFs; (iv) assembly; (v) transport (nuclear import of RPs and AFs, roaming of pre-ribosomes through the nucleolus and nucleus and export of pre-ribosomes to the cytoplasm); and (vi) quality controls and surveillance mechanisms[2]. All steps are integrated, and inhibition of one can strongly affect another.

Synthesis involves all three RNA polymerases (Pol I–III). In humans, three out of four rRNAs are transcribed in the nucleolus by Pol I as a long 47S precursor. Genes encoding 80 RPs and >250 AFs are transcribed by Pol II. Many snoRNAs are processed from pre-mRNA introns; others are synthesized from their own promoters by Pol II or Pol III. 5S rRNA is transcribed by Pol III in the nucleoplasm.

Within the Pol I–transcribed precursors, mature rRNAs are embedded in noncoding spacers: 5′ and 3′ external transcribed spacers (5′ and 3′ ETSs) and internal transcribed spacers 1 and 2 (ITS1 and ITS2). Pre-rRNA processing removes these noncoding spacers accurately, generating the mature 5′ and 3′ termini of rRNAs (**Fig. 2**). Pre-rRNA processing always starts within the noncoding spacers and never at the mature rRNA ends, and it involves both endo- and exoribonucleolytic digestions. In addition, it imparts directionality to ribosome biogenesis and potentially supplies the energy stored in phosphodiester bonds for structural-remodeling events. Alternative pathways act as backup mechanisms, ensuring robustness. For example, in yeast, the 5′ end of 5.8S rRNA can be generated by either the 5′-3′ exoRNase Rat1–Rai1 assisted by Xrn1 or, in a parallel pathway, by Rrp17 (refs. 8,9).

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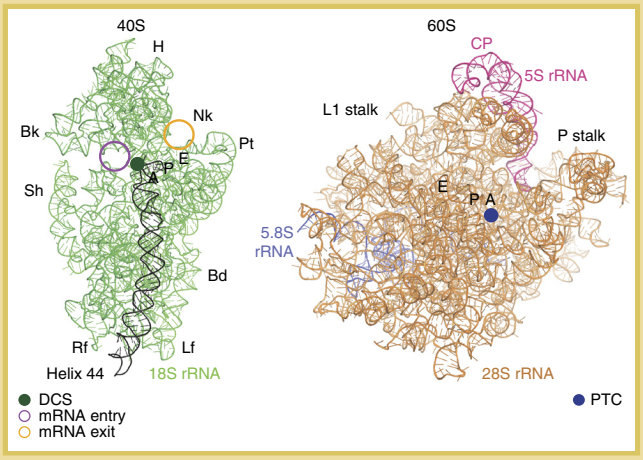
Box 1 Ribosome architecture

The small (40S) and large (60S) ribosomal subunits are depicted from the 'interface view'. mRNA entry (purple circle) and exit (yellow circle) points delineate the mRNA channel. The mRNA is read at the decoding site (DCS; dark-green circle) on the 40S subunit by aminoacylated tRNAs. The incoming tRNA, charged with its cognate amino acid, is recruited to the aminoacyl (A) site. Amino acids are then joined together to form polypeptides at the peptidyl transferase center (PTC; blue circle) located on the 60S subunit. The tRNA carrying the growing nascent peptide chain locates to the peptidyl (P) site, while the exit (E) site holds the deacetylated tRNA before its ejection from the ribosome. Morphological features of each subunit are denoted as follows: H, head; Nk, neck; Pt, platform; Bd, body; Lf, left foot; Rf, right foot; Sh, shoulder; Bk, beak; CP, central protuberance. The DCS lies at the base of helix 44 (black). Yeast rRNAs are shown at atomic resolution (PDB 3U5B and 3U5D).



It took 20 years to identify over 200 *trans*-acting AFs in budding yeast, a reference eukaryotic model organism. These and the many snoRNAs (75 in yeast, ~200 in humans) required to assemble ribosomes make the ribosome-assembly machinery far more complex than the ribosome itself. Most AFs were identified by their essential roles within pre-rRNA processing or pre-subunit export, or by their physical or functional interactions with pre-ribosome components[10]. Some AFs catalyze RNA cleavage (endo- and exoRNases) or have roles in RNA modification (snoRNPs and base methyltransferases), RNP remodeling (helicases, ATPases and GTPases)[11,12] or protein modification (kinases, phosphatases, SUMO conjugases, etc.). Other AFs were recently suggested to test subunit functionality and to act as placeholders that mask important ribosomal sites until subunit maturation is achieved[13–16] (**Fig. 3a**). By design, their displacement is a prerequisite for catalytic activation of the ribosome. Yet, in the absence of known motifs in their protein sequences, the functions of most AFs remain unknown, and further structural work must be conducted to understand precisely what they do.

The description of human pre-rRNA processing has lagged far behind that of budding yeast, partly because of the assumption that processing is evolutionarily conserved. However, 625 human nucleolar proteins were recently tested for functions in ribosome biogenesis; of those, 286 were shown to be required for rRNA processing, including 74 without yeast counterparts[17]. Forty percent of these 286 new processing factors were linked to human diseases, mostly cancers and genetic disorders. Nearly one-third of the human factors identified perform additional or distinct processing functions as compared to those of their yeast homologs[17]. Typically, factors involved in small-subunit processing in yeast are also required for large-subunit maturation in humans and vice versa[17]. For example, the exosome subunit Rrp6 is required for the 3′-end formation of 5.8S rRNA in yeast, and its human homolog EXOSC10 is also needed for 18S rRNA 3′-end maturation[17,18]. These differences could reflect higher coordination between the machineries involved in the processing of the small and large subunits in humans compared to yeast. In fast-growing yeast cells, up to 70% of nascent pre-rRNAs are cleaved cotranscriptionally within ITS1 (ref. 19). This is not known to occur in vertebrates, at least to this extent[20], thus probably offering additional opportunities for interactions between early- and late-acting processing machineries.

The evolutionary trend in ribosome biogenesis is toward increased complexity. A remarkable example is that sequences equivalent to 5.8S and 28S are collinear in bacteria and archaea but are separated by ITS2 in eukaryotes[21]. Across eukaryotes, variable expansions in mature rRNAs occur more often, and there is a greater number and size of noncoding spacers, additional cleavage sites, alternative pathways and new unique AFs as described above. The trend is matched by a 25-fold-higher complexity of the human nucleolar proteome compared to that of yeast, and it correlates with the divergence of a single fibrillar compartment into two morphologically separate nucleolar layers: the fibrillar centers and dense fibrillar components[22,23].

pre-rRNA modification, processing and folding by snoRNAs

snoRNAs are small, abundant, stable RNAs of ancient origin that localize to the nucleolus at steady state[24]. So far, they have been found in all eukaryotes, and equivalents, known as small RNAs (sRNAs), are present in Archaea. snoRNAs act in pre-rRNA modification, processing and folding through Watson-Crick base-pairing with their substrates. There are three classes of snoRNAs: box C/D, box H/ACA and MRP, all of which are active as snoRNPs, in intimate association with conserved core proteins. Assembly of the snoRNPs themselves requires dozens of AFs, as discussed below.

Most box C/D and box H/ACA snoRNPs drive RNA modification[24] (**Box 2**). The conserved boxes are bound by proteins important for snoRNA stability, nucleolar targeting and snoRNP function. Box C/D and H/ACA snoRNAs, ranging in size from 60 to 200 nt and 120 to 250 nt, respectively, are associated with four core proteins, including the enzymes that mediate rRNA modification. For box C/D snoRNPs, this is the methyltransferase Fibrillarin (FBL; NOP1 in yeast), and for box H/ACA, this is the pseudouridine synthase Dyskerin (DKC1 (also known as NAP57); CBF5 in yeast)[24].

Other snoRNPs are involved in pre-rRNA processing. Among those, the RNase MRP is in a class of its own. Composed of the MRP RNA (268 nt in humans, 340 nt in yeast) bound by ten core proteins, MRP is involved in pre-rRNA processing at site A_3 in ITS1 in yeast[25,26], a function that is apparently not conserved in humans[18]. MRP shares eight proteins with RNase P, which is active in tRNA 5′-end maturation, and their RNAs are structurally related[27]. Strikingly, in yeast ITS1, the A_3 cleavage site occupies a position equivalent to that of a tRNA in bacterial pre-rRNAs[21], thus suggesting that the tRNA was lost during evolution while the cleavage site was maintained. Specialized members of both C/D and H/ACA families are also involved in RNA processing in yeast and vertebrates[24]. Of those, the box C/D snoRNA U3, also referred to as the 'SSU-processome'[28], takes part in small-subunit maturation in yeast and humans, whereas U8 is required for large-subunit processing in vertebrates[29].

Finally, snoRNAs probably have largely underestimated roles in pre-rRNA folding because they act through extensive Watson-Crick base-pairing and can have multiple targets on pre-rRNAs, located far apart from each other. This is the case for U3, involved in eukaryotic

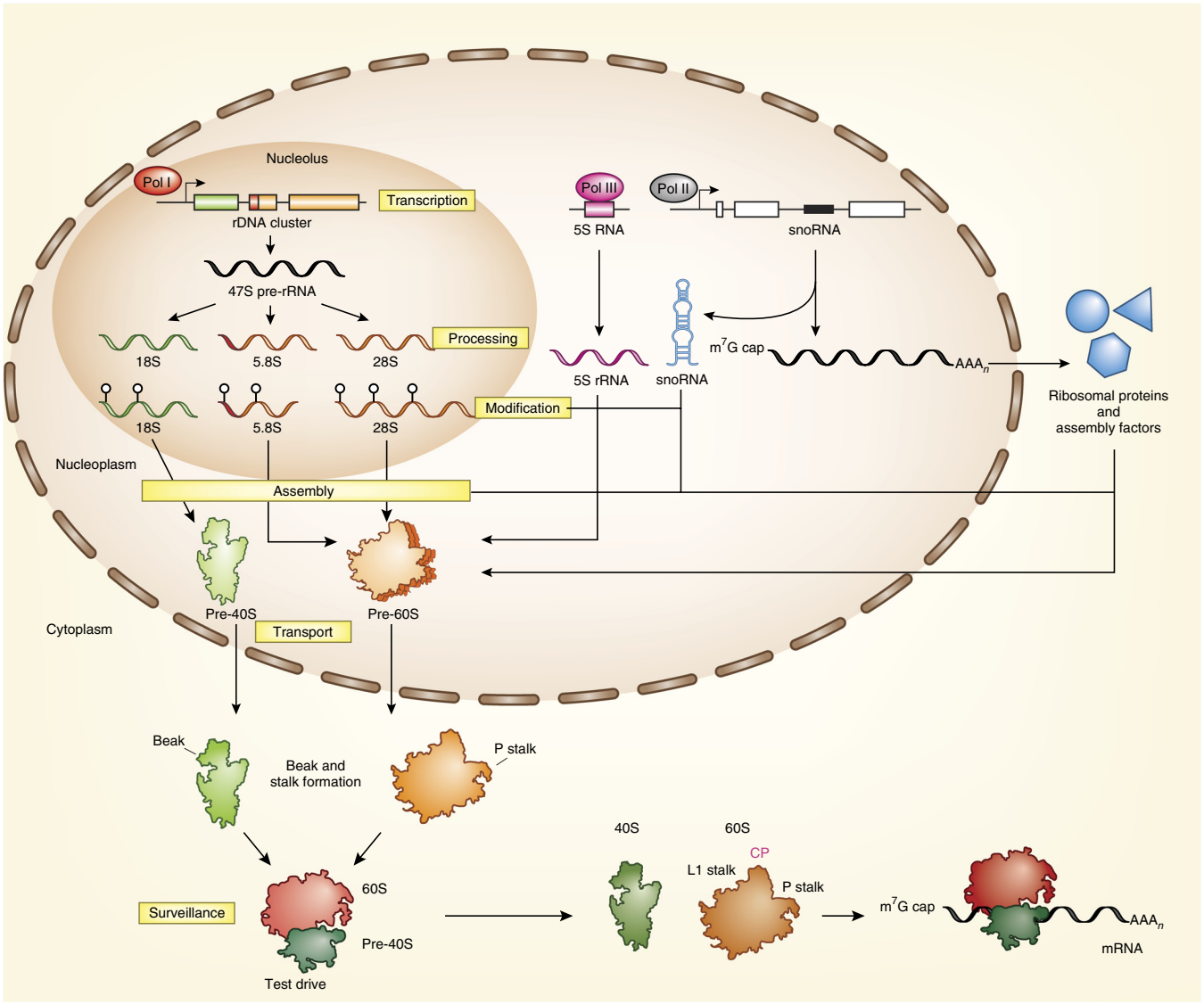


Figure 1 Eukaryotic ribosome biogenesis at a glance. Ribosome biogenesis encompasses six important steps (yellow boxes): (i) transcription of components (rRNAs, mRNAs encoding ribosomal proteins (RPs) and assembly factors (AFs), and snoRNAs); (ii) processing (cleavage of pre-rRNAs); (iii) modification of pre-RNAs, RPs and AFs; (iv) assembly; (v) transport (nuclear import of RPs and AFs; pre-ribosome export to the cytoplasm); and (vi) quality control and surveillance[2]. Three out of four rRNAs are transcribed in the nucleolus by Pol I as a long 47S precursor (47S pre-rRNA), which is then processed and modified to yield the 18S, 5.8S and 28S rRNAs that are assembled into the pre-40S (green) and pre-60S (orange) ribosomal subunits. 5S rRNA (pink) is transcribed by Pol III in the nucleoplasm and incorporated into maturing 60S subunits, forming the central protuberance (CP). 80 RPs, more than 250 AFs and 200 snoRNAs are transcribed by Pol II. The proteins are synthesized in the cytoplasm and reimported to the nucleus for assembly. Pre-40S subunits are exported to the cytoplasm more rapidly than pre-60S subunits, which require numerous nuclear maturation steps. Several structures important for ribosome function are formed only in the cytoplasm[87], including the beak on the 40S subunit and the stalk on the 60S subunit; both are protruding features that could obstruct subunit export if formed prematurely[88,89]. Pre-40S subunits undergo a 'test drive' to prove functionality before final maturation[90–92].

central-pseudoknot formation, and U8, acting during vertebrate ITS2 maturation[29–31]. The central pseudoknot is a universally conserved long-range interaction within the 18S rRNA that has a crucial role in the overall folding of the small subunit. Both U3 and U8 interact with pre-rRNAs during biogenesis, sequestering complementary sequences and thereby preventing their premature interaction. In *Escherichia coli*, which lacks U3, the timing of central-pseudoknot formation is also regulated through establishment of alternate base-pairing, but that is accomplished by *cis*-acting elements within the pre-rRNA itself[30]. This is also true of ITS2 maturation in yeast, which lacks U8 (ref. 32). The strategies used for central-pseudoknot formation and ITS2 maturation provide two remarkable cases of increased complexity in rRNA processing across evolution.

Parallels between biogenesis of ribosomes and snoRNPs

During ribosome assembly, structural reorganization of RNAs and core proteins occurs, and this is accompanied by a reduction of RNP complexity through the successive and regulated loss of associated AFs. This process triggers catalytic activation of the ribosome (**Fig. 3a**). Thus it seems that, having passed the initial assembly stages, ribosome subunit biogenesis pathways essentially become 'disassembly' pathways (examples in refs. 33–35). The assembly of snoRNPs shares many

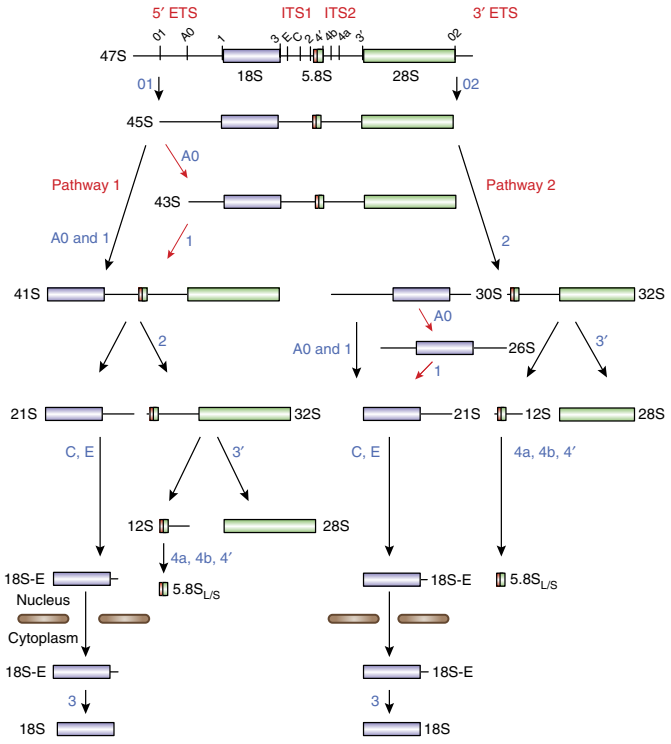


Figure 2 Pre-rRNA processing pathways in human cells. 18S, 5.8S and 28S rRNAs are produced from a single RNA Pol I transcript (47S). The mature sequences are embedded in noncoding 5′ and 3′ external transcribed spacers (ETS) and internal transcribed spacers (ITS1 and ITS2). All cleavage sites are marked on the 47S precursor, and cleavage steps are indicated in blue. 47S is cleaved at sites 01 and 02 on both sides of the molecule to generate the 45S pre-rRNA, which is processed by two alternative pathways. In a minor pathway (pathway 1), site A0 and site 1 are cleaved first to yield the 41S pre-rRNA. Uncoupling of processing at sites A0 and 1 leads to the 43S intermediate (red arrows). The 41S pre-rRNA is digested at site 2 to separate 21S and 32S pre-rRNAs, the precursors destined to form the small and large subunit, respectively. 21S pre-rRNA is cleaved at site E to produce the 18S-E intermediate, which is then processed at site 3 into the mature 18S rRNA in the cytoplasm. Processing of the 32S within ITS2 generates the 12S pre-rRNA and the 28S rRNA. The 12S pre-rRNA is successively trimmed to produce the 5.8S rRNA by a series of exoribonucleolytic digestions. There are two forms of 5.8S rRNA, 5.8S$_S$ and 5.8S$_L$ (5.8$_{L/S}$), with the latter indicated by the red extension (additional information in **Box 3**). In the other major pathway (pathway 2), the 45S pre-rRNA is directly cleaved at site 2 to generate the 30S and 32S pre-rRNAs. Processing of the 30S pre-rRNA at sites A0 and 1 produces 21S, whereas the 26S pre-rRNA arises from uncoupling at cleavage sites A0 and 1 (red arrows). 21S and 32S processing are similar in both pathways. Additional details are in ref. 26 and at http://www.ribogenesis.com/.

similarities with ribosome assembly. Though they are less complex than ribosomes, dozens of AFs are involved in snoRNP production. For instance, as in ribosome biogenesis, small proteinaceous subcomplexes of AFs are sequentially recruited to nascent precursor RNPs; subsequently, there is a progressive loss of associated factors, and there are checkpoints or 'delays' regulating catalytic activation.

The AFs involved in snoRNP biogenesis comprise both class-specific and shared AFs. For example, NAF1 and SHQ1 are specific to H/ACA RNP assembly, whereas Hsp90 and its cochaperone R2TP trigger structural remodeling during synthesis of both C/D and H/ACA RNPs[36]. Furthermore, there is abundant cross-talk in the assembly of distinct RNPs, which sometimes rely on the same AFs. Small Cajal body RNPs (scaRNPs) are structurally related to snoRNPs, but they localize in Cajal bodies and participate in the modification of spliceosomal small nuclear RNAs[37]. SHQ1, involved in H/ACA snoRNPs assembly, is also required for the synthesis of H/ACA scaRNPs and the mammalian H/ACA telomerase RNP[38]. In addition,15.5K (Snu13), the primary box C/D binder, initiates the recruitment of specific core proteins to box C/D snoRNPs, box C/D scaRNPs and spliceosomal U4 snRNP[39,40].

Several ribosome and snoRNP AFs use the principle of 'molecular mimicry', reproducing specific protein-RNA interactions through protein-protein contacts. During ribosome assembly, the adenylate kinase FAP7 interacts with ribosomal protein RPS14 by mimicking its contacts with the rRNA, thus regulating the timing of RPS14 integration in 40S (**Fig. 3b**)[41]. During H/ACA snoRNP assembly, the AF SHQ1 interacts with the pseudouridine synthase CBF5 across the RNA-binding interface, occupying the position of the guide RNA in mature snoRNPs. This precludes interaction of CBF5 with the snoRNA until the snoRNP has adequately matured[42,43] and prevents premature RNA modification (**Fig. 3c**).

Other AFs 'mask' core protein–binding sites on the snoRNA, further regulating the timing of snoRNP assembly. NAF1, a structural homolog of the H/ACA core protein GAR1, has been suggested to act as a 'placeholder' until it is replaced by GAR1 for final snoRNP maturation and catalytic activation[44]. Similarly, NUFIP (yeast Rsa1) has been suggested to hold core proteins together in immature particles and to act as an adaptor between 15.5K-bound RNP precursors and Hsp90–R2TP, which binds to 15.5K (Snu13) in a manner predicted to exclude interactions occurring in mature snoRNPs[45].

rRNA synthesis and processing as a source of ribosome diversity

Being at the core of ribosome function, rRNAs are under tremendous selective pressure. Therefore, their sequence and secondary and tertiary structures are extremely well conserved, although some variation occurs in the expansion segments. Expansions are generally located far from the functional core of the ribosome[46] and therefore are liable to only subtly influence translation. However, sequence diversity at the level of mature rRNAs has been described, involving either regulated expression of specific rDNA genes, activation of cryptic processing or simply nucleotide polymorphism[47–50]. In addition, cases of constitutive differential processing occur in all eukaryotes, such as the short and long forms of 5.8S rRNA, discussed below.

In *Plasmodium*, different rDNA genes are expressed at specific developmental stages, and alternate forms of rRNAs, with sequence heterogeneity in the variable expansions, are incorporated into ribosomes that cannot substitute for function in yeast cells[47,48]. In the naked mole-rat, ribosomes undergo constitutive clipping in the 28S rRNA to result in the excision of a 263-nt fragment from a variable region[49]. Interestingly, clipped mole-rat ribosomes are substantially more accurate than unclipped mouse ribosomes[49].

At multiple places in eukaryotic rRNA-processing pathways, alternative routes can be followed, and there are cases in which cleavages that normally occur concomitantly are uncoupled, thus leading to production of new rRNA intermediates (for example, production of 43S and 26S) (**Fig. 2**). This presumably affects the kinetics of other facets of ribosome biogenesis, such as rRNA modification. This may be why aggressive human breast cancer cells accumulate elevated levels of 43S intermediates and are characterized by altered rRNA modification profiles that make them less prone to translate internal ribosome entry site (IRES)-dependent mRNAs[51].

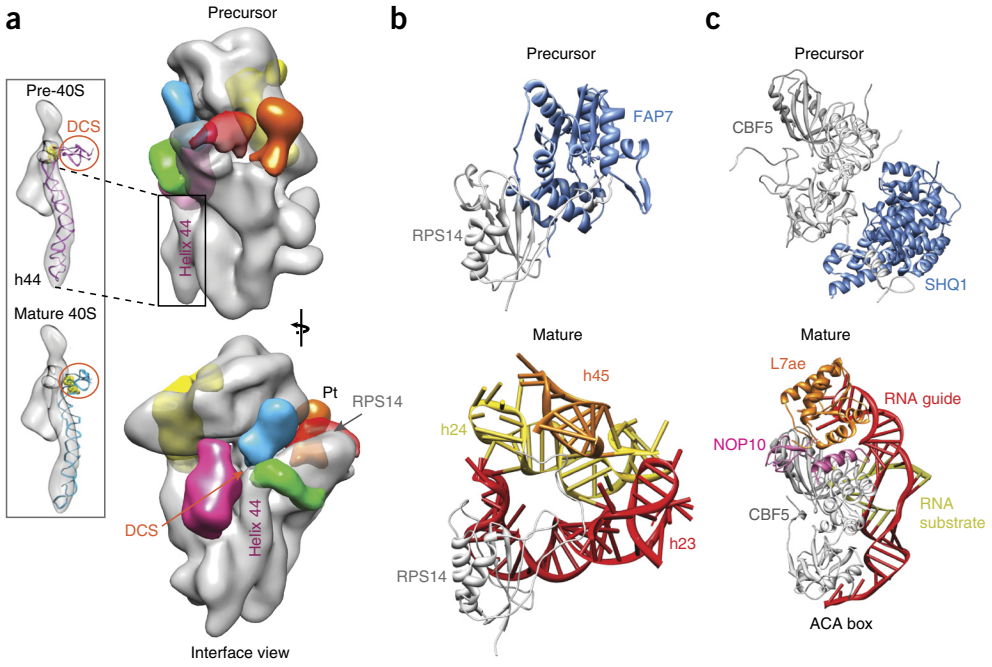


Figure 3 'Placeholders' and molecular mimicry in biogenesis of ribosomes and snoRNPs. (**a**) During ribosomal-subunit biogenesis, placeholders mask important functional sites to prevent premature activity. On the small ribosomal subunit precursor (pre-40S), depicted with rRNA and RPs in gray, AFs (in bright colors) are masking the decoding sites (DCS) at the base of helix (h) 44, as well as tRNA- and mRNA-binding sites (localization of functional sites in **Box 1**). The position of RPS14 discussed in **b** is indicated in the incipient platform (Pt). The inset shows that the DCS is distorted in the precursor subunit and acquires its functional configuration only after AF displacement. Adapted with permission from ref. 13, AAAS. (**b**) Molecular mimicry in ribosome biogenesis. In mature 40S ribosomal subunits, the ribosomal protein RPS14 interacts with three 18S rRNA helices (h23, h24 and h45; bottom). During biogenesis, the rRNA-binding surface of RPS14 is blocked by the adenylate kinase FAP7 to prevent premature integration of RPS14 into the 40S subunit (top). The crystal structure of FAP7–RPS14 was solved in archaea[41] and the RPS14–18S rRNA interaction in budding yeast[46] (PDB 4CVN, 3U5B and 3U5C). (**c**) Molecular mimicry in snoRNP assembly. During H/ACA snoRNP assembly, the AF SHQ1 blocks the snoRNA-binding surface of the pseudouridine synthase CBF5 (top), occupying the position of the guide RNA in mature snoRNPs (bottom). The crystal structure of the CBF5–SHQ1 complex was solved in budding yeast[42] and the CBF5-guide interaction in Archaea[93] (PDB 3HAY and 3ZV0). L7ae (archaeal NHP2) and NOP10 are core H/ACA proteins.

Finally, there are two forms of 5.8S rRNA, differing in length by 7 or 8 nt at the 5′ end[9,25] (**Box 3**). My laboratory has speculated that the 5′ extension of 5.8S rRNA, which is retained in approximately 30% of mature ribosomes, constitutes a remnant of normal processing[9] and provides a 'natural' or constitutive case of *pan*-eukaryotic ribosome diversity, thus generating heterogeneous subpopulations of ribosomes.

rRNA modification heterogeneity in normal and pathophysiological situations

snoRNA-mediated rRNA modification is emerging as a major source of ribosome heterogeneity, and it is becoming clear that not all positions are fully modified at all times. In yeast, only 68% of ribosomes are methylated at position A100 of the 18S rRNA (to form Am100) under standard laboratory growth conditions. This is because of the limited amount of snR51, which normally guides methylation at A100 (ref. 52). The role of Am100 is not known, but, interestingly, unmethylated ribosomes are incorporated into polysomes. Global analysis of yeast rRNA 2′-O methylation by high-throughput sequencing confirmed partial modification, demonstrating that it prevails at ~15% positions (8 out of 54 residues)[50]. Unexpectedly, this work also revealed that inhibiting methylation at specific sites affects modification at others, suggesting some levels of interdependence[50]. Currently open questions include how levels of individual modifications are regulated, i.e., what regulatory *trans*-acting factors and stimulatory growth conditions are involved and whether translation of specific subsets of mRNAs is substantially affected by partial rRNA modification.

Altered rRNA modification levels, leading to functional consequences, have also been reported for human ribosomes. In an aggressive breast cancer cell line, several positions normally targeted for 2′-O methylation appeared to be hypermodified, thus implying the existence of partial methylation under nonpathological conditions[51]. In these cells, the kinetics of processing is affected, and hypermethylation

Box 2 rRNA modifications

rRNAs are modified by methylation on the sugar backbone, by formation of uridine isomers (pseudouridine, Ψ, a C-glycoside isomer of uridine) and by base methylation, acetylation and amino-carboxypropylation[53].
These modifications cluster around functional ribosomal sites, including the decoding site and peptidyl transferase center, and are thought to modulate ribosome function[53]. Sugar methylation and pseudouridine account for most eukaryotic rRNA modifications (55 and 44 in budding yeast; 102 and 93 in humans)[53]. Both modification sites are recognized on pre-rRNAs via Watson-Crick base-pairing with box C/D and box H/ACA small nucleolar RNAs (snoRNAs)[24], which guide the methyltransferase or pseudouridine synthase to the correct position. Base methylations are less frequent (12 in yeast; 10 or 11 estimated in humans) and are catalyzed by enzymes that do not rely on snoRNAs to find and modify their targets. Considering that each human ribosome contains around 100 ribose methylations and 100 pseudouridines, and that each position is subject to individual regulation through specific snoRNA guides, the combinatorial potential is immense. snoRNA-mediated rRNA modification, therefore, affords the most prominent source of ribosome heterogeneity.

correlates with altered translation. Although global translation was not affected, IRES-dependent initiation was reduced four-fold[51]. Translation accuracy was also severely affected, with amino acid misincorporation and stop-codon readthrough[51].

Mutations in the pseudouridine synthase DKC1, a core component of multiple classes of H/ACA RNPs[53], cause X-linked dyskeratosis congenita (X-DC). This ribosomopathy is characterized by hematopoietic failure and increased cancer susceptibility[3]. Interestingly, hematopoietic stem cells with impaired DKC1 function have been shown to be unable to differentiate accurately[54]. The respective contribution of DKC1-containing RNPs to disease etiology currently remains unclear because DKC1 is also part of telomerase. Yet IRES-dependent translation, notably of the tumor suppressor p27 and the antiapoptotic protein XIAP, was shown to be defective in Dkc1 mutant mouse models and in cells from patients with X-DC[55], and decreased *in vitro* IRES binding was observed with ribosomes isolated from hypomorphic Dkc1 mouse embryonic fibroblasts[56]. Yeast cells expressing a catalytically deficient allele of CBF5, the yeast homolog of DKC1, were also impaired in IRES-dependent initiation. Indeed, yeast ribosomes lacking rRNA pseudouridine modifications showed less efficient binding of a reference viral IRES. In addition, reduced A-site and P-site tRNA binding was observed, as well as increased frame-shifting and stop-codon readthough[56]. Effects on reading-frame maintenance were also seen in mutant mouse and human cells.

In X-DC cells expressing various DKC1 mutants, the steady-state levels of individual H/ACA snoRNAs were differentially affected, and these levels directly correlated with variations in the amount of the corresponding rRNA pseudouridine modifications[54]. Thus, cells from patients with X-DC contain heterogeneous populations of ribosomes. However, why some H/ACA RNAs are more sensitive to Dyskerin depletion than others remains to be determined. Interestingly, snoRNA accumulation was differentially affected in cells from different tissues, even if they expressed the same DKC1 mutation. This could provide a possible explanation for the tissue specificity associated with pathological features of X-DC.

snoRNAs and snoRNPs in cancer

In human cells, snoRNA expression levels vary in different tissues according to disease status (for example, in patients with X-DC) and, quite surprisingly, according to circadian rhythm[54,57–59]. Cancers of various histological origins show altered expression of snoRNAs and core snoRNP proteins, sometimes with individual variations from one tumor to another; case reports are available for both C/D and H/ACA snoRNAs[60–62]. In cases in which snoRNA expression is increased, oncogenesis may occur as a result of hyperactivation of ribosome biogenesis, suppression of nucleolar stress, increased production of short regulatory miRNA-like RNAs (discussed below) or generation of differentially modified ribosomes with altered translational capabilities.

Altered levels of the methyltransferase FBL and/or snoRNAs have been reported in breast, cervical, lung and prostate cancers, often correlating with poor patient survival[60–63]. In breast and colon cancer cells, the observation of an inverse correlation between expression levels of the tumor suppressor p53 and FBL has led to the suggestion that p53 represses FBL transcription through direct binding to its first intron[63]. In cancer cells with decreased p53 levels, high levels of FBL led to increased 2′-O methylation with a concomitant reduction in translation fidelity and stimulation of IRES-dependent initiation, notably increasing cancer gene expression[63]. Thus, through FBL upregulation, the inactivation of p53 specifically stimulates translation of pro-oncogenic, antiapoptotic and survival proteins.

An independent study demonstrated that in transformed mammary cells FBL knockdown led to accumulation of p53, owing to protein stabilization and increased IRES-dependent *de novo* synthesis[60]. This indicates that p53 and FBL control each other and that in certain circumstances IRES translation can also be stimulated by decreased rRNA methylation. In addition, p53 regulates the expression of both core snoRNP proteins and snoRNP AFs because the expression of NOLC1 (also known as NOPP140), involved in initial steps of snoRNP assembly, is reduced in p53-knockout cells[64].

snoRNAs are readily detectable in blood plasma and serum samples[65–68]; thus they are likely to be developed as new fluid-based noninvasive biomarkers for improved classification of malignancies and patient stratification.

Other ncRNAs associated with rDNA and snoRNAs

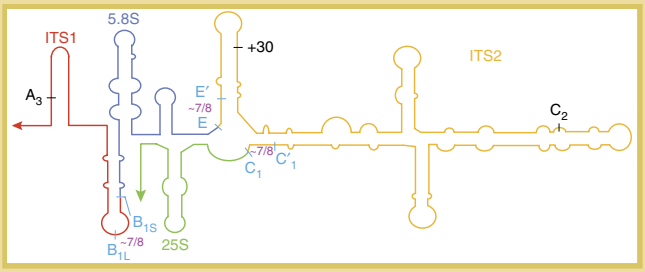
rDNA genes are organized in tandem arrays, and multiple regulatory ncRNAs are produced from rDNA intergenic sequences (IGSs) in yeast and mammals (**Fig. 4**). Strikingly, IGSs have undergone a tremendous size expansion during eukaryotic evolution, from 2.5 kb in budding yeast to 30 kb in humans. The presence of IGS-derived ncRNAs, which seem to be particularly numerous and diverse in human cells, suggests that nature has selected and retained these intergenic expansions because this allows production of small regulatory ncRNAs.

In yeast, sense and antisense transcripts are produced from the IGSs by RNA Pol II; some are targeted for rapid clearance by TRAMP–exosome complexes[69]. Because these ncRNAs are transcribed through DNA elements important for rDNA stability, they are thought to be required for rDNA copy-number maintenance (**Fig. 4a**).

In mice, promoter-associated RNAs (pRNAs) are a class of 150- to 250-nt RNAs overlapping with the rDNA promoter[70,71]. pRNAs are sense transcripts produced from the IGSs by RNA Pol I, and they are incorporated into nucleolar remodeling complexes (NoRCs), whose ATPase activity they regulate[72]. NoRCs recruit enzymes involved in heterochromatin formation, such as DNA methyltransferases, histone

Box 3 Pre-rRNA procesing as a source of ribosome heterogeneity

In yeast and humans, there are two forms of 5.8S rRNA: 5.8S short ($5.8S_S$) and 5.8S long ($5.8S_L$), which differ in size by a 7- or 8-nt 5′ extension (~7/8), ending in yeast at processing site B_{1S} or B_{1L}[9,25]. 5.8S and ITS2 folding brings distant processing sites in pre-rRNA molecules into close vicinity, such as the 5′ and 3′ ends of 5.8S (sites B_{1S}-B_{1L} and E-E′, respectively) and the 5′ end of 25S rRNA (sites C_1-C_1′). This proximity and the striking presence of 7- to 8-nt extensions at all of these three positions suggests that they are matured by a common processing complex[9] and that $5.8S_L$ is, in fact, carrying a remnant of processing or 'processing birthmark'. A_3, +30 and C_2 denote cleavage sites. Adapted with permission from ref. 9, American Society for Microbiology.



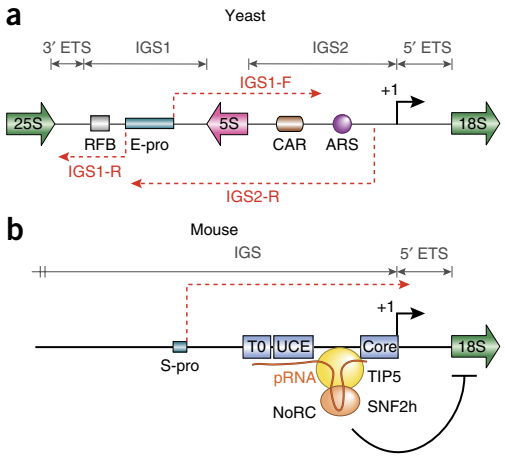


Figure 4 Regulatory cryptic ncRNAs are produced from intergenic rDNA sequences. (**a**) In yeast, three major intergenic cryptic transcripts (IGS1-F, IGS1-R and IGS2-R, shown in red) are produced by RNA Pol II in the sense and antisense directions with respect to the rDNA unit (green)[69]. IGS1-F and IGS1-R are transcribed from the E-pro promoter (blue). IGS1-R passes through the replication-fork barrier (RFB, gray). IGS1-F and IGS2-R are transcribed through the cohesin-associated region (CAR, brown). Both the RFB and CAR elements are important for rDNA stability. Adapted from ref. 69 with permission from EMBO. (**b**) In mice, promoter-associated RNAs (pRNA, orange) are transcribed from a cryptic RNA Pol I promoter (S-pro, blue) located 2 kb upstream of the bona fide rDNA promoter (core). pRNAs are processed to adopt a stem-loop structure and are incorporated into nucleolar remodeling complexes (NoRCs) comprising the ATPase SNF2h and TIP5. NoRCs recruit chromatin modifiers and promote heterochromatin formation and rDNA silencing; these NoRC functions require pRNA, which regulates the ATPase activity of the complex. Adapted with permission from ref. 94, John Wiley and Sons. +1, RNA Pol I transcription start site; ETS, external transcribed spacer; IGS, intergenic sequence; T0, rDNA terminator; UCE, upstream control element; core, core RNA Pol I promoter.

deacetylases and histone methyltransferases, thus promoting rDNA silencing (**Fig. 4b**).

In human cells, diverse IGS ncRNAs induced by heat shock or hypoxic or transcriptional stress act as nucleolar 'detention centers' for specific proteins[73]. In this process of nucleolar immobilization, specific stress-responsive RNAs are thought to 'capture' target proteins expressing a nucleolar localization signal, coined the nucleolar detention sequence (NoDS). Alternatively, spurious IGS transcription may produce various cryptic transcripts that are simply differentially stabilized through binding of proteins whose nucleolar relocalization has been triggered by stress.

In mammals, snoRNAs are generally processed from pre-mRNA introns. snoRNAs can then also be further fragmented into 15- to 35-nt snoRNA-derived RNAs (sdRNAs), some of which have miRNA-like properties[74–77]. Specific sdRNAs indeed require Drosha or Dicer for their synthesis, bind Argonaute and take part in mRNA *trans*-silencing[78,79]. For example, MRP-derived fragments have been suggested to act as miRNAs potentially regulating the expression of genes involved in cartilage-hair hypoplasia[80]. The H/ACA-derived miR-605 regulates steady-state levels of p53 through inhibition of Mdm2 translation[81]. sdRNAs are also involved in regulating alternative pre-mRNA splicing[82,83]. Finally, portions of pre-mRNA introns flanked at both ends by snoRNPs are sometimes stably expressed as snoRNA-derived long noncoding RNAs (sno-lncRNAs)[84]. sno-lncRNAs have notably been associated with Prader-Willi syndrome, in which they might function as 'molecular sinks', titrating Fox2, which is important for alternative-splicing regulation[84].

Concluding remarks and prospects

Recent work has highlighted that ribosome biogenesis is far more complex in human cells than anticipated from work in yeast. Although the general architecture of the pre-rRNA processing pathway is evolutionarily conserved[26], specific steps proceed differently. Unsurprisingly, human cells have evolved unique factors, of which more than 75 have been uncovered so far[17].

In addition, it is becoming increasingly clear that cells express more than a single type of ribosome. Distinct ribosomes have been referred to as 'specialized'[6]. The concept of 'renegade' ribosomes further expresses the notion that ribosomes can deviate from the norm to only a limited extent, so that core functions of the ribosome are not affected. Renegade ribosomes must indeed over-ride numerous quality-control steps and evade extremely efficient and ubiquitous surveillance mechanisms selected during evolution to promote rapid clearance of defective particles[2].

How frequently renegade ribosomes occur in cells and whether they are tissue specific must be further clarified along with their exact impact on differential translation, normal cell physiology, developmental processes and disease etiology. Another open question remains: what are the exact circumstances (for example, microcellular environment, stress, hypoxia, etc.) causing differential rRNA modification that may lead to cancer? At this stage, it is also unclear whether rRNA modification heterogeneity simply reflects the relative abundance of matching guide snoRNAs or whether it involves additional regulatory components. Mature snoRNP activity might be controlled, for example, by post-translational modification of core proteins. snoRNPs clearly require specific catalytic activation during assembly, and this activation step is likely to be a target of regulation. This highlights the importance of further characterizing snoRNP biogenesis pathways.

Finally, there is emerging evidence of direct connections between transcriptional and post-transcriptional steps of ribosome biogenesis involving ribosome AFs and snoRNP components. *Trans*-acting factors shared between ribosome assembly, snoRNP biogenesis and pre-mRNA splicing have been known for some time, but core snoRNP proteins have recently been shown to have further unexpected roles in the regulation of gene expression. FBL can act as an rDNA histone methyltransferase, regulating rRNA synthesis through glutamine methylation of histone H2A[85]. In addition, Dyskerin together with SMUG1 functions as a sentinel in rRNA surveillance. SMUG1 is a DNA-repair enzyme involved in controlling levels of oxidized rRNAs[86], which are particularly enriched in Alzheimer's disease[2]. Whether the new functions of core proteins in epigenetics and rRNA surveillance involve intact snoRNPs remains to be determined, but exciting times surely lie ahead.

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