Received: 30 July 2021 | Revised: 24 February 2022 | Accepted: 19 March 2022

DOI: 10.1002/wrna.1732

ADVANCED REVIEW

WIREs RNA WILEY

The story of rRNA expansion segments: Finding functionality amidst diversity

Nivedita Hariharan[1,2] | **Sumana Ghosh**[3] | **Dasaradhi Palakodeti**[1]

[1]Technologies for the Advancement of Science, Institute for Stem Cell Science and Regenerative Medicine (inStem), Bangalore, India

[2]The University of Trans-disciplinary Health Sciences and Technology, Bangalore, India

[3]Manipal Academy of Higher Education, Manipal, India

Correspondence
Dasaradhi Palakodeti, Technologies for the Advancement of Science, Institute for Stem Cell Science and Regenerative Medicine (inStem), Bangalore, India.
Email: dasaradhip@instem.res.in

Funding information
Council of Scientific and Industrial Research, India; Department of Science and Technology, Ministry of Science and Technology, India, Grant/Award Number: DST/SJF/LSA-02/2015-16

Edited by: Purusharth Rajyaguru and Jeff Wilusz, Editor-in-Chief

Abstract

Expansion segments (ESs) are multinucleotide insertions present across phyla at specific conserved positions in eukaryotic rRNAs. ESs are generally absent in bacterial rRNAs with some exceptions, while the archaeal rRNAs have microexpansions at regions that coincide with those of eukaryotic ESs. Although there is an increasing prominence of ribosomes, especially the ribosomal proteins, in fine-tuning gene expression through translation regulation, the role of rRNA ESs is relatively underexplored. While rRNAs have been established as the major catalytic hub in ribosome function, the presence of ESs widens their scope as a species-specific regulatory hub of protein synthesis. In this comprehensive review, we have elaborately discussed the current understanding of the functional aspects of rRNA ESs of cytoplasmic eukaryotic ribosomes and discuss their past, present, and future.

This article is categorized under:

RNA Structure and Dynamics > Influence of RNA Structure in Biological Systems

Translation > Ribosome Structure/Function

Translation > Regulation

KEYWORDS

expansion segments, ribosome, rRNA, translation regulation

1 | INTRODUCTION

Protein repertoire of a cell, which is one of the key determinants of its identity and behavior, is dynamically regulated at multiple levels of gene expression. While many facets of gene expression regulation are still being uncovered, transcription, and posttranscriptional processes have been regarded as the main drivers of gene expression control. However, of late, translation regulation has emerged as a key mechanism for fine-tuning gene expression, especially during cell state transitions (Ingolia et al., 2011; Schwanhäusser et al., 2011). Several studies have attributed translation regulation to the *cis*-regulatory elements of mRNAs and the *trans*-acting translation factors. Ribosome, the cellular protein-synthesizing hub that is ubiquitous across all domains of life, has often been stereotyped as a passive participant in this

Abbreviations: ER, endoplasmic reticulum; ES, expansion segment; LSU, large subunit; mRNA, messenger RNA; r-protein, ribosomal protein; rRNA, ribosomal RNA; SSU, small subunit; tRNA, transfer RNA.

Nivedita Hariharan and Sumana Ghosh contributed equally to this study.

process. The discovery of ribosomes was quite serendipitous. In the early 1940s, Claude (Claude, 1938, 1940, 1944; Claude & Hoerr, 1943) isolated a lipid and ribonucleoprotein-rich fraction capable of protein synthesis (Keller et al., 1954) that was termed the "microsome." In 1955, George Palade visualized microsomes on endoplasmic reticulum (ER) membranes using an electron microscope (Palade, 1955). The ribonucleoprotein part of the microsome was later named the "Ribosome" (Dintzis, 2006). For many years, work on the ribosomes was focused on ribosomal proteins (r-proteins), since they were thought to be the catalytic component, whereas the ribosomal RNAs (rRNAs) were relegated to the role of a scaffold for the r-proteins. The establishment of rRNA as the primary catalytic component of the ribosome came through the discovery of ribozymes, along with various other studies in the 1970s and 1980s (F. H. Crick, 1968; F. H. C. Crick, 1962; Guerrier-Takada et al., 1983; Herr et al., 1979; Kearsey & Craig, 1981; Noller & Chaires, 1972; Ofengand et al., 1979; Shine & Dalgarno, 1974a; Steitz & Jakes, 1975; Zaug et al., 1983). Ribosomes contain a common core set of r-proteins and three or four rRNAs spread across two subunits (the large subunit or LSU and the small subunit or SSU; Table 1). In the translating 80S ribosome, the LSU catalyzes peptide bond formation, while the SSU associates with mRNA during initiation complex formation and mediates tRNA–mRNA interaction (Aitken & Lorsch, 2012; Schluenzen et al., 2000). Even with such important core functions in translation, no regulatory role was ascribed to the ribosome for a long time. However, recent studies indicate that the ribosomes could also participate in the regulation of gene expression in multiple ways (Xue & Barna, 2012). Research on ribosome's regulatory role has also been more focused toward r-proteins than rRNA. However, rRNA presents many interesting evolutionary features across species, which makes them a potential candidate to regulate translation. The advent of sequencing technology and the insights that rRNA could be crucial for protein synthesis spurred many groups to study the conservation and evolutionary differences of rRNA among different species (Clark et al., 1984; Hassouna et al., 1984; Veldman et al., 1981; Ware et al., 1983). It was noted that the 25S/28S eukaryotic rRNA was longer than the 23S bacterial rRNA. For instance, the 23S rRNA in *Escherichia coli* was 2904 nt long, while the 25S rRNA in *Saccharomyces cerevisiae* was 3392 nt long. This increase in length was even higher in amphibians, birds, mammals, and could be attributed to GC-rich insertions at specific positions along the rRNA. Such insertions were also observed in the eukaryotic 18S rRNA relative to the 16S bacterial rRNA and were termed as expansion segments (ESs) (Clark et al., 1984).

TABLE 1 Table showing the ribosome size and overall composition across kingdoms

Type of ribosome		Size of ribosome	Size and composition of large subunit (LSU)	Size and composition of small subunit (SSU)
Cytosolic	Bacterial (e.g., *Escherichia coli*)	70S	50S (23S rRNA,5S rRNA + 33 proteins)	30S (16S rRNA + 21 proteins)
	Archaeal	70S	50S (23S rRNA, 5S rRNA + 35–40 proteins depending on the organism)	30S (16S rRNA + 25–28 proteins depending on the organism)
	Eukaryote (*Saccharomyces cerevisiae*)	80S	60S (25S rRNA, 5S rRNA, 5.8S rRNA + 45 proteins)	40S (18S rRNA + 33 proteins)
	Eukaryote (e.g., *Homo sapiens*)	80S	60S (28S rRNA, 5S rRNA, 5.8S rRNA + 46 proteins)	40S (18S rRNA + 33 proteins)
Mitochondrial	Kinetoplastids (e.g., *Trypanosoma brucei*)	50S	40S (12S rRNA + 77 proteins)	28–30S (9S rRNA + 56 proteins)
	Mammal (e.g., *Homo sapiens*)	55S	39S (16SrRNA, CP tRNA, 52 proteins)	28S (12S rRNA + 30 proteins)
	Fungi (e.g., *Saccharomyces cerevisiae*)	74S	54S (21S rRNA + 46 proteins)	37S (15S rRNA + ~36 proteins)
	Higher plants (e.g., *Brassica oleracea* var. *botrytis*, or cauliflower)	78S	50S (26S rRNA, 5S rRNA + 45 proteins)	33S (18S rRNA + 37 proteins)
Chloroplast	Plants	70S	50S (23S rRNA, 5S rRNA, 4.5S rRNA + 31 ribosomal protein)	30S (16S rRNA + 21 ribosomal protein)

ESs are taxon-specific nucleotide stretches interspersing otherwise universally conserved blocks of rRNA. Although ESs of cytosolic ribosomes are the most studied, they are also present in the rRNA of mitochondrial and chloroplast ribosomes (Bieri et al., 2017; Brimacombe, 1981; Tomal et al., 2019). Mainly considered to be a distinctive feature of eukaryotic ribosomes, such species-specific insertions have also been identified in the rRNAs of some bacterial ribosomes (Kushwaha & Bhushan, 2020; Penev et al., 2020; Stepanov & Fox, 2021; Tirumalai et al., 2020). Interestingly, archaeal ribosomes have also been reported to harbor microexpansions in the rRNA regions that coincide with eukaryotic ESs (Armache et al., 2010; Bowman et al., 2020). Initially considered to lack any function in the ribosome, ESs were thought to be tolerated in the mature rRNA because of their possible lack of interference in any core ribosomal function (Clark et al., 1984; Gerbi, 1986; Ware et al., 1983). Despite their discovery many decades back, researchers still do not possess more than a rudimentary understanding of what ESs can do. However, there has been growing evidence suggesting the potential roles that rRNA ESs could play in translation. In this review, we have summarized literature citing individual examples that bring out the features of cytosolic rRNA ESs and their known or potential functions.

2 | THEORIES ON EVOLUTION OF EXPANSION SEGMENTS

The origin and evolution of ESs is still a puzzle, although a few theories and experimental studies have tried to unravel it. ESs could either be remnants of a longer ancestral rRNA sequence or they could be recent insertions (Caetano-Anollés, 2015; Petrov et al., 2014) over the more conserved rRNA core. According to a widely accepted model for rRNA evolution, called the "accretion model," the architecture of primitive ribosomes was laid out before the separation of kingdoms, on which iterative accretion of rRNA fragments has occurred during evolution. Based on this model, the ESs could have evolved either by elongation of an already existing helix or by the formation of essentially nonintrusive helical branches in ancestral helical trunks (Bowman et al., 2020; Petrov et al., 2015). To understand the architectural evolution of rRNAs, Yokoyama and Suzuki 2008 randomly inserted a 31-nt long RNA segment into the rRNA of *E. coli* and mapped the sites of insertion. They showed that in both 16S and 23S rRNA, most of the functional insertions showed a tendency to coincide with the regions of ESs found in the respective yeast (*S. cerevisiae*) rRNAs and the insertions were generally present on the ribosomal surface, avoiding any highly conserved functional domains. In this study, the authors have proposed that the ESs might have evolved from the prokaryotic core rRNA through a process of random genetic insertion and selection. Armache et al. have reported the presence of microexpansion segments (5–20 nt) in the archaeon *Pyrococcus furiosus*, some of which coincided with regions where eukaryotic ESs were present (Armache et al., 2010; Bowman et al., 2020) and these archaeal microexpansions could also be an important link to understand the evolution of eukaryotic ESs.

The accretion of eukaryotic ESs can potentially be explained by constructive neutral evolution (CNE) (Muñoz-Gómez et al., 2021; Stoltzfus, 1999), which proposes that complexity can arise or increase neutrally and need not always be the result of adaptive fitness and positive selection. The effective neutrality could occur because of the changes that are either slightly advantageous or disadvantageous, whose costs are small enough to escape natural selection and spread out in a population (especially one with a small effective population size) by random genetic drift. In the context of eukaryotic genome expansion, Lynch et al. have proposed (Lynch & Marinov, 2015) that nucleotide insertions that are possibly mildly advantageous or disadvantageous, can be tolerated by large cells that replicate slowly with small effective population sizes. This is because such insertions possibly cause a very low energetic burden in larger cells, such as the eukaryotic cells and probably are not eliminated by natural selection. It is conceivable that the forces which shape the overall genome architecture might have permitted expansions in rRNA too, which may have then been stabilized because of possible gain of function (Penev et al., 2020). Once an insertion has been stabilized, compensatory slippage occurring during genome replication could have contributed prominently to the sequence diversity of ESs (Hancock & Dover, 1990). This is reminiscent of the possible evolution of ribosomes, in which the fortuitous interaction of proteins that stabilized the sporadic defects in pure ribozymes, combined with natural selection could have resulted in the emergence of a more efficient ribosome (Lukeš et al., 2011; Muñoz-Gómez et al., 2021).

However, there is a possibility that the growth by accretion model may not be universal. In rare cases, homologous expansions can occur in parallel. For example, the ES39L in all studied Lokiarchaeota are larger than in any other archaea while some even exhibit ES39L larger than that of most eukaryotes (Penev et al., 2020). In this study, Penev et al. have proposed two scenarios that could explain this anomaly. One scenario is based on a three-domain tree of life and predicts that the Last Archaeal and Eukaryotic Common Ancestor (LAECA) had a larger ES39L, which could have shrunk in most archaea and was parallelly developed in Asgard archaea and eukaryotes. The other scenario assumes a

close common ancestry of Asgard archaea and eukarya, as stated in the "Two domain tree" model. This model states that eukaryotes consistently originate from within the archaea (Williams et al., 2020). These findings raise the possibility that supersized ESs existed on the ribosomal surface before the last eukaryotic common ancestor, opening the question of whether ribosomal complexity is more deeply rooted than previously thought (Penev et al., 2020).

3 | CHARACTERISTICS OF EXPANSION SEGMENTS

ESs were defined as multinucleotide insertions seen in eukaryotic rRNAs relative to *E. coli* rRNA (Ware et al., 1983). They were also referred to as "insertions," "divergent domains," or "variable regions" in some of the early studies by other groups (Gorski et al., 1987; Michot & Bachellerie, 1987; Musters et al., 1991; Sweeney et al., 1994; Ware et al., 1983). Even though ESs were initially defined as insertions in eukaryotic rRNAs in comparison to bacterial rRNAs, they have also been identified in the rRNAs of a few disparate bacterial and archaeal ribosomes (Kushwaha & Bhushan, 2020; Penev et al., 2020; Stepanov & Fox, 2021; Tirumalai et al., 2020). A recent computational study (Stepanov & Fox, 2021) reported the presence of 5S rRNA ESs in a few disparate bacterial and archaeal genomes. They found that the presence of the observed expansions in 5S rRNA did not correlate with any phylogenetic branching across these two superkingdoms and suggested that these ESs could either potentially confer some niche-specific adaptive advantage to those species or they could be nonfunctional insertions undergoing neutral evolution. Another notable observation from this study was that some bacterial genomes harbor both the normal and expanded versions of the 5S rRNA. It would be interesting to understand whether both of these versions get incorporated in to functional ribosomes and whether they lead to any functional specialization through detailed experimental studies. Because of the very limited number of studies available on bacterial and archaeal ESs, this review focuses on the eukaryotic cytosolic ESs. Initial studies (Gerbi, 1996) comparing rRNA sequences from across eukaryotes revealed that the cytosolic ribosomes in eukaryotes have about 12 ESs in their SSU rRNA and 41 in the LSU rRNAs (ES1-3L and a part of ES4L in 5.8S rRNA and the rest in 25/28S rRNA) when compared with the *E.* coli ribosomes (Figure 1A–F). However, it is important to note that a subset of these segments may be present in a particular eukaryotic species (Ramesh & Woolford Jr., 2016; Yokoyama & Suzuki, 2008).

In this section, we will discuss the various defining features of the ESs of eukaryotic cytosolic ribosomes. We have followed the usual naming convention for ESs and have denoted ESs as ESXN (where X represents the number of the ES, and N indicates either the large subunit, L, or the small subunit, S).

3.1 | Expansion segments vary in length and sequence but are present at the same relative position on the rRNA

The earliest insights on ESs came from two parallel studies (Schibler et al., 1975; Wellauer et al., 1974) that observed double and triple loops with the same morphology across vertebrates in rRNA electron micrographs. The loops varied in length but were present at the same relative position on the rRNA across all observed organisms. Some of these RNA stem-loops were later proposed to be ES7L and ES27L (Clark et al., 1984). Further, studies that compared LSU rRNA sequences from *E. coli* (bacterium), *S. cerevisiae* (yeast), *Physarum polycephalum* (slime mold), *Mus musculus* (mammal), *Rattus norvegicus* (mammal), and *Xenopus laevis* (amphibian) (Brimacombe, 1981; Clark et al., 1984; Hassouna et al., 1984; Veldman et al., 1981; Ware et al., 1983) showed that the eukaryotic 28S rRNA was longer than its bacterial counterpart and this increase in length was due to nucleotide insertions of varying sizes at specific conserved positions on the rRNA. Except at these specific sites, homologous regions were dispersed throughout the rRNA, with a high degree of structure conservation facilitated by compensatory base-pair changes. Interestingly, the mammalian and kinetoplastid (protozoan) mitochondrial rRNAs exhibited segment deletions at the same positions where the eukaryotic expansions were observed (Branlant et al., 1981; Brimacombe, 1981; Gerbi, 1986; Mankin & Kopylov, 1981; Tomal et al., 2019). These observations led to the idea of a common conserved structural core across bacteria and eukaryotes, over which insertions/deletions may occur.

Although the insertion points of ESs coincide across taxa, the sizes of ESs may vary. This was apparent from early studies (Clark et al., 1984; Ware et al., 1983) that compared the expansions in the *X. laevis* 28S rRNA with that of *S. cerevisiae*, *P. polycephalum*, and *E. coli*. It was evident that a few ESs generally show a phylogenetically linked increase in size with increasing organismal complexity (bacteria < archaea < single celled and invertebrate eukaryotes

< vertebrates) (Figure 1I). For instance, the largest expansion segment—ES7L ranges from around 20 nucleotides in bacteria, 80 nucleotides in archaea to about 210 nucleotides in non-vertebrates, and over 870 nucleotides in mammals (Petrov et al., 2014). Notably, the bulk of the increase in LSU rRNA size in vertebrates comes from just two ESs, ES7L

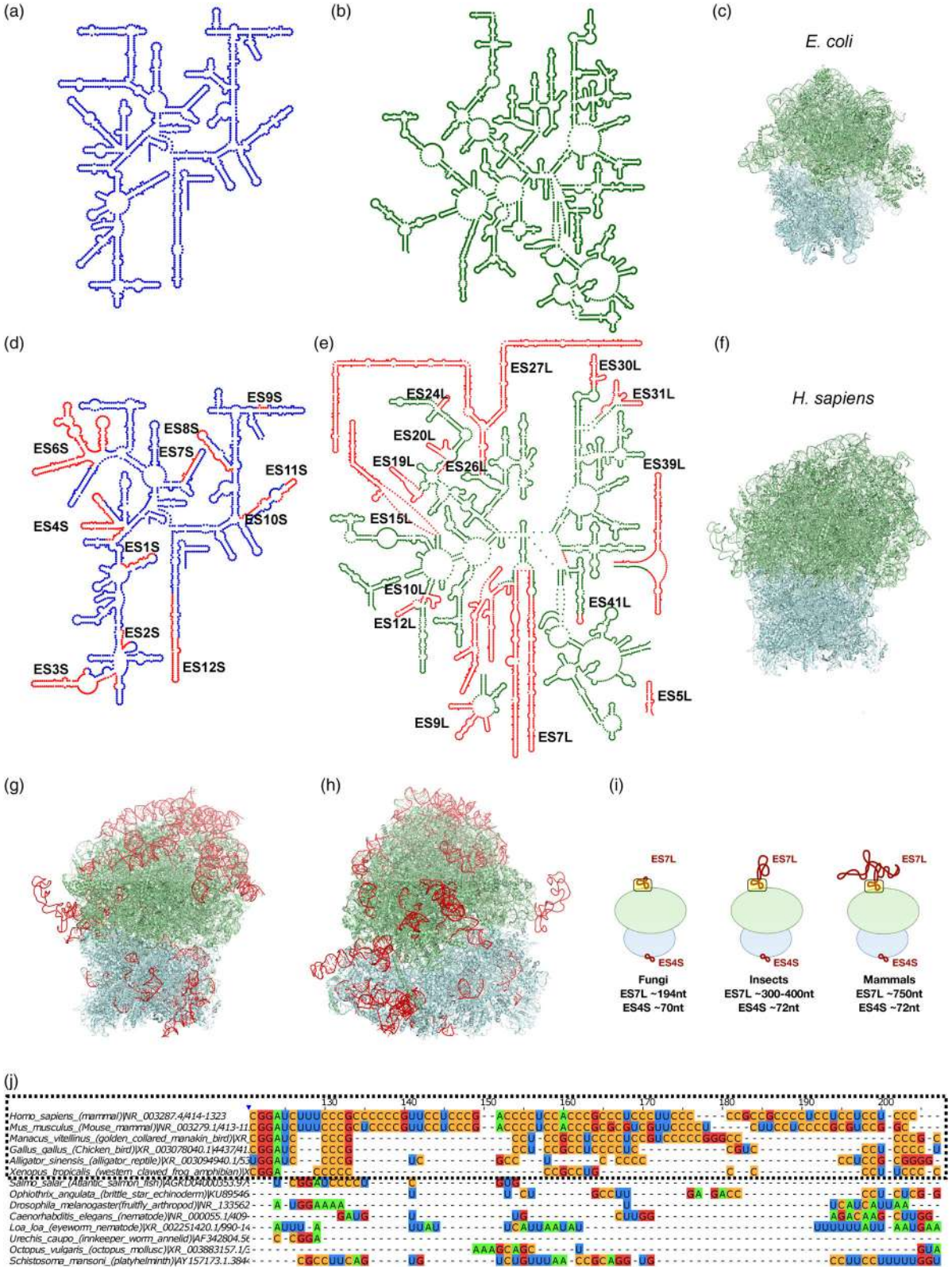

FIGURE 1 Legend on next page.

and ES27L. Another interesting aspect is that the ES accretion is significantly more pronounced in the large subunit than in the small subunit Petrov et al., 2015. The 25S–28S LSU rRNA has expanded by about 500 nucleotides in plants and fungi and about 2400 nucleotides in vertebrates over the bacterial 23S rRNA. Whereas expansion in 18S rRNA appears to have saturated and does not differ appreciably across eukaryotes, with about 1540 nucleotides in bacteria to about 1800 nucleotides in yeast and 1870 nucleotides in human (Parker et al., 2014, 2015). Although earlier studies (Yokoyama & Suzuki, 2008) have reported the presence of ES1-3L in 5.8S rRNA, a detailed characterization to understand their variability across eukaryotes is yet to be conducted. Complexity of translation and its regulation has increased from bacteria and archaea to eukaryotes and even among eukaryotes, the translational apparatus has diversified (Hernández et al., 2012). However, it will be interesting to understand whether ESs contribute to this increased complexity.

The increase in ES size also correlates with an increase in GC content. Sequence comparison of ESs from different organisms revealed the presence of inverted and direct repeats, which may have potentially caused DNA strand slippage during replication leading to their higher variability in eukaryotes (Clark et al., 1984; Hassouna et al., 1984). Compensatory slippage and natural selection may have then led to the accumulation of the same nucleotide repeats called homoiterons (Parker et al., 2015) and other repetitive motifs over the course of ES evolution (Hancock & Dover, 1990). This biased expansion probably occurred in both plants and metazoa, with GC enrichment being prominent. An extensive bioinformatic investigation by Parker et al. (2015) showed that the LSU ESs exhibited an abundance of GC homoiterons, which increased drastically in vertebrates (Figure 1J), especially in tetrapods, and this accumulation is much smaller in SSU ESs (Chandramouli et al., 2008; Clark et al., 1984; Hassouna et al., 1984; Parker et al., 2014, 2015; Wakeman & Maden, 1989. This contrasts with the ESs of single-celled and invertebrate eukaryotes such as *P. polycephalum* (slime mold), *Tetrahymena thermophila* (protozoan), and *S. cerevisiae* (yeast). *Drosophila melanogaster* (arthropod), which have a lower GC content and those of metazoan mitochondrial rRNAs which are largely reduced in size and have only a few GC homoiterons (Armache et al., 2010; Ben-Shem et al., 2011; Klinge et al., 2011). However, ESs may not always follow a monotonic increase in size or number across eukaryotes. For example, ES15L is shorter in angiosperms (flowering plants) and ES39L is larger in euglenozoans (protozoa) and tetrapods (four-limbed animals) when compared with other eukaryotes (Parker et al., 2015). Metazoans also contain additional ESs in the LSU like the ES30L and ES43L, which are lacking in yeast and *Tetrahymena* (Anger et al., 2013). The microsporidium *Vairimorpha necatrix* rRNAs have lost almost all eukaryote-specific ESs and are in fact smaller than their *E. coli* counterparts (Barandun et al., 2019). The length and sequence variation in ESs can also occur within a species, including in different tissue types or developmental stages of an organism, though the extent of such variability and its relevance is yet to be understood (Kuo et al., 1996; Leffers & Andersen, 1993; Locati et al., 2017; Parks et al., 2018; Tseng et al., 2008; Ware et al., 1983). Hence, variability in the number, nucleotide bias, and length of ESs is an interesting aspect of ribosomal evolution that opens up the possibility of species-specific ribosomal gain-of-function and supports the hypothesis of specialized ribosomes.

FIGURE 1 (a,b) Secondary structures of the 16S and 23S rRNA of *E. coli* ribosome. (c) Front view of the bacterial 70S ribosome (PDB: 7N1P; 10.2210/pdb7n1p/pdb; Rundlet et al., 2021). (d,e) Secondary structures of the 18S and 28S rRNA of human ribosome with the ESs highlighted in red. The co-ordinates of the ESs were taken from Parker et al., 2015. (f) Front view of the human 80S ribosome (PDB: 4UG0; 10.2210/pdb4UG0/pdb; Khatter et al., 2015). The human ribosomes are 4.3 MDa and are nearly twice as large when compared with *E. coli* ribosomes which are 2.3 MDa (Melnikov et al., 2012). Secondary structure figures of the rRNAs from both *E. coli* and human were created using RiboVision web server (Bernier et al., 2014). (g,h) Front view (g) and back view (h) of the human 80S ribosome (PDB: 4UG0; 10.2210/pdb4UG0/pdb; Khatter et al., 2015) showing the ESs (highlighted in red; it is important to note that some regions of long flexible ESs are only modeled and/or partially resolved based on secondary structure predictions and cryo-EM data, while some regions are not yet resolved). As evident from the figure, the ESs are usually solvent-exposed and highly accessible; these structures were rendered using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). (i) Representative cartoon showing the trend of expansion in ESs in the LSU versus the SSU. ESs are present at the same relative position across taxa. ES7L, an ES of the LSU, increases in length progressively from single-celled eukaryotes to mammals. In fact, the majority of LSU rRNA size increase in vertebrates comes from two ESs—ES7L and ES27L. The yellow box highlights the base of the ES7L which is similar in structure across the different species, but the growth of ES7L occurs by iterative accretion of RNA fragments onto the more conserved basal structure. In contrast, the ES4S in the SSU does not show much difference in their lengths across eukaryotes. (j) Multiple sequence alignment (MSA) of a region of ES7L across different eukaryotic species shows the level of sequence variation among them. The ES7L in vertebrates (highlighted with a dotted box) is more expanded with insertions of GC homoiterons (G-red, C-orange) as opposed to the invertebrate eukaryotes. MSA was built using Clustal Omega (Sievers et al., 2011) in Jalview Waterhouse et al., 2009 using sequences mined from the databases SILVA (Quast et al., 2013) and NCBI nucleotide (NCBI, 1988)

3.2 | Expansion segments and their structure

Although ESs show wide fluctuations in length and primary sequence across eukaryotes, they are predicted to form remarkably conserved secondary structures (Figure 1A,B,D,E) within kingdoms (Hancock et al., 1988). In the case of ES7L that shows a wide size and sequence variation across taxa, studies have proposed the presence of a conserved "structural core" at its base, with the increase in its length coming from extensions at the apexes of a common set of helical elements (Hassouna et al., 1984; Michot & Bachellerie, 1987). This also suggested the presence of different evolutionary rates within subareas of ESs, with the basal core changing slower than the more recently evolved tracts. This aspect of ESs has helped in understanding the phylogenetic relationships among organisms in several studies (Belshaw & Quicke, 1997; Campbell et al., 1993; Gonzalez et al., 1990; Pélandakis et al., 1991; Ruiz Linares et al., 1991; Subbotin et al., 2007).

The structures of many ESs are usually solved with electron-density maps from ribosome structural studies coupled with predicted secondary structures of these ESs. But concrete structures for some ESs, especially the distal ends of long ESs are either unavailable or are modeled based on secondary structure predictions and not resolved yet in cryo-EM reconstructions due to their flexible nature. According to several eukaryotic ribosomal structural studies (Ben-Shem et al., 2011; Chandramouli et al., 2008; Petrov et al., 2015), many ESs are usually highly accessible as they are mainly present on the solvent-accessible ribosomal surface while being excluded from the functional regions such as the peptidyl transferase center, the decoding center, the core of the subunit interface, and tRNA-binding sites (Figure 1G,H). In fact, the evolution of ESs is postulated to have resulted in the presence of two delineable layers over the ribosomal core. The first layer is viewed to consist of a tightly intertwined network of eukaryote-specific ribosomal proteins as well as rRNA, resulting in an RNA–protein cluster and multiple RNA–RNA tertiary interactions, respectively. This is proposed to be followed by a second layer consisting of tentacle-like highly mobile rRNA elements, which may be majorly formed by the large ESs of the 28S rRNA (Armache et al., 2010; Melnikov et al., 2012; Parker, Balasubramaniam, Sallee, & Parker, 2018). The two ribosomal subunits also differ markedly in the spatial distribution of their rRNA ESs.

As shown in Figure 1G,H, most of the ESs in the SSU are predicted to be generally clustered at the spur or foot region, with the exception of ES9S which is positioned at the head (Armache et al., 2010; Ben-Shem et al., 2011). Whereas in the LSU, most of the ESs are predicted to be usually located at the top, back, and sides of the ribosome, leaving the conserved portion of the inter-subunit interface and exit tunnel regions relatively unperturbed (Anger et al., 2013; Armache et al., 2010.

The modeled and resolved structural regions of ESs from different eukaryotes have showed that many ESs may have both helical regions and disordered single-stranded regions, which interact with ribosomal proteins, other ESs and could also potentially associate with extraribosomal proteins or mRNA (Anger et al., 2013; Ben-Shem et al., 2011; Chandramouli et al., 2008; Leppek et al., 2020).

Recent work based on computational analysis (Mestre-Fos, Penev, Richards, et al., 2019) has shown that the ESs may also have the propensity to form tertiary G-quadruplex structures, as was observed in ES7L, ES27L, ES3S, ES6S, and ES12S. Further, *in vitro* studies (Mestre-Fos, Penev, Richards, et al., 2019; Mestre-Fos, Penev, Suttapitugsakul, et al., 2019) have also shown that ES7L, ES3S, and ES12S of human ribosomes can form G-quadruplexes. The occurrence of ribosomal G-quadruplexes has also been validated *in vivo* in HEK293 cells (Mestre-Fos et al., 2020). Data from this study (Mestre-Fos, Penev, Richards, et al., 2019) also suggests that ES6S can switch between G-quadruplex and duplex conformations in response to protein association and environmental conditions. Another interesting observation by Mestre-Fos, Penev, Suttapitugsakul, et al. 2019 is the possible occurrence of inter-ribosomal G-quadruplexes in polysomes, which needs detailed investigation. The G-quadruplex forming tracts from ESs are found specifically in chordates and their sequences and exact locations on the rRNA tentacles vary across phylogeny (Mestre-Fos, Penev, Suttapitugsakul, et al., 2019).

The rRNA ESs could potentially interact with the r-proteins, rRNAs and other ESs. ESs may have co-evolved with the eukaryote-specific r-proteins or their extensions to facilitate these interactions (Melnikov et al., 2018), which can contribute toward forming certain important sites of the eukaryotic ribosome. For example, ES3S and ES6S have been predicted to form a 7–9-bp helix at the base of SSU via base-pairing in many eukaryotes (Alkemar & Nygård, 2003) and such an interaction between these ESs have been validated by structural studies on ribosomes in yeast (Armache et al., 2010; Ben-Shem et al., 2011). This region contributes to the binding site for the eukaryotic translation initiation factors eIF3 and eIF4G (Alkemar & Nygård, 2004; Anger et al., 2013). Unlike in yeast, the bottom part of ES6S does not interact with ES3S in the 40S ribosomal subunits in the thermophilic fungus *Thermomyces lanuginosus*. This is because ES3S is not completely single-stranded, as a part of it forms a helix which could prevent the ES6S and ES3S tertiary

interaction in the organism (Nilsson et al., 2007). The spatial concentration and interaction of certain ESs near important ribosomal features like the L1 stalk, the central protuberance, and the P stalk is speculated to act as docking sites for many factors that may be involved in co-translational targeting, processing, folding of the nascent chain, and the signal recognition particle (Ben-Shem et al., 2011). Thus, we speculate that any structural variation in these regions might lead to functional differences during translation.

The ESs can also create new structural features in the ribosome in a species-specific manner. For instance, both the small and large ribosomal subunits of the protozoan *Trypanosoma cruzi* are larger than that of yeast, mostly due to the rRNA expansions (Gao et al., 2005). Particularly, its 40S subunit appears more expanded than the SSU of bacteria, archaea and even other eukaryotes. This has been attributed to two large ESs in the *T. cruzi* 18S rRNA, the ES6S and ES7S. Part of ES6S-ES7S creates a large helical structure (the "turret"), located at the most lateral side of the 40S subunit. The upper end of the turret appears as a sharp, freestanding spiral called the "spire," close to the mRNA exit channel. The lower portion of the turret extends to the bottom of the SSU, where it bends by almost 90° and forms a bridge with the 60S subunit. This is a unique type of connection between the small and large subunits of the *T. cruzi* ribosome. It is speculated that this modified 40S subunit of *T. cruzi* could help in the translation of distinctively structured mRNAs that are found in this organism (Gao et al., 2005).

ESs can also contribute toward forming ribosomal intersubunit bridges in eukaryotes. Through evolution, the ribosomal interface has gained novel eukaryotic intersubunit bridges, which practically doubles the specific interaction surface between the subunits. In the newly added bridges, nearly all the participating components are eukaryote-specific. Although proteins play the dominant role, the rRNA expansions are also important in forming these bridges. For instance, in the ribosomes of *S. cerevisiae*, the bridge eB12 below the mRNA exit tunnel is formed through multiple interactions between the C-terminal alpha-helix of L19e and the base of helix E in ES6S (Ben-Shem et al., 2011). Extension of ESs in eukaryotes may also result in the evolution of species-specific inter-subunit bridges. In *D. melanogaster*, helix ES27L-C (C being one of the three arms of the ES27L) is extended compared with yeast ES27L, leading to the formation of a metazoan-specific intersubunit bridge (eB16) through interaction with r-protein S8e (Anger et al., 2013). Also, the formation of such bridges can depend on the conformation adopted by the ESs. For example, although human ES27L is larger than those of yeast and *Drosophila*, contact with S8e is not observed in the human 80S because it has been predicted to adopt a conformation extending toward the L1 stalk (ES27L-in), which is different than in yeast and *Drosophila* (Anger et al., 2013). Sometimes, the intersubunit bridges may be rearranged based on the conformation of the ESs. In *Drosophila* ribosomes, there is a dynamic interplay of structural rearrangements between ES27L and ES31L, depending on which ES27L can interact with S8e to form the bridge eB17 (Anger et al., 2013). Though a speculation at this point, the increase in the intersubunit interaction surface resulting from the evolution of eukaryote-specific bridges, may improve the stability of the 80S ribosome and could potentially lead to an efficient translation.

4 | FUNCTIONAL ROLES OF EXPANSION SEGMENTS

Since their discovery, there has been a lot of ambiguity regarding the significance of ESs. Of particular interest was the observation from Gerbi's group that there were ESs present in *P. polycephalum* or in yeast that were absent in *X. laevis* and that even among most of the shared ESs, there was very little sequence or length conservation. These observations led to the perspective that they possibly do not have a role in ribosome function (Clark et al., 1984; Ware et al., 1983). However, different evolutionary rates within ESs and closely related secondary structures among the species compared, led to the speculation that these ESs could potentially have some role either in ribosome biogenesis or in translation (Hassouna et al., 1984; Michot & Bachellerie, 1987). The earliest experimental studies on ESs involved the insertion or deletion of nucleotides in various ESs. Musters et al. (Musters et al., 1990) inserted a short oligonucleotide in to the ES3S (denoted as V3) and ES9S (denoted as V8) region in the 17S yeast rRNA and reported that while the ES9S insertion was incorporated in the functional 40S subunits, insertion in the ES3S interfered with the 40S subunit formation. In another study, the same group (Musters et al., 1991) also reported that the deletion of most of ES19L (denoted as V9) in yeast 26S rRNA or its replacement by the equivalent segment from the mouse 28S rRNA did not affect the formation of functional 60S subunits carrying the mutant rRNA and led the authors to suggest that ES19L probably do not have a role in the functioning of yeast 60S subunits. A study in *T. thermophila* (Sweeney et al., 1994) showed that deletion of ES27L (denoted as D8) or its replacement with an unrelated sequence of similar size led to defects in ribosome biogenesis in the protozoan, while replacement with the ES27L segment from *S. cerevisiae* (yeast), *Dictyostelium discoideum* (slime mold), and *Caenorhabditis elegans* (nematode) did not result in any defect. The authors proposed that even though the primary sequences of the ES27L were different among these organisms,

they may potentially have a similar tertiary structure which may allow them to function interchangeably. Another study in yeast (Jeeninga et al., 1997) showed that mutations in ES7L (denoted as V3) and ES27L (denoted as V13) led to defects in ribosome biogenesis and growth too. Unfortunately, despite the interesting results from these earlier reports, systematic experimental studies on the function of ESs have been extremely scarce, with most of the scant available information coming from more recent studies. The perturbation of the ESs either in parts or as a whole could lead to diverse outcomes and some studies have also speculated their probable function from bioinformatics analyses and structural data. In this section, we highlight some of the important studies on the function of ESs and bring out the potential roles they could be playing in a cell (Figure 2).

4.1 | Ribosome biogenesis

Some of the earliest studies (Jeeninga et al., 1997; Sweeney et al., 1994) probing function of ESs showed that deletion or mutational perturbations of ES27L and ES7L in *Tetrahymena thermophila* or in yeast caused lethality due to a defect in ribosome biogenesis. More recently, high-resolution cryo-EM studies of pre-ribosomal structures in yeast showed that ESs facilitate ribosome biogenesis by interacting with a range of assembly factors. ES27L binds the nuclear export and quality control factor Arx1 in later stages of 60S assembly in yeast (Bradatsch et al., 2012; Leidig et al., 2014; Wu et al., 2016). Even the release of Arx1 from the pre-ribosomal particle is facilitated by the release factor Rei1 which binds to ES41L along with RPL22 (Greber et al., 2012). The Arx1, along with its recycling factors Rei1 and Jjj1, forms an Arx1–Rei1–Jjj1 complex that also interacts with ES24L. In addition, ES27L interacts with the Erb1–Ytm1 complex in the nucleolus, which are involved in pre-rRNA processing during ribosome biogenesis (Wegrecki et al., 2015).

ES7L also binds to several ribosome assembly factors like Erb1, Rrp1, Rpf1, Mak16, and Nsa1 in yeast (Kater et al., 2017; Wang et al., 2021; Zhou et al., 2019). Wang et al. showed that replacement of ES7L in *S. cerevisiae* (fungus) with that of *Candida albicans* (fungus) led to severe growth defects and specific 27S pre-rRNA processing deficiencies, which was attributed to reduced binding of the ribosome assembly factor Noc2 to the rRNA. Substituting yeast Noc2 with its *C. albicans* ortholog rescued the defects. This indicated that specific rRNA ESs and their cognate assembly factors may have potentially co-evolved to regulate ribosome biogenesis (Wang et al., 2021). Noc2 interacting partners Noc1/Mak21 and Rrp5 are also likely to associate with ES7L in yeast (Gómez Ramos et al., 2016; Hierlmeier et al., 2013; Lebaron et al., 2013) and may coordinate with each other for proper pre-rRNA processing. ES7L also potentially helps in the recruitment and assembly of certain ribosomal proteins like Rpl6 and Rpl4 in yeast (Ohmayer et al., 2015; Stelter et al., 2015; Wang et al., 2021). A few other known examples of ES-assembly factor interactions are the yeast ES6S interaction with the snoRNA, snR30 (Fayet-Lebaron et al., 2009), Rrp5 (Lebaron et al., 2013), and Rrp7 (Lin et al., 2013); and ES4L binding to Rlp7 (Dembowski et al., 2013).

Ramesh and Woolford Jr. (Ramesh and Woolford Jr. 2016) showed that a deletion of 12 LSU ESs led to improper pre-rRNA processing and growth defects in yeast. They categorized the ESs as "early acting" (ES39L, ES5L, ES7L, ES15L); "middle acting" (ES3L, ES20L, ES26L), and "late acting" (ES9L, ES10L, ES12L, ES31L, ES41L) and also observed that most of these specific sets of ESs clustered in specific regions of the ribosome based on their stage of function in the ribosome biogenesis pathway. Hence, the different ESs may bind to stage-specific ribosome biogenesis assembly factors.

Extensive functional and more detailed structural analysis needs to be extended to all ESs across different species, which will lead to a better understanding of the significance of these enigmatic ESs in ribosome biogenesis. ESs interact with multiple assembly factors either directly or indirectly at different stages of assembly. They may even have an alternative mechanism in the biogenesis pathway, but it has not been investigated yet. Furthermore, since most of the above experiments have been done in yeast, whether the variation in ESs gives added regulation in ribosome biogenesis in other eukaryotes remains to be investigated.

4.2 | Role in translation and protein folding

The tentacle-like extensions of ESs on the ribosomal surface may act as docking sites for many *trans*-acting translation factors. They may not be crucial for the core ribosome function but may be important for fine-tuning translational and posttranslational processes. For instance, ES3S–ES6S interaction region in *T. cruzi* is thought to be a binding site for initiation factors eIF3 and eIF4G (Alkemar & Nygård, 2004; Anger et al., 2013). Additionally, these interactions form

eukaryote-specific intersubunit bridges whose main function is unknown, but it is speculated that they may help counter the ratcheting of the SSU and reset the ribosome for the next round of protein synthesis (Chandramouli et al., 2008). A recent study (Díaz-López et al., 2019) proposed a role for the ES6S region in cap-dependent translation initiation of structured mRNAs in eukaryotes. The authors observed that ES6S provided an extended binding channel in the 48S

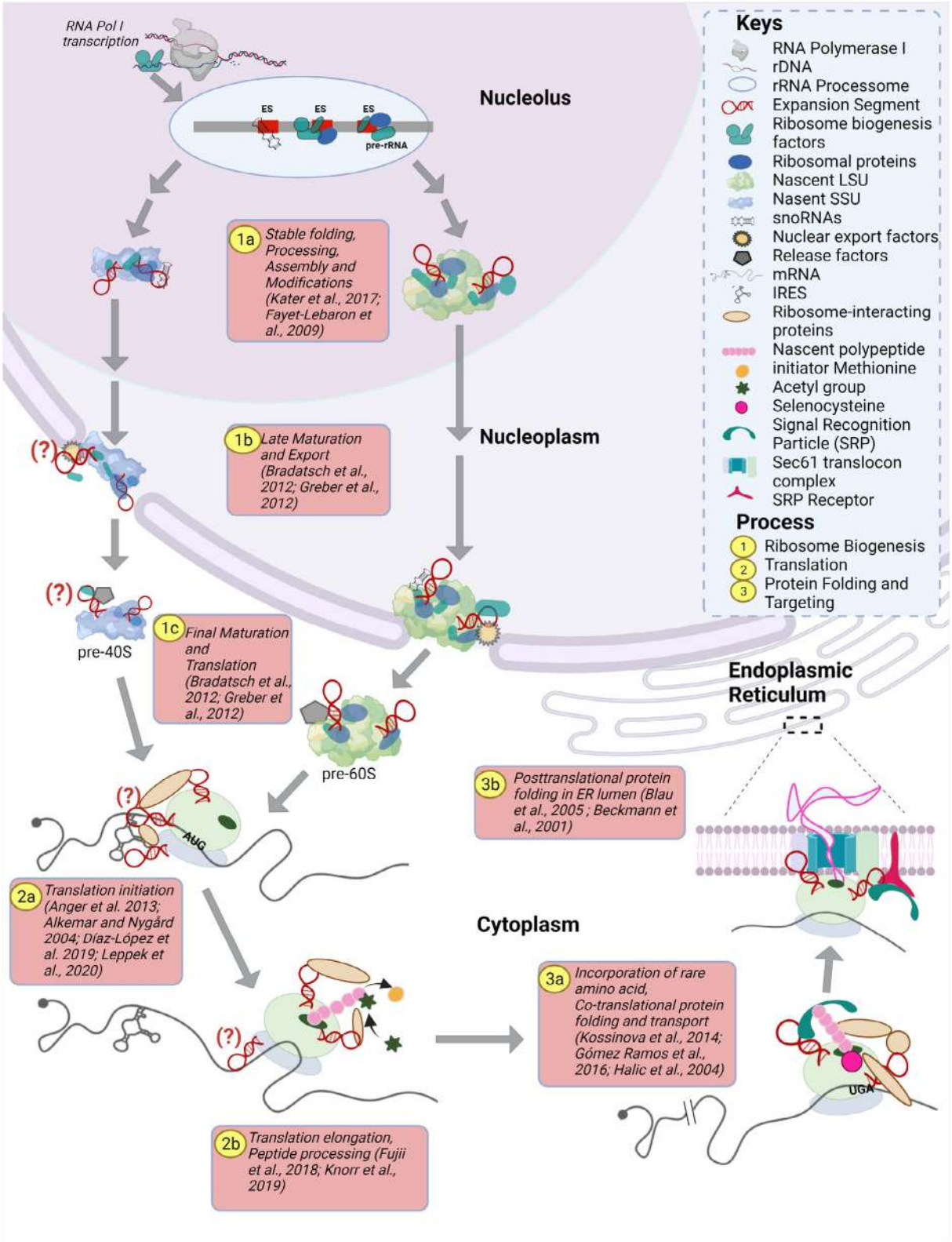

FIGURE 2 Legend on next page.

preinitiation complex for the eIF4A-mediated unwinding and scanning of human mRNAs with highly structured 5′UTR and suggested that this possibly improved the processivity of the scanning complex along the mRNA. Another example of the role of an ES in translation is the interaction of human ES27L with Ebp1, the human homolog of Arx1. Although the exact function of Ebp1 is still elusive, it is speculated to play a role in translation by inhibiting the phosphorylation of a eukaryotic translation initiation protein called eIF2α and in cell cycle (Wild et al., 2020).

Further, ES7L G-quadruplexes bind many helicases and hnRNPs *in-vitro*, where hnRNPs are a family of RNA-binding proteins with functions including pre-mRNA processing and transport of mRNAs to ribosomes (Mestre-Fos, Penev, Suttapitugsakul, et al., 2019), but whether these interactions also occur *in vivo* is not yet known. However, ribosomal G-quadruplexes have been shown to interact with heme *in vivo* in HEK293 cells (human) (Mestre-Fos et al., 2020) and may possibly regulate heme bioavailability and protein hemylation.

ES has also been shown to act as a binding site for peptide processing factors and this is the first demonstrated function of ES in translation regulation. A recent study (Fujii et al., 2018) in yeast showed that deletion of only one of the three ES27L arms resulted in defects in translational fidelity upon exposure to paromomycin, a translational error-inducing drug. This defect in translational fidelity was traced back to the inability of Map1 to bind to the deletion-containing ES27L. Map1 is a methionine aminopeptidase that cleaves off the initiator methionine from the N-terminal end of the nascent emerging polypeptides. These findings were concurred by a similar study that explored the effect of reductive stress using DTT treatment in an ES27L deletion yeast mutant (Shankar et al., 2020). The authors reported the formation of protein aggregates, which they attributed to reduced processing of nascent polypeptides due to the lack of association of N-terminal processing enzymes, which could not be counteracted by chaperones due to DTT stress (Shankar et al., 2020). Moreover, a recent Cryo-EM study by Knorr et al. in yeast captured the interaction between the ribosome nascent chain complex and NatA (*N*-acetyl aminotransferase), an enzyme responsible for the acetylation of the N-terminal of a nascent polypeptide. NatA interacts with ES27L, ES39L and ES7L, and these multiple interactions stabilize the NatA ensemble on the ribosome for efficient substrate modification by Naa10, the catalytic unit of NatA (Fujii et al., 2018; Knorr et al., 2019).

ESs are also involved in the interaction of ribosomes with ribosome binding proteins necessary for selenocysteine incorporation (Kossinova et al., 2014). The insertion of rare amino acid selenocysteine (Sec) into a polypeptide chain during its synthesis occurs in response to the stop codon UGA redefined as the codon for Sec. One pivotal trans-acting factor in this process is SECIS Binding Protein (SBP2), which potentially binds the 60S subunit or 80S ribosome through helix E of ES7L in human. Across species that are known to encode selenocysteine-containing proteins, any sequence/structure changes in ES7L-E are accompanied by concomitant amino acid changes in SBP2 and this is speculated to maintain the ability of the protein to interact with the ribosome. This exemplifies the correlation between the structure of an ES with a protein of the translation apparatus. There are probably other such instances yet to be discovered, which could help us understand the subtle differences they impart to the process of translation among eukaryotes.

FIGURE 2 Figure summarizing the various known and potential functions played by ESs in a cell. *1. Ribosome biogenesis*: ESs may play a role in the different stages of ribosome biogenesis. ESs in the pre-ribosomal complexes act as binding sites for several ribosome assembly factors (Bradatsch et al., 2012; Dembowski et al., 2013; Gómez Ramos et al., 2016; Greber et al., 2012; Hierlmeier et al., 2013; Kater et al., 2017; Lebaron et al., 2013; Lin et al., 2013; Wang et al., 2021) and snoRNA (like snR30) (Fayet-Lebaron et al., 2009). ESs also interact with nuclear export factors (like the ARX1 in yeast) to help transport the pre-ribosomal complexes from the nucleus to the cytoplasm (Bradatsch et al., 2012; Greber et al., 2012). In the cytoplasm, the ESs further bind to factors that help in the release of the export factors and other assembly factors to facilitate the maturation of the ribosomal subunits Bradatsch et al., 2012; (?) indicates potential interaction that is yet to be supported by experimental data. *2. Translation*: ESs play myriad roles in protein synthesis. ESs in the SSU form binding sites for the eukaryotic translation initiation factors and also helps in the scanning of mRNA for proper positioning of the ribosome (Alkemar & Nygård, 2004; Anger et al., 2013; Díaz-López et al., 2019). ESs in the LSU may bind ribosome-binding proteins for several processes like initiator methionine cleavage from the nascent polypeptide (Fujii et al., 2018) or acetylation of nascent polypeptides (Knorr et al., 2019). (?) indicates the possible interaction of ESs with mRNA, which is supported by computational analysis (Parker, Balasubramaniam, Sallee, & Parker, 2018) and some experimental data (Leppek et al., 2020). More evidence is required to establish the veracity and physiological significance of such interactions. *3. Protein folding and targeting*: ESs may help in the co-translational folding of emerging polypeptides since they act as binding sites for chaperones (Gómez Ramos et al., 2016). They may also help in selenocysteine incorporation (Kossinova et al., 2014). They may help in further peptide folding and processing since they can interact with factors/proteins which help in the attachment of ribosomes to the ER membrane, thereby helping in the peptide folding in the ER lumen (Blau et al., 2005; Beckmann et al., 2001; Halic et al., 2004. This figure has been created with BioRender.com

ES7L could also potentially interact with chaperones, quality control proteins and aminoacyl tRNA synthetases, based on an in vitro pulldown study done in yeast (Gómez Ramos et al., 2016). Another study (Ghosh et al., 2020) showed that even a single point mutation on yeast ES7L (A501U) caused sequestration of ribosomes into cytosolic compartments shared with misfolded soluble proteins. The authors proposed that the aberrant nascent polypeptide chains generated by faulty ribosomes localize to inclusion bodies, where they may be refolded or degraded. This observed misfolding may be due to the inability of the mutated ES7L to act as a scaffold for chaperones, which might disturb the chaperone-assisted co-translational folding of the nascent polypeptide chains and generate aberrant polypeptides. Such studies give an insight into how the ESs can define ribosomal integrity, co-translational protein maturation events, and consequently cellular fitness.

4.3 | Role in binding to ER membrane and protein translocation

In some eukaryotes, it has been shown that certain ESs may have the ability to interact with the endoplasmic reticulum (ER) membrane. The presence of dense homoiterons in ESs is speculated to help in the attachment of cytosolic ribosomes to the ER membrane or to the cytoskeletal matrix and facilitate interaction with different groups of translation-related proteins (Parker et al., 2015). However, a detailed experimental characterization is needed to validate this hypothesis. ES27L has been proposed to play a role in tethering ribosomes to the ER. The cytosolic domain of a ribosome-associated ER membrane protein—ERj1p called ERj1C, has been shown to contact ES27L in complexes formed between dog pancreatic ribosomes and recombinant mouse ERj1C (Blau et al., 2005). The interaction between ES27L and the ER membrane may help in stabilizing the binding of the ribosome to the ER and any conformational changes in ES27L is thought to trigger the dissociation of the ribosome from the ER (Pfeffer et al., 2012). Beckmann et al. observed that ES27L in yeast *in vitro* assembled ribosome-nascent chain complexes (RNCs), along with ES24L, also binds to the protein translocon complex Sec61, which transports proteins to the ER in eukaryotes.

Therefore, based on this study, it has been suggested that both ES24L and ES27L may play an important role in the process of co-translational protein translocation, by serving as an attachment site for the protein-conducting channel in the ER (Beckmann et al., 2001).

The Signal Recognition Particle (SRP), which identifies the signal sequence on nascent polypeptides emerging from the translating ribosome, may also contact the ESs along with other portions of the ribosome for a stable interaction. For instance, in wheatgerm-canine RNCs, it has been shown that the N-terminal part of the SRP54 M domain interacts with ES24L (helix 59), while the 7S RNA of the S domain of SRP interacts with ES39L (along with RPL16) (Halic et al., 2004).

4.4 | Interaction with mRNA and small RNAs

In 2002, Mauro and Edelman proposed that ribosomal subunits may regulate translation via differential mRNA binding in the "Ribosome Filter Hypothesis" (Mauro & Edelman, 2002). The alternative "Ribosome Concentration Hypothesis" (Mills & Green, 2017) considers ribosomes as molecular machines that passively translate mRNAs without any role in regulating translation and speculates that the change in ribosome concentration within a cell could lead to differential binding of the mRNA to the ribosomes. Although these opposite views (Ribosome Filter Hypothesis vs. Ribosome Concentration Hypothesis) are yet to be resolved, we think that the two scenarios can co-exist and could be an interesting line of future investigation. The differential binding of the mRNAs to the ribosome could also potentially occur due to variability in r-proteins or rRNA. Till date, concrete mRNA–rRNA interactions due to base-pair complementarity have been shown only in a few cases for both bacteria and eukaryotes (Dresios et al., 2006; Pánek et al., 2013; Panopoulos & Mauro, 2008; Parker, Balasubramaniam, & Parker, 2018; Parker, Balasubramaniam, Sallee, & Parker, 2018; Shine & Dalgarno, 1974b; Steitz & Jakes, 1975; Tranque et al., 1998). However, it is speculated that rRNA ESs may be good candidates for interactions with mRNA because they are solvent-exposed and are predicted to contain structurally dynamic flexible regions (Pánek et al., 2013).

One of the ways by which rRNA segments could interact with mRNA is through sequence complementarity and a few studies have analyzed this prospect either computationally or experimentally. A bioinformatic study by Pánek et al. (Panel at al., 2013) using sequences from yeast and human, put forth the concept of "sticky rRNA regions," which are rRNA segments that can potentially interact with mRNA through base-pairing and regulate translation. Their

inspection of the sticky regions in 18S rRNAs revealed that they predominantly map to the solvent-exposed SSU ESs. Parker, Balasubramaniam, Sallee, and Parker (Parker, Balasubramaniam, Sallee, and Parker 2018) reported extensive complementarity between ESs and human mRNAs through computational analysis. Their data showed that ESs of the 28S rRNA exhibited a higher degree of complementarity to mRNAs as compared with the 28S and 18S core as well as 18S ESs, both in terms of number and density. These complementary regions were enriched in the 5′UTR region of mRNAs and were GC-rich. The mere presence of complementary stretches between mRNA and ESs does not indicate an interaction between them, though it has been hypothesized that these interactions may help with the proper positioning of ribosomes on the mRNA or may impact the rate of translation. The analysis also shows that ESs carry sense matches to the transcripts, which are speculated to possibly aid in the competitive detachment of mRNP protein components from the mRNA, thereby facilitating the entrance of mRNAs into the mRNA tunnel of the ribosome The computational predictions and ideas from these studies could be focal points of future investigations.

Experimental evidence showing interactions between mRNA and eukaryotic ESs are almost non-existent and is an area that needs extensive investigation. A study by Chappell and Mauro (Chappell and Mauro 2003) showed experimentally that a 22 nucleotide 5′UTR IRES element, which included a region complementary to the solvent-exposed ES6S of 18S rRNA, enhanced the translation of Rbm3 mRNAs in various mammalian cell lines and in a cell free translation system. The authors have suggested that an interaction through base-pairing between this IRES module and the ES6S may be a possible binding mechanism used by the Rbm3 mRNA to recruit ribosomes directly and enhance translation initiation of the downstream ORF, though this proposed interaction is yet to be experimentally validated. In this case, if the interaction can be validated, the ribosome can potentially act as a filter and selectively enhance translation of Rbm3 transcripts as postulated by the "ribosome filter hypothesis" (Mauro & Edelman, 2002). Corroborative to this hypothesis, the first evidence of a direct interaction between an mRNA and an ES came from a recent study (Leppek et al., 2020) showed using cryo-EM that the 5′UTR of Hoxa9 mRNA has a modular P4 stem-loop that directly binds to ES9S of the human ribosome and allows cap-independent translation initiation of the downstream ORF. The evolutionarily distant yeast ribosomes, which possess a different ES9S sequence compared with mammalian ribosomes, cannot bind to this Hox 5′UTR element. The same group (Leppek et al., 2021) also developed a technology called Variable Expansion segment-Ligand Chimeric Ribosome-ImmunoPrecipitation RNA-seq (VELCRO-IP RNA-seq) and showed that ES9S can interact with a large array of mRNAs other than HOXa9 mRNA in mouse embryonic stem cells.

VELCRO involves the generation of chimeric ribosomes in which the species-specific ES under investigation replaces its native counterpart in the yeast ribosome. Such chimeric ribosomes can be coupled with a pulldown followed by RNA sequencing (VELCRO-IP RNA-seq) to check rRNA-mRNA interactions genome-wide. Their data also revealed additional ES9S-interacting mRNA fragments mapping to the CDS and 3′UTR regions, whose functional impact is yet to be investigated. The authors have speculated that such RNA elements in these regions may be involved in diverse functions related to RNA metabolism such as mRNA localization or decay, which needs to be experimentally explored. These interesting results highlight the potential of ESs in guiding species-specific and gene-specific translation.

The interaction between mRNA and ESs could also be structure-based. For instance, ES7S in 18S rRNA intercalates IRES stem-loops IIId and IIIe and helix hIII1 of the HCV (Hepatitis C Virus) IRES and facilitates a network of interactions between these three elements, that may be crucial to stabilize the IRES for efficient ribosome recruitment (Yamamoto et al., 2015). Another example of a potential structure-based interaction is the presence of an ES6S–ES7S based helical structure called "turret" in the SSU of protozoan *T. cruzi* that may possibly interact with a unique feature in the 5′UTR of the unusually structured mRNAs seen in the protozoan (Gao et al., 2005). Such interactions may also involve base-pairing (Quade et al., 2015; Yamamoto et al., 2015) and needs further probing. It would be interesting to holistically identify and characterize the species-specific changes in the other components of translation machinery alongside changes in rRNA ESs. We speculate that the presence of specially structured mRNAs and unique ES-based structure in *Trypanosomes* could indicate that ESs can potentially evolve in parallel with mRNAs and translation factors resulting in species-specific elements of translation regulation.

Another under-explored avenue of investigation is the potential interaction of rRNA-derived fragments (rRFs) (Guan & Grigoriev, 2021; Lambert et al., 2019) and especially the ones that originate from within the ESs, with various protein-coding transcripts. rRFs have been observed across many eukaryotes and their function is not clear yet. A recent computational meta-analysis (Guan & Grigoriev, 2021) using data from human and mouse suggested that rRFs may bind to both coding and intronic regions of many transcripts and it is speculated that such an interaction may add another layer of translation regulation. Therefore, we think a comprehensive exploration of rRFs from ESs as potential regulators of translation across eukaryotes would improve our current understanding of translation regulation.

ESs can also bind to small RNAs. Kiss and coworkers (Fayet-Lebaron et al., 2009) showed that the yeast ES6S contains two short conserved motifs that can form six base-pair perfect helices with two motifs of snR30, which is a snoRNA (small nucleolar RNA) involved in 18S rRNA processing (Bally et al., 1988; Morrissey & Tollervey, 1993). One of the two snR30 interaction sites in ES6S is also computationally predicted to form a long-range tertiary interaction with ES3S in many eukaryotes (Alkemar & Nygård, 2003, 2004, 2006). The binding of snR30 to ES6S could thus prevent this interaction, while its dissociation could lead to a conformational switch in pre-ribosomes (Strunk & Karbstein, 2009). Another bioinformatic study (Parker, Balasubramaniam, & Parker, 2018) has reported the presence of up to twofold enhanced complementarity in LSU ESs toward GC-rich human microRNAs when compared with all the other rRNA regions. The authors have proposed that given the considerable abundance of rRNAs in mammalian tissues, interactions between ESs and microRNAs could potentially help control miRNA balance, possibly serving to lower the availability of GC-rich miRNAs, which will, in turn, prevent the binding of these miRNAs to their cognate mRNAs, thereby preventing mRNA degradation. This hypothesis needs experimental verification too. Overall experimental data available for the interactions between ESs and other classes of RNA is still very sparse and would definitely benefit from extensive systematic studies, as the functional implications of such interactions are quite diverse (Box 1).

5 | CONCLUSION

Almost four decades since the discovery of ESs (Clark et al., 1984; Hassouna et al., 1984; Veldman et al., 1981; Ware et al., 1983), researchers have been unveiling tantalizing glimpses into the potential of these enigmatic insertions across phyla. However, ESs are still a "black box" in the field of ribosomal biology. Despite their established or potential functions in some cellular processes, we still have no understanding of the mechanistic intricacies of the influence of ESs. Another potential avenue of investigation is to extensively capture the structure of ribosomal ESs in various molecular complexes and organisms. This is relevant because a profile of the dynamic conformational changes of ESs at different stages of translation could help in understanding the communication among the various functional centers of the ribosome. It would also be interesting to study the coevolution of ESs with various ribosome biogenesis and translation factors, as it may reveal nuanced differences in these processes among different eukaryotic species. This understanding could make us better equipped to devise effective therapeutic targets or strategies in drug design.

One other interesting area that needs widespread focus is the extent of intraindividual and interindividual variability present in ESs. The presence of variable ES regions among the tandem rDNA repeats within a cell or even among different tissues could have physiological implications. Although more than 80% of the transcribed RNA in a yeast cell is rRNA (Lewis & Tollervey, 2000; Warner, 1999), it is unclear which rDNA loci harboring potentially impactful variants in ESs and other regions get transcribed at any given time or under a specific condition. Such variability in sequence and spatio-temporal expression, in conjunction with variable rRNA modifications, could expand the pool of heterogeneous ribosomes that are functionally specialized. Such "specialized ribosomes" may interact with subsets of mRNAs, regulate their translation and improve the robustness of cellular response under various conditions. We suggest that a comprehensive database that collates all the information on ESs (like nomenclature, sequence, predicted/known structure, posttranscriptional modifications, polymorphisms, and known/potential function), both within a species and across different species would be a very useful resource for future research. By no means exhaustive, we think that the aspects mentioned above are important to propel the field forward.

A major hindrance in the research on ESs is the paucity of specific tools to probe their nature and function. Several groups have developed techniques to understand rRNA function, which can be used to study ESs. But the presence of tandem rRNA repeats spread across different chromosomes in vertebrates poses a unique challenge that needs to be addressed. Despite their potential limitations, some of the techniques developed earlier can be a starting point for the development of innovative techniques for the study of ESs. For instance, Burman and Mauro (Burman and Mauro 2012) established an 18S rRNA expression system, which can be used to express an exogenous mutant rRNA while simultaneously blocking the endogenous ribosomes with an antibiotic. Although this technique has been successfully applied to murine cells to investigate the importance of the transcribed spacers flanking the 18S rRNA in rRNA pre-processing, they can potentially be useful in probing the function of ESs as well. However, this system would require (1) Selection of an inhibitor of endogenous ribosomes that will be suitable for the species or cell line under investigation. (2) Identification of the mutations in rRNA that would confer resistance to the inhibitor. (3) Verification of the extent of incorporation of the mutated rRNA in to functional ribosomal subunits; and (4) assessment of the level of resistance conferred by the introduced mutations. Another technique using Psoralen-cross linking followed by RNA sequencing has been used to understand diverse groups of RNA–

BOX 1 Expansion segments of the mitochondrial and chloroplast ribosomes

The mitochondria (present in all eukaryotes) and chloroplasts (present only in plants) are two vital membrane-bound organelles present in the eukaryotic cell. Both contain their own genomes and ribosomes for the expression of their transcripts (Schwarz & Kössel, 1979, 1980; Tomal et al., 2019). In this section, we will give a brief overview of the mitochondrial and chloroplast ribosomes and discuss their ESs.

The *mitochondrial ribosomes or the mitoribosomes* are responsible for the translation of proteins important for oxidative phosphorylation and ATP synthesis. The mitoribosomes have undergone extensive remodeling in many aspects compared with the ribosomal core. This class of ribosomes is highly heterogeneous across the different eukaryotic kingdoms. (Tomal et al., 2019). The evolution of mitoribosomes can be explained by an early constructive phase and a later reductive phase. The evolution of mitoribosomes might have occurred in the following path: rRNA rich but protein poor (in bacteria, from where the mitochondria could have possibly derived later, according to the endosymbiont theory) to rRNA rich and protein-rich (in the plant, fungi, most algae, and protists mitoribosome) to rRNA poor and protein-rich (metazoa and kinetoplastid mitoribosome) (van der Sluis et al., 2015).

The mtrRNA of mammalian and kinetoplastid (Trypanosomes) mitoribosome is highly decreased in size compared with bacterial ribosomes. This is due to extensively reduced mtrRNA because of several amputations of the rRNA sequence (Greber & Ban, 2016; Tomal et al., 2019). Sometimes, but not always, these amputations coincide with the positions where the eukaryotic ESs are inserted into the core structure (Gerbi, 1986; Mankin & Kopylov, 1981).

Although there have been a few reductions in the lengths of mtrRNA in plants (Waltz et al., 2019), the accrual of ESs have resulted in their overall growth, making them one of the largest mitoribosomes. The mitoribosomal ESs extensively remodel parts of the mtSSU and mtLSU and form interactions with newly acquired or extended mitochondrial ribosomal proteins, which might possibly impart specialized functions. For instance, the large mtSSU head extension of plant mitoribosomes is primarily shaped by a 370 nt ES of helix h39 in 18S rRNA, which is proposed to bind plant-specific mitoribosomal proteins and translation factors, though this needs to be investigated (Tomal et al., 2019; Waltz et al., 2019). The fungal mitoribosomes also have an expanded rRNA. In yeast, the mtSSU is short, but the mtLSU is the longest of all known species due to the presence of 11 ESs (Amunts et al., 2014; Tomal et al., 2019). However, only a few of these insertions correlate with those of the eukaryotic ESs. Similarly, in *Neurospora crassa*, the mitoribosome consists of 16 ESs, with 15 of them in the mtLSU. Most of these ESs, along with the mitochondria-specific proteins, help in the remodeling of the mitoribosome (Amunts et al., 2014; van der Sluis et al., 2015) and coordinate the binding of Nicotinamide adenine dinucleotide (NAD) and the natural inhibitor of mitochondrial ATP synthase IF1 (Itoh et al., 2020).

For the small subunit, the yeast mtSSU is longer than the SSU of the *E. coli* ribosome but has experienced both rRNA reductions and insertions. The yeast mtSSU is thus much less expanded compared with yeast mtLSU but is much more expanded than the mammalian mitoribosome (Desai et al., 2017).

The ESs in fungal mitoribosomes may also help in forming contact points between the mitoribosome and the inner mitochondrial membrane (IMM). The yeast mtLSU has two membrane contact sites which are formed by the mitochondrial inner membrane protein Mba1 and a 21S mtLSU rRNA ES 96-ES1 (this ES is preceded by helix 96 of the 21S rRNA in the mitoribosome). However, such ES mediated contact site is absent in humans even when the mitoribosome contacts the IMM (Englmeier et al., 2017), thereby showing that the same function may be performed by different components depending on the species (Tomal et al., 2019).

The *chloroplast ribosome or chlororibosome* is around 10 Angstrom larger than the *E. coli* ribosome but smaller than the mitoribosome (Tomal et al., 2019). The majority of chloroplast encoded proteins are targeted to the thylakoid membranes and include components required for photosynthesis. On a structural level, chloroplast ribosomes closely resemble bacterial ones, and the chlororibosomal rRNA did not expand as much as the plant mitochondrial ribosome (van der Sluis et al., 2015). Compared with the bacterial ribosome, there is a loss of certain rRNA regions due to rRNA truncations, but this is almost balanced by the acquisition of plastid-specific ESs (Bieri et al., 2017). A few of the insertion sites may correlate with the position of ESs of eukaryotic cytosolic ribosomes. For example, insertion at helix 38 of chloroplast 23S correlates with the position of ES12L in the 80S ribosome (Graf et al., 2017). However, most of the insertions are small. Other than slight remodeling of the ribosome by the interactions between these novel insertions and ribosomal proteins (Bieri et al., 2017), the functions of these rRNA insertions in both mitochondrial and chloroplast ribosomes are unknown and require extensive research.

RNA interactions. The majority of the reported interactions are those of mRNA:rRNA, which could be used to delineate the interactivity of ESs (Lu et al., 2018; Nilsen, 2014). However, the functional relevance of such interactions needs to be addressed.

As mentioned earlier, the VELCRO-IP technique is quite versatile and can be applied to the different ESs of different species to find out their mRNA binding partners. This technique will generate a chimeric yeast ribosome in which the species-specific ES will replace its endogenous native counterpart. This pioneering technique will enable us to insert ESs of any species into the yeast ribosome, which will facilitate the study of that ES within the context of assembled ribosomes (Leppek et al., 2021). However, the ribosome is a molecular mammoth whose structure, conformation, and available binding sites are shaped by a complex network of interactions among its constituent r-proteins and rRNA. Hence, inserting an ES of interest from a different species onto the yeast ribosome may perturb its native state and may not reflect the actual structure and function of the ES.

The advent of CRISPR-Cas technology for gene editing is one way to manipulate multigene loci. Since editing hundreds of tandem rDNA repeats spread across several chromosomal loci still poses a huge challenge for gene manipulation, very few studies have attempted to do this through CRISPR. For example, in one study, the researchers have used CRISPR-Cas technology to successfully remove all 62 copies of porcine endogenous retroviruses (PERVs) spread across different chromosomes in porcine kidney epithelial cells (Yang et al., 2015). This study highlights the possibility of editing the rDNA gene clusters spread across chromosomes in eukaryotes, with further optimization of the CRISPR-Cas9 technique.

Species-specific variants in rRNA ESs can be novel therapeutic targets in drug design against eukaryotic pathogens. One study used this approach and showed that drug molecules with high affinity for *Candida albicans* ES7L in vitro induced mortality in the pathogen, but not in human HEK293T cells (Gómez Ramos et al., 2017). Another study showed that blocking ES6S using small molecules, inhibits translation of mRNAs involved in signal transduction and cancer which tend to possess long, structured 5′UTRs (Díaz-López et al., 2019). In this case, ES6S could be a potential target for designing anti-cancer drugs. Hence, the potential of other ESs to be effective therapeutic targets seems promising and needs to be explored. Thus, it is important to study rRNA ESs not only for the fundamental understanding of a complex macromolecule but also for the valuable insights that could very well have far-reaching clinical implications.

ACKNOWLEDGMENTS

We would like to thank Dr. Srikar Krishna Gopinath for his valuable comments and suggestions on the review.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Nivedita Hariharan: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (equal); software (lead); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Sumana Ghosh:** Conceptualization (equal); investigation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Dasaradhi Palakodeti:** Funding acquisition (lead); supervision (lead); writing – review and editing (supporting).

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Nivedita Hariharan https://orcid.org/0000-0001-5468-0580
Dasaradhi Palakodeti https://orcid.org/0000-0003-2219-4785

RELATED WIREs ARTICLES

Understanding the potential of hepatitis C virus internal ribosome entry site domains to modulate translation initiation via their structure and function
An overview of pre-ribosomal RNA processing in eukaryotes
Ribosome heterogeneity and specialization in development

REFERENCES

Aitken, C. E., & Lorsch, J. R. (2012). A mechanistic overview of translation initiation in eukaryotes. *Nature Structural & Molecular Biology*, *19*(6), 568–576.

Alkemar, G., & Nygård, O. (2003). A possible tertiary rRNA interaction between expansion segments ES3 and ES6 in eukaryotic 40S ribosomal subunits. *RNA*, *9*(1), 20–24.

Alkemar, G., & Nygård, O. (2004). Secondary structure of two regions in expansion segments ES3 and ES6 with the potential of forming a tertiary interaction in eukaryotic 40S ribosomal subunits. *RNA*, *10*(3), 403–411.

Alkemar, G., & Nygård, O. (2006). Probing the secondary structure of expansion segment ES6 in 18S ribosomal RNA. *Biochemistry*, *45*(26), 8067–8078.

Amunts, A., Brown, A., Bai, X.-C., Llácer, J. L., Hussain, T., Emsley, P., Long, F., Murshudov, G., Scheres, S. H. W., & Ramakrishnan, V. (2014). Structure of the yeast mitochondrial large ribosomal subunit. *Science*, *343*(6178), 1485–1489.

Anger, A. M., Armache, J.-P., Berninghausen, O., Habeck, M., Subklewe, M., Wilson, D. N., & Beckmann, R. (2013). Structures of the human and Drosophila 80S ribosome. *Nature*, *497*(7447), 80–85.

Armache, J.-P., Jarasch, A., Anger, A. M., Villa, E., Becker, T., Bhushan, S., Jossinet, F., Habeck, M., Dindar, G., Franckenberg, S., Marquez, V., Mielke, T., Thomm, M., Berninghausen, O., Beatrix, B., Söding, J., Westhof, E., Wilson, D. N., & Beckmann, R. (2010). Cryo-EM structure and rRNA model of a translating eukaryotic 80S ribosome at 5.5-A resolution. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(46), 19748–19753.

Bally, M., Hughes, J., & Cesareni, G. (1988). SnR30: A new, essential small nuclear RNA from Saccharomyces cerevisiae. *Nucleic Acids Research*, *16*(12), 5291–5303.

Barandun, J., Hunziker, M., Vossbrinck, C. R., & Klinge, S. (2019). Evolutionary compaction and adaptation visualized by the structure of the dormant microsporidian ribosome. *Nature Microbiology*, *4*(11), 1798–1804.

Beckmann, R., Spahn, C. M., Frank, J., & Blobel, G. (2001). The active 80S ribosome-Sec61 complex. *Cold Spring Harbor Symposia on Quantitative Biology*, *66*, 543–554.

Belshaw, R., & Quicke, D. L. (1997). A molecular phylogeny of the Aphidiinae (Hymenoptera: Braconidae). *Molecular Phylogenetics and Evolution*, *7*(3), 281–293.

Ben-Shem, A., Garreau de Loubresse, N., Melnikov, S., Jenner, L., Yusupova, G., & Yusupov, M. (2011). The structure of the eukaryotic ribosome at 3.0 Å resolution. *Science*, *334*(6062), 1524–1529.

Bernier, C. R., Petrov, A. S., Waterbury, C. C., Jett, J., Li, F., Freil, L. E., Xiong, X., Wang, L., Migliozzi, B. L. R., Hershkovits, E., Xue, Y., Hsiao, C., Bowman, J. C., Harvey, S. C., Grover, M. A., Wartell, Z. J., & Williams, L. D. (2014). RiboVision suite for visualization and analysis of ribosomes. *Faraday Discussions*, *169*, 195–207.

Bieri, P., Leibundgut, M., Saurer, M., Boehringer, D., & Ban, N. (2017). The complete structure of the chloroplast 70S ribosome in complex with translation factor pY. *The EMBO Journal*, *36*(4), 475–486.

Blau, M., Mullapudi, S., Becker, T., Dudek, J., Zimmermann, R., Penczek, P. A., & Beckmann, R. (2005). ERj1p uses a universal ribosomal adaptor site to coordinate the 80S ribosome at the membrane. *Nature Structural & Molecular Biology*, *12*(11), 1015–1016.

Bowman, J. C., Petrov, A. S., Frenkel-Pinter, M., Penev, P. I., & Williams, L. D. (2020). Root of the tree: The significance, evolution, and origins of the ribosome. *Chemical Reviews*, *120*(11), 4848–4878.

Bradatsch, B., Leidig, C., Granneman, S., Gnädig, M., Tollervey, D., Böttcher, B., Beckmann, R., & Hurt, E. (2012). Structure of the pre-60S ribosomal subunit with nuclear export factor Arx1 bound at the exit tunnel. *Nature Structural & Molecular Biology*, *19*(12), 1234–1241.

Branlant, C., Krol, A., Machatt, M. A., Pouyet, J., Ebel, J. P., Edwards, K., & Kössel, H. (1981). Primary and secondary structures of Escherichia coli MRE 600 23S ribosomal RNA. Comparison with models of secondary structure for maize chloroplast 23S rRNA and for large portions of mouse and human 16S mitochondrial rRNAs. *Nucleic Acids Research*, *9*(17), 4303–4324.

Brimacombe, R. (1981). Secondary structure and evolution of ribosomal RNA. *Nature*, *294*(5838), 209–210.

Burman, L. G., & Mauro, V. P. (2012). Analysis of rRNA processing and translation in mammalian cells using a synthetic 18S rRNA expression system. *Nucleic Acids Research*, *40*(16), 8085–8098.

Caetano-Anollés, G. (2015). Ancestral insertions and expansions of rRNA do not support an origin of the ribosome in its peptidyl transferase center [review of *ancestral insertions and expansions of rRNA do not support an origin of the ribosome in its peptidyl transferase center*]. *Journal of Molecular Evolution*, *80*(3-4), 162–165.

Campbell, B. C., Steffen-Campbell, J. D., & Werren, J. H. (1993). Phylogeny of the Nasonia species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. *Insect Molecular Biology*, *2*(4), 225–237.

Chandramouli, P., Topf, M., Ménétret, J.-F., Eswar, N., Cannone, J. J., Gutell, R. R., Sali, A., & Akey, C. W. (2008). Structure of the mammalian 80S ribosome at 8.7 A resolution. *Structure*, *16*(4), 535–548.

Chappell, S. A., & Mauro, V. P. (2003). The internal ribosome entry site (IRES) contained within the RNA-binding motif protein 3 (Rbm3) mRNA is composed of functionally distinct elements. *Journal of Biological Chemistry*, *278*(36), 33793–33800. https://doi.org/10.1074/jbc.m303495200

Clark, C. G., Tague, B. W., Ware, V. C., & Gerbi, S. A. (1984). Xenopus laevis 28S ribosomal RNA: A secondary structure model and its evolutionary and functional implications. *Nucleic Acids Research*, *12*(15), 6197–6220.

Claude, A. (1938). Concentration and purification of chicken tumor I agent. *Science*, *87*(2264), 467–468.

Claude, A. (1940). Particulate components of normal and tumor cells. *Science*, *91*(2351), 77–78.

Claude, A. (1944). The constitution of mitochondria and microsomes, and the distribution of nucleic acid in the cytoplasm of a leukemic cell. *The Journal of Experimental Medicine*, *80*(1), 19–29.

Claude, A., & Hoerr, N. L. (1943). Distribution of nucleic acids in the cell and the morphological constitution of cytoplasm. In J. Cattell (Ed.), *Biological symposia: Frontiers in cytochemistry* (p. 111). The Jacques Cattell Press.

Crick, F. H. (1962). The genetic code. *Scientific American*, *207*(4), 66–77.

Crick, F. H. (1968). The origin of the genetic code. *Journal of Molecular Biology*, *38*(3), 367–379.

Dembowski, J. A., Ramesh, M., McManus, C. J., & Woolford, J. L., Jr. (2013). Identification of the binding site of Rlp7 on assembling 60S ribosomal subunits in *Saccharomyces cerevisiae*. *RNA*, *19*(12), 1639–1647.

Desai, N., Brown, A., Amunts, A., & Ramakrishnan, V. (2017). The structure of the yeast mitochondrial ribosome. *Science*, *355*(6324), 528–531.

Díaz-López, I., Toribio, R., Berlanga, J. J., & Ventoso, I. (2019). An mRNA-binding channel in the ES6S region of the translation 48S-PIC promotes RNA unwinding and scanning. *eLife*, *8*, e48246.

Dintzis, H. M. (2006). The wandering pathway to determining N to C synthesis of proteins: Some recollections concerning protein structure and biosynthesis. *Biochemistry and Molecular Biology Education*, *34*(4), 241–246.

Dresios, J., Chappell, S. A., Zhou, W., & Mauro, V. P. (2006). An mRNA-rRNA base-pairing mechanism for translation initiation in eukaryotes. *Nature Structural & Molecular Biology*, *13*(1), 30–34.

Englmeier, R., Pfeffer, S., & Förster, F. (2017). Structure of the human mitochondrial ribosome studied in situ by cryoelectron tomography. *Structure*, *25*(10), 1574–1581.e2.

Fayet-Lebaron, E., Atzorn, V., Henry, Y., & Kiss, T. (2009). 18S rRNA processing requires base pairings of snR30 H/ACA snoRNA to eukaryote-specific 18S sequences. *The EMBO Journal*, *28*(9), 1260–1270.

Fujii, K., Susanto, T. T., Saurabh, S., & Barna, M. (2018). Decoding the function of expansion segments in ribosomes. *Molecular Cell*, *72*(6), 1013–1020.e6.

Gao, H., Ayub, M. J., Levin, M. J., & Frank, J. (2005). The structure of the 80S ribosome from *Trypanosoma cruzi* reveals unique rRNA components. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(29), 10206–10211.

Gerbi, S. (1996). Expansion segments: Regions of variable size that interrupt the universal core secondary structure of ribosomal RNA. In R. A. Zimmermann & A. E. Dahlberg (Eds.), *Ribosomal RNA structure, evolution, processing, and function in protein biosynthesis* (pp. 71–87). Telford-CRC Press.

Gerbi, S. A. (1986). The evolution of eukaryotic ribosomal DNA. *Bio Systems*, *19*(4), 247–258.

Ghosh, A., Williams, L. D., Pestov, D. G., & Shcherbik, N. (2020). Proteotoxic stress promotes entrapment of ribosomes and misfolded proteins in a shared cytosolic compartment. *Nucleic Acids Research*, *48*(7), 3888–3905.

Gómez Ramos, L. M., Degtyareva, N. N., Kovacs, N. A., Holguin, S. Y., Jiang, L., Petrov, A. S., Biesiada, M., Hu, M. Y., Purzycka, K. J., Arya, D. P., & Williams, L. D. (2017). Eukaryotic ribosomal expansion segments as antimicrobial targets. *Biochemistry*, *56*(40), 5288–5299.

Gómez Ramos, L. M., Smeekens, J. M., Kovacs, N. A., Bowman, J. C., Wartell, R. M., Wu, R., & Williams, L. D. (2016). Yeast rRNA expansion segments: Folding and function. *Journal of Molecular Biology*, *428*(20), 4048–4059.

Gonzalez, I. L., Sylvester, J. E., Smith, T. F., Stambolian, D., & Schmickel, R. D. (1990). Ribosomal RNA gene sequences and hominoid phylogeny. *Molecular Biology and Evolution*, *7*(3), 203–219.

Gorski, J. L., Gonzalez, I. L., & Schmickel, R. D. (1987). The secondary structure of human 28S rRNA: The structure and evolution of a mosaic rRNA gene. *Journal of Molecular Evolution*, *24*(3), 236–251.

Graf, M., Arenz, S., Huter, P., Dönhöfer, A., Nováček, J., & Wilson, D. N. (2017). Cryo-EM structure of the spinach chloroplast ribosome reveals the location of plastid-specific ribosomal proteins and extensions. *Nucleic Acids Research*, *45*(5), 2887–2896.

Greber, B. J., & Ban, N. (2016). Structure and function of the mitochondrial ribosome. *Annual Review of Biochemistry*, *85*, 103–132.

Greber, B. J., Boehringer, D., Montellese, C., & Ban, N. (2012). Cryo-EM structures of Arx1 and maturation factors Rei1 and Jjj1 bound to the 60S ribosomal subunit. *Nature Structural & Molecular Biology*, *19*(12), 1228–1233.

Guan, L., & Grigoriev, A. (2021). Computational meta-analysis of ribosomal RNA fragments: Potential targets and interaction mechanisms. *Nucleic Acids Research*, *49*(7), 4085–4103.

Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., & Altman, S. (1983). The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell*, *35*, 849–857.

Halic, M., Becker, T., Pool, M. R., Spahn, C. M. T., Grassucci, R. A., Frank, J., & Beckmann, R. (2004). Structure of the signal recognition particle interacting with the elongation-arrested ribosome. *Nature*, *427*(6977), 808–814.

Hancock, J. M., & Dover, G. A. (1990). "Compensatory slippage" in the evolution of ribosomal RNA genes. *Nucleic Acids Research*, *18*(20), 5949–5954.

Hancock, J. M., Tautz, D., & Dover, G. A. (1988). Evolution of the secondary structures and compensatory mutations of the ribosomal RNAs of *Drosophila melanogaster*. *Molecular Biology and Evolution*, *5*(4), 393–414.

Hassouna, N., Michot, B., & Bachellerie, J. P. (1984). The complete nucleotide sequence of mouse 28S rRNA gene. Implications for the process of size increase of the large subunit rRNA in higher eukaryotes. *Nucleic Acids Research*, *12*(8), 3563–3583.

Hernández, G., Proud, C. G., Preiss, T., & Parsyan, A. (2012). On the diversification of the translation apparatus across eukaryotes. *Comparative and Functional Genomics*, *2012*, 256848.

Herr, W., Chapman, N. M., & Noller, H. F. (1979). Mechanism of ribosomal subunit association: Discrimination of specific sites in 16 S RNA essential for association activity. *Journal of Molecular Biology*, *130*(4), 433–449.

Hierlmeier, T., Merl, J., Sauert, M., Perez-Fernandez, J., Schultz, P., Bruckmann, A., Hamperl, S., Ohmayer, U., Rachel, R., Jacob, A., Hergert, K., Deutzmann, R., Griesenbeck, J., Hurt, E., Milkereit, P., Baßler, J., & Tschochner, H. (2013). Rrp5p, Noc1p and Noc2p form a protein module which is part of early large ribosomal subunit precursors in *S. cerevisiae*. *Nucleic Acids Research*, *41*(2), 1191–1210.

Ingolia, N. T., Lareau, L. F., & Weissman, J. S. (2011). Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. *Cell*, *147*(4), 789–802.

Itoh, Y., Naschberger, A., Mortezaei, N., Herrmann, J. M., & Amunts, A. (2020). Analysis of translating mitoribosome reveals functional characteristics of translation in mitochondria of fungi. *Nature Communications*, *11*(1), 5187.

Jeeninga, R. E., Van Delft, Y., de Graaff-Vincent, M., Dirks-Mulder, A., Venema, J., & Raué, H. A. (1997). Variable regions V13 and V3 of *Saccharomyces cerevisiae* contain structural features essential for normal biogenesis and stability of 5.8S and 25S rRNA. *RNA*, *3*(5), 476–488.

Kater, L., Thoms, M., Barrio-Garcia, C., Cheng, J., Ismail, S., Ahmed, Y. L., Bange, G., Kressler, D., Berninghausen, O., Sinning, I., Hurt, E., & Beckmann, R. (2017). Visualizing the assembly pathway of nucleolar pre-60S ribosomes. *Cell*, *171*(7), 1599–1610.

Kearsey, S. E., & Craig, I. W. (1981). Altered ribosomal RNA genes in mitochondria from mammalian cells with chloramphenicol resistance. *Nature*, *290*(5807), 607–608.

Keller, E. B., Zamecnik, P. C., & Loftfield, R. B. (1954). The role of microsomes in the incorporation of amino acids into proteins. *Journal of Histochemistry & Cytochemistry*, *2*(5), 378–386.

Khatter, H., Myasnikov, A. G., Natchiar, S. K., & Klaholz, B. P. (2015). Structure of the human 80S ribosome. *Nature*, *520*(7549), 640–645.

Klinge, S., Voigts-Hoffmann, F., Leibundgut, M., Arpagaus, S., & Ban, N. (2011). Crystal structure of the eukaryotic 60S ribosomal subunit in complex with initiation factor 6. *Science*, *334*(6058), 941–948.

Knorr, A. G., Schmidt, C., Tesina, P., Berninghausen, O., Becker, T., Beatrix, B., & Beckmann, R. (2019). Ribosome–NatA architecture reveals that rRNA expansion segments coordinate N-terminal acetylation. *Nature Structural & Molecular Biology*, *26*(1), 35–39.

Kossinova, O., Malygin, A., Krol, A., & Karpova, G. (2014). The SBP2 protein central to selenoprotein synthesis contacts the human ribosome at expansion segment 7L of the 28S rRNA. *RNA*, *20*(7), 1046–1056.

Kuo, B. A., Gonzalez, I. L., Gillespie, D. A., & Sylvester, J. E. (1996). Human ribosomal RNA variants from a single individual and their expression in different tissues. *Nucleic Acids Research*, *24*(23), 4817–4824.

Kushwaha, A. K., & Bhushan, S. (2020). Unique structural features of the mycobacterium ribosome. *Progress in Biophysics and Molecular Biology*, *152*, 15–24.

Lambert, M., Benmoussa, A., & Provost, P. (2019). Small non-coding RNAs derived from eukaryotic ribosomal RNA. *Non-Coding RNA*, *5*(1), 16. https://doi.org/10.3390/ncrna5010016

Lebaron, S., Segerstolpe, A., French, S. L., Dudnakova, T., de Lima Alves, F., Granneman, S., Rappsilber, J., Beyer, A. L., Wieslander, L., & Tollervey, D. (2013). Rrp5 binding at multiple sites coordinates pre-rRNA processing and assembly. *Molecular Cell*, *52*(5), 707–719.

Leffers, H., & Andersen, A. H. (1993). The sequence of 28S ribosomal RNA varies within and between human cell lines. *Nucleic Acids Research*, *21*(6), 1449–1455.

Leidig, C., Thoms, M., Holdermann, I., Bradatsch, B., Berninghausen, O., Bange, G., Sinning, I., Hurt, E., & Beckmann, R. (2014). 60S ribosome biogenesis requires rotation of the 5S ribonucleoprotein particle. *Nature Communications*, *5*, 3491.

Leppek, K., Byeon, G. W., Fujii, K., & Barna, M. (2021). VELCRO-IP RNA-seq reveals ribosome expansion segment function in translation genome-wide. *Cell Reports*, *34*(3), 108629.

Leppek, K., Fujii, K., Quade, N., Susanto, T. T., Boehringer, D., Lenarčič, T., Xue, S., Genuth, N. R., Ban, N., & Barna, M. (2020). Gene- and species-specific Hox mRNA translation by ribosome expansion segments. *Molecular Cell*, *80*(6), 980–995.

Lewis, J. D., & Tollervey, D. (2000). Like attracts like: Getting RNA processing together in the nucleus. *Science*, *288*(5470), 1385–1389.

Lin, J., Lu, J., Feng, Y., Sun, M., & Ye, K. (2013). An RNA-binding complex involved in ribosome biogenesis contains a protein with homology to tRNA CCA-adding enzyme. *PLoS Biology*, *11*(10), e1001669.

Locati, M. D., Pagano, J. F. B., Girard, G., Ensink, W. A., van Olst, M., van Leeuwen, S., Nehrdich, U., Spaink, H. P., Rauwerda, H., Jonker, M. J., Dekker, R. J., & Breit, T. M. (2017). Expression of distinct maternal and somatic 5.8S, 18S, and 28S rRNA types during zebrafish development. *RNA*, *23*(8), 1188–1199.

Lu, Z., Gong, J., & Zhang, Q. C. (2018). PARIS: Psoralen analysis of RNA interactions and structures with high throughput and resolution. *Methods in Molecular Biology*, *1649*, 59–84.

Lukeš, J., Archibald, J. M., Keeling, P. J., Ford Doolittle, W., & Gray, M. W. (2011). How a neutral evolutionary ratchet can build cellular complexity. *IUBMB Life*, *63*(7), 528–537. https://doi.org/10.1002/iub.489

Lynch, M., & Marinov, G. K. (2015). The bioenergetic costs of a gene. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(51), 15690–15695.

Mankin, A. S., & Kopylov, A. M. (1981). A secondary structure model for mitochondrial 12S rRNA: An example of economy in riRNA structure. *Biochemistry International*, *3*(6), 587–593.

Mauro, V. P., & Edelman, G. M. (2002). The ribosome filter hypothesis. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(19), 12031–12036.

Melnikov, S., Ben-Shem, A., De Loubresse, N. G., Jenner, L., Yusupova, G., & Yusupov, M. (2012). One core, two shells: Bacterial and eukaryotic ribosomes. *Nature Structural & Molecular Biology*, *19*(6), 560–567.

Melnikov, S., Manakongtreecheep, K., & Söll, D. (2018). Revising the structural diversity of ribosomal proteins across the three domains of life. *Molecular Biology and Evolution*, *35*(7), 1588–1598.

Mestre-Fos, S., Ito, C., Moore, C. M., Reddi, A. R., & Williams, L. D. (2020). Human ribosomal G-quadruplexes regulate heme bioavailability. *The Journal of Biological Chemistry*, *295*(44), 14855–14865.

Mestre-Fos, S., Penev, P. I., Richards, J. C., Dean, W. L., Gray, R. D., Chaires, J. B., & Williams, L. D. (2019). Profusion of G-quadruplexes on both subunits of metazoan ribosomes. *PLoS One*, *14*(12), e0226177.

Mestre-Fos, S., Penev, P. I., Suttapitugsakul, S., Hu, M., Ito, C., Petrov, A. S., Wartell, R. M., Wu, R., & Williams, L. D. (2019). G-Quadruplexes in human ribosomal RNA. *Journal of Molecular Biology*, *431*(10), 1940–1955.

Michot, B., & Bachellerie, J. P. (1987). Comparisons of large subunit rRNAs reveal some eukaryote-specific elements of secondary structure. *Biochimie*, *69*(1), 11–23.

Mills, E. W., & Green, R. (2017). Ribosomopathies: There's strength in numbers. *Science*, *358*(6363), eaan2755. https://doi.org/10.1126/science.aan2755

Morrissey, J. P., & Tollervey, D. (1993). Yeast snR30 is a small nucleolar RNA required for 18S rRNA synthesis. *Molecular and Cellular Biology*, *13*(4), 2469–2477.

Muñoz-Gómez, S. A., Bilolikar, G., Wideman, J. G., & Geiler-Samerotte, K. (2021). Constructive neutral evolution 20 years later. *Journal of Molecular Evolution*, *89*(3), 172–182.

Musters, W., Boon, K., van der Sande, C. A., van Heerikhuizen, H., & Planta, R. J. (1990). Functional analysis of transcribed spacers of yeast ribosomal DNA. *The EMBO Journal*, *9*(12), 3989–3996.

Musters, W., Conçalves, P. M., Boon, K., Raué, H. A., van Heerikhuizen, H., & Planta, R. J. (1991). The conserved GTPase center and variable region V9 from Saccharomyces cerevisiae 26S rRNA can be replaced by their equivalents from other prokaryotes or eukaryotes without detectable loss of ribosomal function. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(4), 1469–1473.

National Library of Medicine (US) & National Center for Biotechnology Information. (1988). Nucleotide [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information. https://www.ncbi.nlm.nih.gov/nuccore/

Nilsen, T. W. (2014). Detecting RNA–RNA interactions using psoralen derivatives. *Cold Spring Harbor Protocols*, *2014*(9), 080861.

Nilsson, J., Sengupta, J., Gursky, R., Nissen, P., & Frank, J. (2007). Comparison of fungal 80 S ribosomes by cryo-EM reveals diversity in structure and conformation of rRNA expansion segments. *Journal of Molecular Biology*, *369*(2), 429–438.

Noller, H. F., & Chaires, J. B. (1972). Functional modification of 16S ribosomal RNA by kethoxal. *Proceedings of the National Academy of Sciences of the United States of America*, *69*(11), 3115–3118.

Ofengand, J., Liou, R., Kohut, J., 3rd, Schwartz, I., & Zimmermann, R. A. (1979). Covalent cross-linking of transfer ribonucleic acid to the ribosomal P site. Mechanism and site of reaction in transfer ribonucleic acid. *Biochemistry*, *18*(20), 4322–4332.

Ohmayer, U., Gil-Hernández, Á., Sauert, M., Martín-Marcos, P., Tamame, M., Tschochner, H., Griesenbeck, J., & Milkereit, P. (2015). Studies on the coordination of ribosomal protein assembly events involved in processing and stabilization of yeast early large ribosomal subunit precursors. *PLoS One*, *10*(12), e0143768.

Palade, G. E. (1955). A small particulate component of the cytoplasm. *The Journal of Biophysical and Biochemical Cytology*, *1*(1), 59–68.

Pánek, J., Kolár, M., Vohradský, J., & Shivaya Valásek, L. (2013). An evolutionary conserved pattern of 18S rRNA sequence complementarity to mRNA 5′UTRs and its implications for eukaryotic gene translation regulation. *Nucleic Acids Research*, *41*(16), 7625–7634.

Panopoulos, P., & Mauro, V. P. (2008). Antisense masking reveals contributions of mRNA-rRNA base pairing to translation of Gtx and FGF2 mRNAs. *The Journal of Biological Chemistry*, *283*(48), 33087–33093.

Parker, M. S., Balasubramaniam, A., & Parker, S. L. (2018). The expansion segments of human 28S rRNA match MicroRNAs much above 18S rRNA or core segments. *MicroRNA (Shariqah, United Arab Emirates)*, *7*(2), 128–137.

Parker, M. S., Balasubramaniam, A., Sallee, F. R., & Parker, S. L. (2018). The expansion segments of 28S ribosomal RNA extensively match human messenger RNAs. *Frontiers in Genetics*, *9*, 66.

Parker, M. S., Sah, R., Balasubramaniam, A., Sallee, F. R., Park, E. A., & Parker, S. L. (2014). On the expansion of ribosomal proteins and RNAs in eukaryotes. *Amino Acids*, *46*(7), 1589–1604.

Parker, M. S., Sallee, F. R., Park, E. A., & Parker, S. L. (2015). Homoiterons and expansion in ribosomal RNAs. *FEBS Open Bio*, *5*, 864–876.

Parks, M. M., Kurylo, C. M., Dass, R. A., Bojmar, L., Lyden, D., Vincent, C. T., & Blanchard, S. C. (2018). Variant ribosomal RNA alleles are conserved and exhibit tissue-specific expression. *Science Advances*, *4*(2), eaao0665.

Pélandakis, M., Higgins, D. G., & Solignac, M. (1991). Molecular phylogeny of the subgenus sophophora of Drosophila derived from large subunit of ribosomal RNA sequences. *Genetica*, *84*(2), 87–94.

Penev, P. I., Fakhretaha-Aval, S., Patel, V. J., Cannone, J. J., Gutell, R. R., Petrov, A. S., Williams, L. D., & Glass, J. B. (2020). Supersized ribosomal RNA expansion segments in Asgard archaea. *Genome Biology and Evolution*, *12*(10), 1694–1710.

Petrov, A. S., Bernier, C. R., Hsiao, C., Norris, A. M., Kovacs, N. A., Waterbury, C. C., Stepanov, V. G., Harvey, S. C., Fox, G. E., Wartell, R. M., Hud, N. V., & Williams, L. D. (2014). Evolution of the ribosome at atomic resolution. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(28), 10251–10256.

Petrov, A. S., Gulen, B., Norris, A. M., Kovacs, N. A., Bernier, C. R., Lanier, K. A., Fox, G. E., Harvey, S. C., Wartell, R. M., Hud, N. V., & Williams, L. D. (2015). History of the ribosome and the origin of translation. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(50), 15396–15401.

Pfeffer, S., Brandt, F., Hrabe, T., Lang, S., Eibauer, M., Zimmermann, R., & Förster, F. (2012). Structure and 3D arrangement of endoplasmic reticulum membrane-associated ribosomes. *Structure*, *20*(9), 1508–1518.

Quade, N., Boehringer, D., Leibundgut, M., van den Heuvel, J., & Ban, N. (2015). Cryo-EM structure of hepatitis C virus IRES bound to the human ribosome at 3.9-Å resolution. *Nature Communications*, *6*, 7646.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(Database issue), D590–D596.

Ramesh, M., & Woolford, J. L., Jr. (2016). Eukaryote-specific rRNA expansion segments function in ribosome biogenesis. *RNA*, *22*(8), 1153–1162.

Ruiz Linares, A., Hancock, J. M., & Dover, G. A. (1991). Secondary structure constraints on the evolution of Drosophila 28 S ribosomal RNA expansion segments. *Journal of Molecular Biology*, *219*(3), 381–390.

Rundlet, E. J., Holm, M., Schacherl, M., Natchiar, S. K., Altman, R. B., Spahn, C. M. T., Myasnikov, A. G., & Blanchard, S. C. (2021). Structural basis of early translocation events on the ribosome. *Nature*, *595*(7869), 741–745.

Schibler, U., Wyler, T., & Hagenbüchle, O. (1975). Changes in size and secondary structure of the ribosomal transcription unit during vertebrate evolution. *Journal of Molecular Biology*, *94*(3), 503–517.

Schluenzen, F., Tocilj, A., Zarivach, R., Harms, J., Gluehmann, M., Janell, D., Bashan, A., Bartels, H., Agmon, I., Franceschi, F., Yonath, A. (2000). Structure of functionally activated small ribosomal subunit at 3.3 Å resolution. *Cell*, *102*(5), 615–623. https://doi.org/10.1016/s0092-8674(00)00084-2

Schwanhäusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., & Selbach, M. (2011). Global quantification of mammalian gene expression control. *Nature*, *473*(7347), 337–342.

Schwarz, Z., & Kössel, H. (1979). Sequencing of the 3′-terminal region of a 16S rRNA gene from Zea mays chloroplast reveals homology with *E. coli* 16S rRNA. *Nature*, *279*(5713), 520–522.

Schwarz, Z., & Kössel, H. (1980). The primary structure of 16S rDNA from Zea mays chloroplast is homologous to *E. coli* 16S rRNA. *Nature*, *283*(5749), 739–742.

Shankar, V., Rauscher, R., Reuther, J., Gharib, W. H., Koch, M., & Polacek, N. (2020). rRNA expansion segment 27Lb modulates the factor recruitment capacity of the yeast ribosome and shapes the proteome. *Nucleic Acids Research*, *48*(6), 3244–3256.

Shine, J., & Dalgarno, L. (1974a). The 3′-terminal sequence of Escherichia coli 16S ribosomal RNA: Complementarity to nonsense triplets and ribosome binding sites. *Proceedings of the National Academy of Sciences of the United States of America*, *71*(4), 1342–1346.

Shine, J., & Dalgarno, L. (1974b). Identical 3′-terminal octanucleotide sequence in 18S ribosomal ribonucleic acid from different eukaryotes. A proposed role for this sequence in the recognition of terminator codons. *Biochemical Journal*, *141*(3), 609–615.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D., & Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal omega. *Molecular Systems Biology*, *7*, 539.

Steitz, J. A., & Jakes, K. (1975). How ribosomes select initiator regions in mRNA: Base pair formation between the 3′ terminus of 16S rRNA and the mRNA during initiation of protein synthesis in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*, *72*(12), 4734–4738.

Stelter, P., Huber, F. M., Kunze, R., Flemming, D., Hoelz, A., & Hurt, E. (2015). Coordinated ribosomal L4 protein assembly into the pre-ribosome is regulated by its eukaryote-specific extension. *Molecular Cell*, *58*(5), 854–862.

Stepanov, V. G., & Fox, G. E. (2021). Expansion segments in bacterial and archaeal 5S ribosomal RNAs. *RNA*, *27*(2), 133–150.

Stoltzfus, A. (1999). On the possibility of constructive neutral evolution. *Journal of Molecular Evolution*, *49*(2), 169–181.

Strunk, B. S., & Karbstein, K. (2009). Powering through ribosome assembly. *RNA*, *15*(12), 2083–2104.

Subbotin, S. A., Sturhan, D., Vovlas, N., Castillo, P., Tambe, J. T., Moens, M., & Baldwin, J. G. (2007). Application of the secondary structure model of rRNA for phylogeny: D2-D3 expansion segments of the LSU gene of plant-parasitic nematodes from the family Hoplolaimidae Filipjev, 1934. *Molecular Phylogenetics and Evolution*, *43*(3), 881–890.

Sweeney, R., Chen, L., & Yao, M. C. (1994). An rRNA variable region has an evolutionarily conserved essential role despite sequence divergence. *Molecular and Cellular Biology*, *14*(6), 4203–4215.

Tirumalai, M. R., Kaelber, J. T., Park, D. R., Tran, Q., & Fox, G. E. (2020). Cryo- electron microscopy visualization of a large insertion in the 5S ribosomal RNA of the extremely halophilic archaeon Halococcus morrhuae. *FEBS Open Bio*, *10*(10), 1938–1946.

Tomal, A., Kwasniak-Owczarek, M., & Janska, H. (2019). An update on mitochondrial ribosome biology: The plant mitoribosome in the spotlight. *Cell*, *8*(12), 1562.

Tranque, P., Hu, M. C., Edelman, G. M., & Mauro, V. P. (1998). rRNA complementarity within mRNAs: A possible basis for mRNA-ribosome interactions and translational control. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(21), 12238–12243.

Tseng, H., Chou, W., Wang, J., Zhang, X., Zhang, S., & Schultz, R. M. (2008). Mouse ribosomal RNA genes contain multiple differentially regulated variants. *PLoS One*, *3*(3), e1843.

van der Sluis, E. O., Bauerschmitt, H., Becker, T., Mielke, T., Frauenfeld, J., Berninghausen, O., Neupert, W., Herrmann, J. M., & Beckmann, R. (2015). Parallel structural evolution of mitochondrial ribosomes and OXPHOS complexes. *Genome Biology and Evolution*, *7*(5), 1235–1251.

Veldman, G. M., Klootwijk, J., de Regt, V. C., Planta, R. J., Branlant, C., Krol, A., & Ebel, J. P. (1981). The primary and secondary structure of yeast 26S rRNA. *Nucleic Acids Research*, *9*(24), 6935–6952.

Wakeman, J. A., & Maden, B. E. (1989). 28 S ribosomal RNA in vertebrates. Locations of large-scale features revealed by electron microscopy in relation to other features of the sequences. *Biochemical Journal*, *258*(1), 49–56.

Waltz, F., Nguyen, T.-T., Arrivé, M., Bochler, A., Chicher, J., Hammann, P., Kuhn, L., Quadrado, M., Mireau, H., Hashem, Y., & Giegé, P. (2019). Small is big in Arabidopsis mitochondrial ribosome. *Nature Plants*, *5*(1), 106–117.

Wang, X., Yue, Z., Xu, F., Wang, S., Hu, X., Dai, J., & Zhao, G. (2021). Coevolution of ribosomal RNA expansion segment 7L and assembly factor Noc2p specializes the ribosome biogenesis pathway between *Saccharomyces cerevisiae* and *Candida albicans*. *Nucleic Acids Research*, *49*(8), 4655–4667.

Ware, V. C., Tague, B. W., Clark, C. G., Gourse, R. L., Brand, R. C., & Gerbi, S. A. (1983). Sequence analysis of 28S ribosomal DNA from the amphibian *Xenopus laevis*. *Nucleic Acids Research*, *11*(22), 7795–7817.

Warner, J. R. (1999). The economics of ribosome biosynthesis in yeast. *Trends in Biochemical Sciences*, *24*(11), 437–440.

Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M., & Barton, G. J. (2009). Jalview version 2: a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, *25*(9), 1189–1191.

Wegrecki, M., Rodríguez-Galán, O., de la Cruz, J., & Bravo, J. (2015). The structure of Erb1-Ytm1 complex reveals the functional importance of a high-affinity binding between two β-propellers during the assembly of large ribosomal subunits in eukaryotes. *Nucleic Acids Research*, *43*(22), 11017–11030.

Wellauer, P. K., Dawid, I. B., Kelley, D. E., & Perry, R. P. (1974). Secondary structure maps of ribosomal RNA: II. Processing of mouse L-cell ribosomal RNA and variations in the processing pathway. *Journal of Molecular Biology*, *89*(2), 397–407.

Wild, K., Aleksić, M., Lapouge, K., Juaire, K. D., Flemming, D., Pfeffer, S., & Sinning, I. (2020). MetAP-like Ebp1 occupies the human ribosomal tunnel exit and recruits flexible rRNA expansion segments. *Nature Communications*, *11*(1), 776.

Williams, T. A., Cox, C. J., Foster, P. G., Szöllősi, G. J., & Martin Embley, T. (2020). Phylogenomics provides robust support for a two-domains tree of life. *Nature Ecology & Evolution*, *4*(1), 138–147. https://doi.org/10.1038/s41559-019-1040-x

Wu, S., Tutuncuoglu, B., Yan, K., Brown, H., Zhang, Y., Tan, D., Gamalinda, M., Yuan, Y., Li, Z., Jakovljevic, J., Ma, C., Lei, J., Dong, M.-Q., Woolford, J. L., Jr., & Gao, N. (2016). Diverse roles of assembly factors revealed by structures of late nuclear pre-60S ribosomes. *Nature*, *534*(7605), 133–137.

Xue, S., & Barna, M. (2012). Specialized ribosomes: A new frontier in gene regulation and organismal biology. *Nature Reviews. Molecular Cell Biology*, *13*(6), 355–369.

Yamamoto, H., Collier, M., Loerke, J., Ismer, J., Schmidt, A., Hilal, T., Sprink, T., Yamamoto, K., Mielke, T., Bürger, J., Shaikh, T. R., Dabrowski, M., Hildebrand, P. W., Scheerer, P., & Spahn, C. M. (2015). Molecular architecture of the ribosome-bound hepatitis C virus internal ribosomal entry site RNA. *The EMBO Journal*, *34*(24), 3042–3058.

Yang, L., Güell, M., Niu, D., George, H., Lesha, E., Grishin, D., Aach, J., Shrock, E., Xu, W., Poci, J., Cortazio, R., Wilkinson, R. A., Fishman, J. A., & Church, G. (2015). Genome- wide inactivation of porcine endogenous retroviruses (PERVs). *Science*, *350*(6264), 1101–1104.

Yokoyama, T., & Suzuki, T. (2008). Ribosomal RNAs are tolerant toward genetic insertions: Evolutionary origin of the expansion segments. *Nucleic Acids Research*, *36*(11), 3539–3551.

Zaug, A. J., Grabowski, P. J., & Cech, T. R. (1983). Autocatalytic cyclization of an excised intervening sequence RNA is a cleavage–ligation reaction. *Nature*, *301*(5901), 578–583.

Zhou, D., Zhu, X., Zheng, S., Tan, D., Dong, M.-Q., & Ye, K. (2019). Cryo-EM structure of an early precursor of large ribosomal subunit reveals a half-assembled intermediate. *Protein & Cell*, *10*(2), 120–130.

How to cite this article: Hariharan, N., Ghosh, S., & Palakodeti, D. (2022). The story of rRNA expansion segments: Finding functionality amidst diversity. *WIREs RNA*, e1732. https://doi.org/10.1002/wrna.1732