Functions of Ribosomal Proteins in Assembly of Eukaryotic Ribosomes In Vivo*

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Abstract

The proteome of cells is synthesized by ribosomes, complex ribonucleoproteins that in eukaryotes contain 79–80 proteins and four ribosomal RNAs (rRNAs) more than 5,400 nucleotides long. How these molecules assemble together and how their assembly is regulated in concert with the growth and proliferation of cells remain important unanswered questions. Here, we review recently emerging principles to understand how eukaryotic ribosomal proteins drive ribosome assembly in vivo. Most ribosomal proteins assemble with rRNA cotranscriptionally; their association with nascent particles is strengthened as assembly proceeds. Each subunit is assembled hierarchically by sequential stabilization of their subdomains. The active sites of both subunits are constructed last, perhaps to prevent premature engagement of immature ribosomes with active subunits. Late-assembly intermediates undergo quality-control checks for proper function. Mutations in ribosomal proteins that affect mostly late steps lead to ribosomopathies, diseases that include a spectrum of cell type–specific disorders that often transition from hypoproliferative to hyperproliferative growth.

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INTRODUCTION

Ribosomes are complex ribonucleoprotein (RNP) machines that catalyze protein synthesis in all cells. Ribosomes consist of two subunits; the large subunit (LSU) is about twice the size of the small subunit (SSU). The SSU functions as the decoding center to bring together messenger RNAs (mRNAs) and aminoacyl–transfer RNAs (tRNAs) to translate the genetic code. Coordinated conformational changes within the SSU also allow for translocation of the tRNA/mRNA pair through the ribosome. The eukaryotic SSU contains an 18S ribosomal RNA (rRNA) (1,800 nucleotides in yeast) and 33 different ribosomal proteins (r-proteins), which are organized into three distinct structural subdomains: the body, which contains the 5′ domain of 18S rRNA; the platform, which contains the central domain; and the head, which contains the 3′ major domain (**Figure 1*a*,*b***). The eukaryotic LSU, which houses the peptidyltransferase center (PTC) that catalyzes peptide bond formation, contains 5S rRNA (121 nucleotides in yeast), 5.8S rRNA (158 nucleotides in yeast), 25S–28S rRNA (25S rRNA; 3,396 nucleotides in yeast), and 46 (in yeast) or 47 (in human) r-proteins. The 25S rRNA is composed of six different rRNA domains, which are more intertwined with each other than are domains in the SSU. Thus, the LSU was originally described as largely monolithic, with only a few notable structural features, such as the central protuberance (CP) and the L1 and acidic stalks (**Figure 1*c*,*d***). However, we have now learned that the LSU is indeed partitioned into neighborhoods that assemble sequentially (see the section titled Assembly of 60S Ribosomal Subunits, below). These correspond at least in part to the previously defined secondary-structure domains.

How these rRNAs and r-proteins associate with each other to form functional ribosomes has been a challenging and important problem investigated almost since the discovery of ribosomes (1, 2). We now know that in eukaryotes ribosome biogenesis begins with the transcription of two precursor rRNAs (pre-rRNAs) in the nucleolus—one for 5S rRNA and another for 18S, 5.8S, and 25S rRNAs—and the synthesis in the cytoplasm of all r-proteins and *trans*-acting factors that assist ribosome biogenesis. Upon import of these proteins into the nucleus, the pre-rRNAs undergo complex interconnected pathways of modification, folding, binding to r-proteins, and

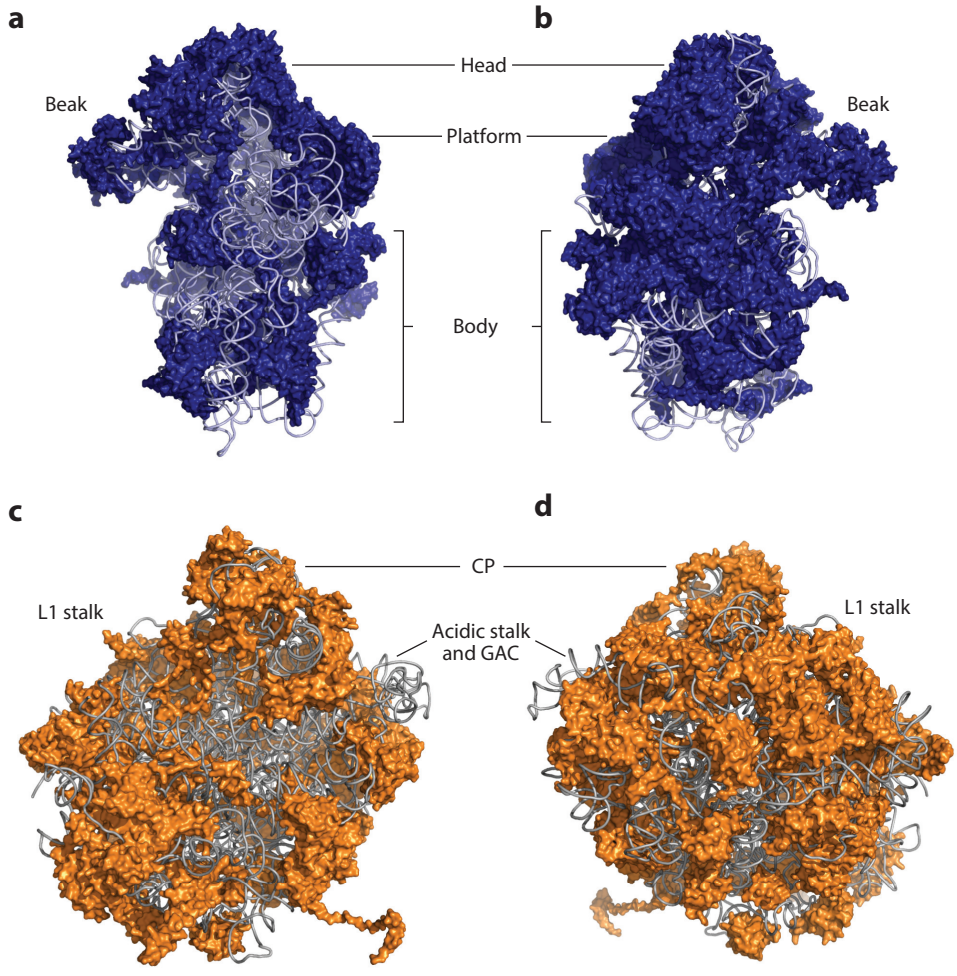


Figure 1

Crystal structure of (*a*,*b*) the small subunit (SSU) and (*c*,*d*) the large subunit (LSU) from *Saccharomyces cerevisiae* at 3.0-Å resolution. (*a*,*c*) The subunit interface of the SSU and LSU, respectively. (*b*,*d*) The solvent-exposed surface of the SSU and LSU, respectively. Abbreviations: CP, central protuberance. GAC, GTPase-activation center. The crystal structure is adapted from Protein Data Bank codes 3U5B, 3U5C, 3U5D, and 3U5E.

removal of spacer sequences. Assembly continues upon release of preribosomal particles from the nucleolus to the nucleoplasm and is completed upon export to the cytoplasm (**Figure 2**). To meet a growing and dividing cell's high demand for ribosome production, assembly must occur efficiently and with high fidelity. In yeast, more than 200 assembly factors (3, 4) and 77 small nucleolar RNAs (snoRNAs) (5–7) associate transiently with nascent ribosomes to facilitate these processes. More than 500 assembly factors and 300 snoRNAs have been found in higher eukaryotes (5, 6). Assembly of properly functioning subunits is enabled by quality-control mechanisms that identify and eliminate improperly constructed particles that might subvert the pool of active subunits (reviewed in Reference 8). Thus, the ribosome has become a well-studied model to understand assembly and function of the many different RNPs found in cells.

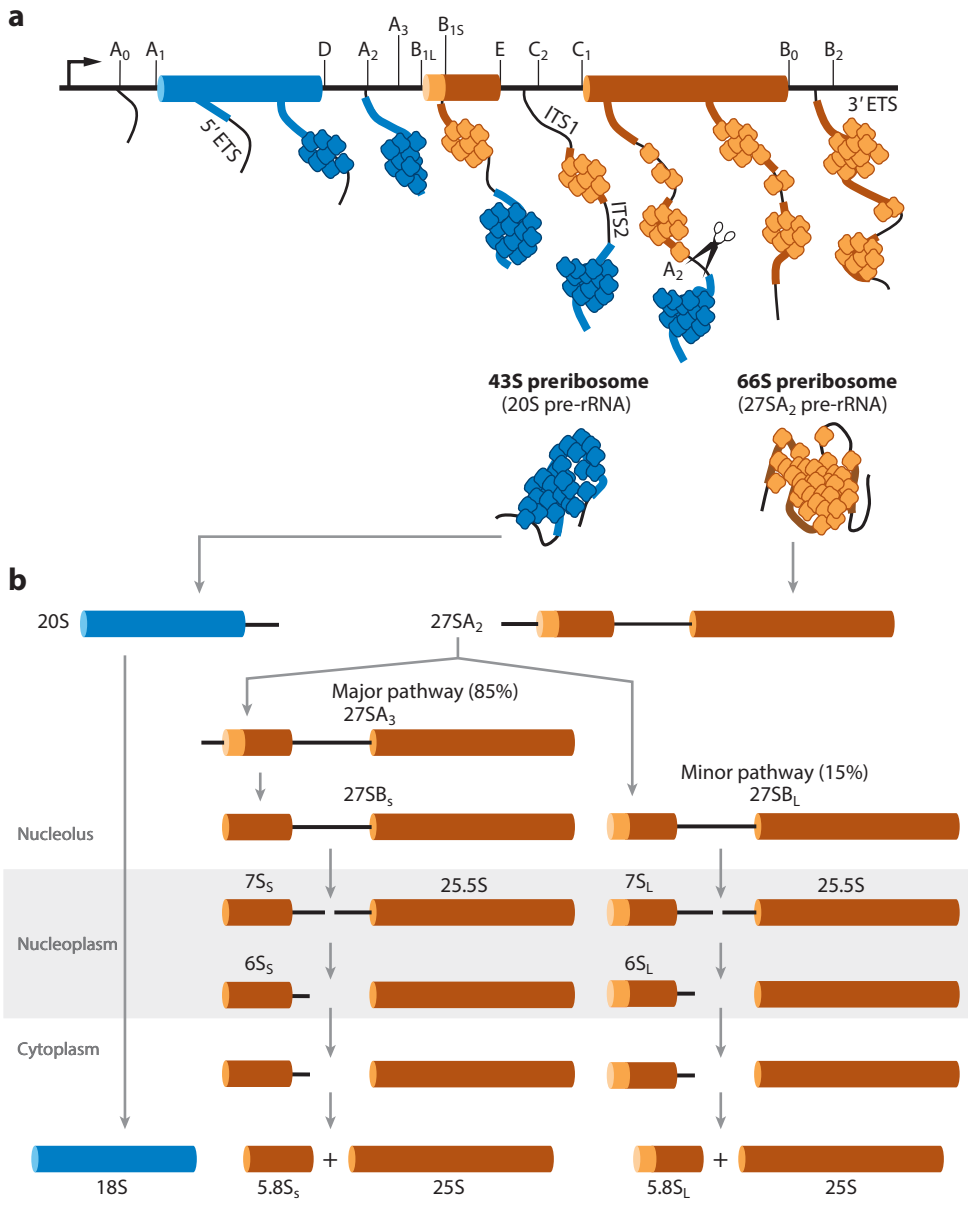


Figure 2

Cotranscriptional precursor rRNA (pre-rRNA) processing in *Saccharomyces cerevisiae*. (*a*) As RNA polymerase I transcribes a ribosomal DNA (rDNA) repeat, nascent pre-rRNAs are cleaved cotranscriptionally at the A_2 site, releasing a 43S preribosome containing 20S pre-rRNA. The 66S preribosome containing $27SA_2$ pre-rRNA is released upon completion of transcription. The pre-rRNA processing sites are indicated along the rDNA gene, and the external and internal transcribed spacer sequences are indicated on the nascent transcript. (*b*) The pre-rRNAs then undergo a series of exo- and endonucleolytic cleavages to remove the spacer sequences, finally liberating mature 18S, 5.8S, and 25S rRNAs. Not shown is the flanking 5S gene, transcribed in the opposite direction.

Understanding ribosome biogenesis is important for human health. Because ribosome function is closely tied to the proper growth and proliferation of cells, dysregulation of ribosome biogenesis has profound consequences (9). Loss-of-function mutations in most r-proteins or assembly factors are lethal in model organisms and presumably embryonic lethal when homozygous in humans (e.g., 10–13; also see **http://omim.org/**). The more likely occurrence of partial loss-of-function mutations leads to so-called ribosomopathies in humans. These diseases include a wide variety of cell type–specific hypo- or hyperproliferative disorders, which manifest as developmental defects, diverse hematological dysfunctions, or cancers (9, 14, 15). Thus, basic research on ribosome biogenesis is necessary to dissect the molecular mechanisms underlying these pathologies and to identify therapeutic strategies for their treatment.

Structural and functional analyses have revealed that, despite the presence of so many r-proteins, the ribosome is a ribozyme (16). The immediate environment of the PTC is devoid of r-proteins and functions as an RNA-based catalyst to promote peptide bond formation. What, then, are the roles of the r-proteins? In the 1970s, pioneering work by Mizushima & Nomura (17) and Nierhaus & Dohme (18) to reconstitute bacterial ribosomal subunits (r-subunits) in vitro enabled investigations of how r-proteins participate in the assembly of ribosomes. At the same time, bacterial genetic experiments identified mutations in r-proteins that conferred resistance to antibiotics that block specific steps of protein synthesis, suggesting that r-proteins have roles in ribosome function (e.g., 19). However, these antibiotics typically bind rRNA, not r-proteins, indicating that r-proteins play more indirect roles in ribosome function, mediated by their cooperative interactions with rRNA (for a review, see Reference 20).

Initial investigations of assembly and function of eukaryotic ribosomes focused mostly on the processing of pre-rRNAs and on the discovery and functional characterization of *trans*-acting assembly factors (for reviews, see References 3 and 21–24), with less emphasis on the analysis of r-proteins. In contrast, the last 10 years have witnessed a significant increase in efforts to systematically investigate the roles of r-proteins in ribosome assembly in yeast and cultured mammalian cells (e.g., 25–30). This progress has been enabled by more powerful tools to analyze how r-proteins work together with assembly factors to drive r-subunit biogenesis, including better methods to inspect pre-rRNA folding and pre-RNP structure (e.g., 31–37). Here, we review this more recent research to understand the roles of r-proteins in assembly of eukaryotic ribosomes, focusing primarily on yeast, in which subunit biogenesis has been most extensively studied. Note that the lessons learned from yeast appear to be broadly applicable to the study of human ribosomes, although some of the details differ.

WHAT MIGHT THE STRUCTURE OF THE RIBOSOME TELL US ABOUT R-PROTEIN FUNCTION?

High-resolution X-ray and cryo–electron microscopy (cryo-EM) structures of ribosomes from eukaryotes such as yeast, *Drosophila*, *Tetrahymena*, plants, and mammals have been invaluable in developing more detailed models for the roles of r-proteins in ribosome structure and function and, with appropriate caveats, ribosome assembly (e.g., 38–44; reviewed in Reference 45). These structures, as well as those of bacterial and archaeal ribosomes (e.g., 46–49), have revealed that each subunit contains a core of rRNA with globular domains of r-proteins bound at or partially buried below the surface (**Figure 1**). Perhaps reflecting more diverse regulation of translation in eukaryotes, their ribosomes are larger and more complex than those of prokaryotes, although the common core is highly conserved in all forms of life (45, 50, 51). There are both eukaryote-specific r-proteins not found in prokaryotes (**Table 1**) and many more r-proteins in eukaryotes that contain specific tails

extending from their globular domains, typically predicted to be intrinsically disordered (e.g., 38). Several of these extensions protrude deep into the rRNA. However, most of them thread across the surface of the subunits and contact multiple domains of rRNA, suggesting that they play a pivotal role in bringing and/or keeping rRNA domains together. In addition to the eukaryote-specific r-proteins and r-protein extensions, there are extra sequences embedded in eukaryotic rRNA, known as expansion segments (ES). The ES are clustered in several neighborhoods of both the SSU and the LSU, and many contact eukaryote-specific r-protein extensions, suggesting that they may have coevolved (38). Although the structure of each subunit must be established by interactions among the r-proteins and the rRNAs, the importance of such networks of interactions with r-proteins has been tested in only a few cases, which focused more on effects on ribosome function than on assembly (e.g., 52–54). In developing models for the roles of r-proteins in ribosome assembly, the known locations of r-proteins within mature subunits have provided powerful platforms that are guiding our thinking. Although one assumes that the locations of most r-proteins are similar in assembling particles, one keeps in mind examples of the differences discussed in the next sections (32, 55–57).

LESSONS LEARNED FROM STUDYING IN VITRO RECONSTITUTION OF BACTERIAL RIBOSOMES

Following the initial experiments to reconstitute r-subunits in vitro under equilibrium conditions (reviewed in Reference 2), investigators studied the kinetics of rRNA folding and r-protein–rRNA interactions with sophisticated biophysical approaches (58–63). Although most of these more recent experiments focused on in vitro reconstitution of the SSU, the following principles that emerged guide our thinking about assembly of both subunits in vivo.

1. In the absence of r-proteins, and in the presence of high Mg^{2+} concentrations, rRNAs rapidly fold into secondary and tertiary structures resembling those found in mature subunits in vivo. However, there are multiple different rRNA folding pathways, some of which form kinetically trapped or unstable structures.
2. Binding of r-proteins to rRNA helps to overcome these problems by guiding the proper folding of rRNA and by stabilizing productive conformers.
3. In vitro, each rRNA secondary-structure domain of the SSU can fold and bind r-proteins independently of other domains, suggesting that multiple different folding and assembly pathways can be followed.
4. Binding of individual r-proteins to rRNA occurs in stages. The molecules first form labile encounter complexes, followed by generation of one or more intermediates, until the native complex is formed. Thus, initial interactions are weak and then strengthened as assembly proceeds. During these transitions, both the r-proteins and the rRNA can undergo structural changes—a mutually induced fit mechanism.
5. Association of r-proteins with rRNA has both local and long-range effects on rRNA folding and RNP formation.
6. These conformational changes create hierarchical and cooperative assembly pathways. Association of early, "primary binding" r-proteins organizes binding sites for subsequent assembly of secondary, then tertiary r-proteins. In addition, primary binders tend to associate with the 5′ domain of 16S rRNA and tertiary binders with its 3′ domain.

Together, these principles reveal that, although largely hierarchical, assembly occurs through multiple parallel and alternative pathways, and that assembly tends to occur in a 5′-to-3′ direction with respect to rRNA.

Table 1 Ribosomal proteins (r-proteins) from the eukaryotic ribosome

Yeast r-protein name[a] (☺ 😐 ☹)	Human r-protein name[b] (disease)	Bacterial r-protein name	Main rRNA contacts[c]	r-Protein contacts	Timing of stable assembly[d]	Most prominent assembly defects upon depletion (yeast)	References for timing and/or defects
Small subunit							
S0A (YGR214W) S0B (YLR048W)	SA (CA)	S2	Head: H35 Platform: H26	S2, S17, S21	Medium nucle(ol)ar	Delay of A_2 No 18S rRNA	27, 28, 171, 167
S1A (YLR441C) S1B (YML063W)	S3A	NA	Platform: H23	S14, S26	Early nucle(ol)ar	Inhibition of A_1–A_2 No 20S pre-rRNA No export	27, 28
S2 (YGL123w)	S2	S5	Body: H1, H2 Head: H35, H36	S0, S9, S21, S22, S3, S30	Medium nucle(ol)ar	Delay of A_2 No 18S rRNA	27, 28
S3 (YNL178W)	S3	S3	Head: H34, H38, H41,	S10, S20, S29, S17, Asc1, S2	Late nucle(ol)ar, cytoplasmic positioning	Delay of A_2 No 18S rRNA	27, 28, 32, 57
S4A (YJR145C) S4B (YHR203C)	S4	NA	Body: H7, H9, H15	S9, S24, S6	No data	Unknown	172
S5 (YJR123W)	S5	S7	Head: H29, J43/28, H40, H41a	S16, S25, S28	Medium nucle(ol)ar	Delay of A_0–A_2 No 18S rRNA No export	27, 28
S6A (YPL090C) S6B (YBR181C)	S6	NA	Body: H6, H7, H8, ES3B, H10	S4	Early nucle(ol)ar	Inhibition of A_1–A_2 No 20S pre-rRNA No export	27, 28
☹ S7A (YOR096W) S7B (YNL096C)	S7 Φ (DBA)	NA	Platform: ES6A, ES6E	S13, S22, S27	Late nucle(ol)ar	Delay of A_1–A_2 18S rRNA formation	27
S8A (YBL072C) S8B (YER102W)	S8	NA	Body: ES3A, H9, H11, H13, J6/7	S11	Early nucle(ol)ar	Inhibition of A_1–A_2 No 20S pre-rRNA No export	27
S9A (YPL081W) S9B (YBR189W)	S9	S4	Body: H3, H12, H17, ES6A, ES6D	S2, S4, S30, S24	Early nucle(ol)ar	Inhibition of A_1–A_2 No 20S pre-rRNA No export	27, 28
S10A (YOR293W) S10B (YMR230W)	S10 Φ (DBA)	NA	Head: H33 J32/33, H34	S3, S12	Cytoplasmic	Delay of A_1–A_2 No 18S rRNA	27, 28, 57, 97
S11A (YDR025W) S11B (YBR048W)	S11	S17	Body: H9, H11, H20, ES6C	S8, S23, S4, S13, S22	Early nucle(ol)ar	Inhibition of A_1–A_2 No 20S pre-rRNA No export	27, 28

(*Continued*)

Table 1 (*Continued*)

Yeast r-protein name[a] (☺ 😐 ☹)	Human r-protein name[b] (disease)	Bacterial r-protein name	Main rRNA contacts[c]	r-Protein contacts	Timing of stable assembly[d]	Most prominent assembly defects upon depletion (yeast)	References for timing and/or defects
☹ S12 (YOR369C)	S12	NA	Head: H33	S10, S31	Unknown	Unknown	None
S13 (YDR064W)	S13	S15	Platform: H20, H22	S7, S27	Early nucle(ol)ar	Inhibition of A_0–A_2 No 20S pre-rRNA No export	27, 28
S14A (YCR031C) S14B (YJL191W)	S14 (5q−) Φ	S11	Platform: H23, H24, H45	S1, S26	Early nucle(ol)ar, cytoplasmic positioning	Inhibition of A_0–A_2 No 20S pre-rRNA No export	27, 28, 97, 166
S15 (YOL040C)	S15 (DBA) Φ	S19	Head: H33, H42, J30/31, J32/30	S18	Late nucle(ol)ar	Delay of A_0–A_2 No 18S rRNA	27, 28
S16A (YMR143W) S16B (YDL083C)	S16	S9	Head: H39, ES9, H40, H41a, H43	S19, Asc1	Medium nucle(ol)ar	Inhibition of A_0–A_2 No 20S pre-rRNA No export	27, 28
S17A (YML024W) S17B (YDR447C)	S17 (DBA) Φ	NA	Head: H37, H38, H40	S0, S3, Asc1	Late nucle(ol)ar	Unknown	28
S18A (YDR450W) S18B (YML026C)	S18	S13	Head: H41a, H42	S15, S19, S25	Late nucle(ol)ar	Delay of A_2 No 18S rRNA No export	27
S19A (YOL121C) S19B (YNL302C)	S19 (DBA) Φ	NA	Head: ES9, H41	S16, S18	Late nucle(ol)ar	Delay of A_2 No 18S rRNA No export	27, 28
S20 (YHL015W)	S20	S10	Head: H31, H34, H39, H41	S3, S29	Late nucle(ol)ar/ cytoplasmic	Delay of A_2 No 18S rRNA	27, 28, 57
S21A (YKR057W) S21B (YJL136C)	S21	NA	Platform: ES6A, H27	S0, S2, S22	Medium nucle(ol)ar	Delay of A_2 No 18S rRNA	167
S22A (YJL190C) S22B(YLR367W)	S15A	S8	Body: H12 Platform: ES6A, ES6E, H25	S7, S21, S23, S27, S11	No data	No data	None
S23A (YGR118W) S23B (YPR132W)	S23	S12	Body: H3, H5, H18Platform: H19, H44	S11, S22, S30	Early nucle(ol)ar	Inhibition of A_1–A_2 No 20S pre-rRNA No export	27
S24A (YER047W) S24B (YIL069C)	S24 (DBA) Φ	NA	Body: H6, H8, H15, H17, Platform: ES6D	S4, S9	Early nucle(ol)ar	Inhibition of A_1–A_2 No 20S pre-rRNA No export	27, 28

😐 S25A (YGR027C) S25B (YLR333C)	S25	NA	Head: H41a, J41/42, J42/29	None	No data	No data	None
S26A (YGL189C) S26B (YER131W)	S26 Φ (DBA)	NA	Head: H26, H27, H28 Platform: J23/22, H45, 3′ end	S1	Cytoplasmic	Delay of A_1–A_2 No 18S rRNA	27, 28, 57, 97
S27A (YKL156W) S27B (YHR021C)	S27	NA	Platform: H22, H26	S7, S13, S22	Early nucle(ol)ar	Inhibition of A_0–A_2 No 20S pre-rRNA No export	27, 28
S28A (YOR267C) S28B (YLR264W)	S28	NA	Head: H29, H43	S5	Medium nucle(ol)ar	Delay of A_1–A_2 No 18S rRNA	27, 28
S29A (YLR388W) S29B (YDL061C)	S29	S14	Head: H31, H32, J31/32, H34	S3, S20	Late nucle(ol)ar/ cytoplasmic	Unknown	28, 57
S30A (YLR287C-A) S30B (YOR182C)	S30	NA	Body: H16, H17, H18	S9, S23	Late nucle(ol)ar	Delay of A_0–A_2 18S rRNA formation	27
S31 (YLR167W)	S27A Φ (DBA)	NA	Head: H33 Long tail contacts: H32, H34	S12	Late nucle(ol)ar/ cytoplasmic	Delay of A_1–A_2 18S rRNA formation	27, 28, 79
😐 Asc1 (YMR116C)	RACK1	NA	Head: H39, H40	S3, S16, S17	Cytoplasmic	Unknown	57, 97
Large subunit							
L1A (YPL220W) L1B (YGL135W)	L10A	L1	Not in structure	None	Early nucle(ol)ar	Slight delay of C_2 25S/5.8S rRNA formation	25, 29, 168
L2A (YFR031C-A) L2B (YIL018W)	L8	L2	II: H33, H34, H35a III: ES19, H55/56J IV: H66, H67, H68 V: H74, H75, ES31b, H93	L8, L43	Late nucle(ol)ar	Mild delay of C_2 Strong delay of E No export	25, 29, 104
L3 (YOR063W)	L3	L3	II: H35, H41/42J; IV: H61, H62 V: H72, H73, H90, H92, H94, ES39d H95/96J, H99, H100, H101	L16, L23, L24	Early nucle(ol)ar	Inhibition of A_3, B_{1S}, and C_2 No 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104, 112

(*Continued*)

Table 1 *(Continued)*

Yeast r-protein name[a] (☺ 😐 ☹)	Human r-protein name[b] (disease)	Bacterial r-protein name	Main rRNA contacts[c]	r-Protein contacts	Timing of stable assembly[d]	Most prominent assembly defects upon depletion (yeast)	References for timing and/or defects
L4A (YBR031W) L4B (YDR012W)	L4	L4	I: H4, H19/20J, H3/23J, H23, ES7b, ES7c II: H26, H27, H28, H32, H45, H46, H47 V: H73/74J, H74 5.8S: H3, H4	L7, L13, L15, L18, L26, L37	Early nucle(ol)ar	Inhibition of A_3, B_{1S}, and C_2 No 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104, 169
L5 (YPL131W)	L5 Φ (DBA)	L18	II: H38, ES12 V: H83, H84/85J, H85, H87 5S: H2, H3	L10, L11, L21	Early nucle(ol)ar, late positioning	Mild delay of C_2 Strong of delay E No 25S/5.8S rRNA No 5S rRNA No export	25, 29, 33, 56, 104, 173
L6A (YML073C) L6B (YLR448W)	L6	NA	II: ES27b, ES27c, H45, H46 VI: ES39	L14, L32, L33	Early nucle(ol)ar	Inhibition of A_3, B_{1S}, and C_2	25, 104
L7A (YGL076C) L7B (YPL198W)	L7	L30	I: ES7b, ES7c II: H38, ES12, H39, H40, H41, H45 5S: H4	L4, L18, L20, L21	Early nucle(ol)ar	Inhibition of A_3, B_{1S}, and C_2 No 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 31, 104
L8A (YHL033C) L8B (YLL045C)	L7A	NA	I: ES4, H15 III: ES19 V: H76/79J, H79, ES31, ES31c 5.8S: ES4	L2, L15, L25, L27, L36	Early nucle(ol)ar	Inhibition of A_3, B_{1S}, and C_2 No 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 31, 104
L9A (YGL147C) L9B (YNL067W)	L9 Φ (DBA)	L6	II: H42 V: H91 VI: H97, ES39	L14, L16, L20, L40	Medium nucle(ol)ar	Mild delay of C_2 Few 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104
L10 (YLR075W)	L10 Φ (Autism)	L16	II: H38, H39, H42 V: H80, H81, H89 5S: H1/2/5J, H4	L5, L21	Cytoplasmic	Slight delay of C_2 25S/5.8S rRNA formation No export	25, 29, 72
L11A (YPR102C) L11B (YGR085C)	L11 Φ (DBA)	L5	V: H83, H84, H85 5S: H1, H2, H3	L5, L42	Early nucle(ol)ar, late positioning	Delay of C_2 and E No 5.8S rRNA No 5S rRNA	25, 33, 104, 174

☹ L12A (YEL054C) L12B (YDR418W)	L12	L11	Not in structure	P0	Late nucle(ol)ar	Slight delay of C_2 25S/5.8S rRNA formation	25
L13A (YDL082W) L13B (YMR142C)	L13	NA	I: H11, H12, H13, H14, H15 II: H27, H28, H29, ES9, H32, H37 V: H88 5.8S: H4	L4, L15, L18, L28, L35, L36	Early nucle(ol)ar	Slight delay of C_2 25S/5.8S rRNA formation No export	25, 29, 104
L14A (YKL006W) L14B (YHL001W)	L14	NA	I: ES7b II: H41 VI: ES39	L6, L9, L16, L20	Nucle(ol)ar	Unknown	25
L15A (YLR029C) L15B (YMR121C)	L15 (DBA) Φ	NA	I: ES4, H11, H12, H13, H15, H21, H22 II: H27, H28, H35a III: H52 IV: H66 V: H75, H76, H79 5.8S: ES4	L4, L8, L13, L35, L36, L42	Nucle(ol)ar	Unknown	25
L16A (YIL133C) L16B (YNL069C)	L13A	L13	I: H25 II: H41 V: H72, H73 VI: H94, H97, ES39d	L3, L9, L14, L20, L33	Early nucle(ol)ar	Inhibition of A_3, B_{1S}, and C_2 No 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104, 169
L17A (YKL180W) L17B (YJL177W)	L17	L22	I: H2, H24, ES7 II: H35, H26a III: H49a, H50 IV: H26a V: H73 VI: ES39, H99, H100 5.8S: H2	L33	Medium nucle(ol)ar	Mild delay of C_2 Few 7S pre-rRNA No 25S/5.8S rRNA No export	25, 26, 29, 104
L18A (YOL120C) L18B (YNL301C)	L18	NA	I: H13 II: H27, H29, H30, H31, H36, H38, H41, H45 V: H86, H88	L4, L7, L13, L28	Early nucle(ol)ar	Inhibition of A_3, B_{1S}, and C_2 No 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104

(*Continued*)

Table 1 *(Continued)*

Yeast r-protein name[a] (☺ 😐 ☹)	Human r-protein name[b] (disease)	Bacterial r-protein name	Main rRNA contacts[c]	r-Protein contacts	Timing of stable assembly[d]	Most prominent assembly defects upon depletion (yeast)	References for timing and/or defects
L19A (YBR084C-A) L19B (YBL027W)	L19	NA	II: H34 III: H47, H48, H50, H53, H56, H57, H58, H59, H60 IV H62, H63 VI: H96	None	Medium nucle(ol)ar	Strong delay of C_2 Few 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104
L20A (YMR242C) L20B (YOR312C)	L18A	NA	II: ES27b, H41, H42 VI: ES39 5S: H4, H45/J	L4, L7, L9, L14, L16, L21	Early nucle(ol)ar	Inhibition of A_3, B_{1S}, and C_2 No 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104
L21A (YBR191W) L21B (YPL079W)	L21	NA	II: H38, ES12 V: H81, H85, H86, H88 5S: H1	L5, L7, L10, L20, L29	Late nucle(ol)ar	Mild delay of C_2 Strong delay of E No export	25, 29, 104
😐 L22A (YLR061W) L22B (YFL034C-A)	L22	NA	III: H57, H59	None	Nucle(ol)ar	Not done	25
L23A (YBL087C) L23B (YER117W)	L23	L14	IV: H61, H71 V: H90, H92 VI: H95, H96	L3, L24	Medium nucle(ol)ar	Strong delay of C_2 Few 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104
☺ L24A (YGL031C) L24B (YGR148C)	L24	NA	IV: H63 VI: H96, ES41	L3, L23	Cytoplasmic	60S deficit	25, 175
L25 (YOL127W)	L23A	L23	I: ES4, H15 III: H50, H51, ES19, H53 V: H79 5.8S: ES3	L8, L35, L39	Medium nucle(ol)ar	Strong delay of C_2 Few 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104, 109
☺ L26A (YLR344W) L26B (YGR034W)	L26 Φ (DBA)	L24	I: H4, H19, H20, H24 5.8S; H2/4J, H7	L4, L37, L39	Medium nucle(ol)ar	Very slight delay of C_2 25S/5.8S rRNA formation Slight export defect	25, 104, 107
L27A (YHR010W) L27B (YDR471W)	L27	NA	III: ES20a, H55, H58 V: ES31c	L8, L30, L34	Medium nucleolar	Strong delay of C_2 Few 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104

L28 (YGL103W)	L27A	L15	I: H11, H12, H13, H21, H22 II: H25.1, H27, H29, H32, H36, H37, H38, H46 V: H86, H88	L13, L18, L32, L36	Late nucle(ol)ar	Mild delay of C_2 25S/5.8S rRNA formation No export	25, 29, 104
☺ L29 (YFR032C-A)	L29	NA	II: H30, H31, H37, ES12, H38, H39 V: H80, H81, H86	L21	Cytoplasmic	60S deficit	25, 129
L30 (YGL030W)	L30	NA	III: H58 V: ES31	L27, L34, L43	Nucle(ol)ar	Unknown	25, 176
☹ L31A (YDL075W) L31B (YLR406C)	L31	NA	III: H47 IV: H61 VI: H96, H101	None	Medium nucle(ol)ar	Slight delay of C_2 25S/5.8S rRNA formation	25, 104
L32 (YBL092W)	L32	NA	I: H25, ES7c II: H25.1/26J, H36, H40, H41, H36/45J, H46	L6, L28	Early nucle(ol)ar	Inhibition of A_3, B_{1S}, and C_2 Few 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104
L33A (YPL143W) L33B (YOR234C)	L35A Φ (DBA)	NA	I: H25, ES7b, ES7c II: H40, H41 VI: ES39	L6, L16, L17	Early nucle(ol)ar	Inhibition of A_3, B_{1S}, and C_2 No 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104, 110
L34A (YER056C-A) L34B (YIL052C)	L34	NA	II: H33, H49 III: H53, ES20, H51, H54, H55, H56, H57, H58 V: ES31	L27, L30	Medium nucle(ol)ar	Strong delay of C_2 Few 7S pre-rRNA No 25S/5.8S rRNA No export	25, 29, 104
L35A (YDL191W) L35B (YDL136W)	L35	L29	I: ES4, H15, H16, H175.8S: H4/5J, H6, H7	L13, L15, L25, L37	Medium nucle(ol)ar	Strong delay of C_2 Few 7S pre-rRNA No 25S/5.8S rRNA No export	25, 26, 29, 108
L36A (YMR194W) L36B (YPL249C-A)	L36 Φ (DBA)	NA	I: H12, H15, H16/21J, H21/22J, H22 IV: H68 V: H76	L8, L13, L15, L18, L28	Late nucle(ol)ar/ cytoplasmic	Inhibition of A_3, B_{1S}, and C_2 No 7S pre-rRNA No 25S/5.8S rRNA	25, 104, 177

(*Continued*)

Table 1 (*Continued*)

Yeast r-protein name[a] (☺ 😐 ☹)	Human r-protein name[b] (disease)	Bacterial r-protein name	Main rRNA contacts[c]	r-Protein contacts	Timing of stable assembly[d]	Most prominent assembly defects upon depletion (yeast)	References for timing and/or defects
L37A (YLR185W) L37B (YDR500C)	L37	NA	I: H11, H18, H23 II: H32, H33, H35 III: H49/50J IV: H65 5.8S: H4/5J, H5, H7, H8	L4, L26, L35, L39	Medium nucle(ol)ar	Mild delay of C_2 Few 7S pre-rRNA No 25S/5.8S rRNA No export	25, 26, 104
☺ L38 (YLR325C)	L38	NA	III: H53, H54, H58	None	Nucle(ol)ar	Unknown	
😐 L39 (YJL189W)	L39	NA	III: H49/50J, H50, H51 5.8S: H6, H7, H8	L25, L26, L37	Late nucle(ol)ar	Slight delay of C_2 and E 25S/5.8S formation	25
L40A (YIL148W) L40B (YKR094C)	L40	NA	II: H42 V: H89, H91 VI: H97	L9	Cytoplasmic	Slight delay of C_2 25S/5.8S rRNA formation No export	29, 106
☺ L41A (YDL184C) L41B (YDL133C-A)	L41	NA	IV: H62, H70/71J, H71	None	Unknown	Not done	178
L42A (YNL162W) L42B (YHR141C)	L36A	NA	I: H11, H13, H21 II: H31 IV: H68 V: H74, H75, H82/83J, H86, H87, H88	L15, L11	Cytoplasmic	Unknown	25
L43A (YPR043W) L43B (YJR094W-A)	L37A	NA	II: H34 III: H55/56J, H58 IV: H62, H64/65J, H67 V: ES31	L2, L30	Late nucle(ol)ar	Mild delay of C_2 Strong delay of E No export	25, 29, 104

P0 (YLR340W)	P0	L10	Not in structure	L12, P1, P2	Late nucle(ol)ar/ Cytoplasmic	Slight delay of C_2 No export	25, 104
☹ P1A (YDL081C) P1B (YDL130W)	P1	NA	Not in structure	P0, P2	Cytoplasmic	Slight delay of C_2 25S/5.8S rRNA formation	25
☹ P2A (YOL039W) P2B (YDR382W)	P2	NA	Not in structure	P0, P1	Cytoplasmic	Slight delay of C_2 25S/5.8S rRNA formation	25
NA	L28	NA	NA	NA	Unknown	NA	None

[a]Faces denote growth-defect phenotypes upon deletion of the corresponding nonessential genes (happy, slight; normal, moderate; sad, severe).

[b]The symbol Φ denotes that mutations in the corresponding genes have been linked to ribosomopathies.

[c]Roman numerals in the large-subunit panel of the table refer to the domain of 25S ribosomal RNA (rRNA) secondary structure. The letter J indicates the junction between helices.

[d]The timing of assembly of r-protein was determined by coimmunoprecipitation with precursor ribosomal RNA (pre-rRNA) processing intermediates.

Abbreviations: CA, congenital asplenia; DBA, Diamond–Blackfan anemia; NA, not applicable, as there is no bacterial ortholog for these proteins; 5q−, 5q− syndrome.

It is important to emphasize that the mechanisms of ribosome assembly in vivo likely differ in some ways from those observed in vitro. In vitro reconstitution experiments were done with r-proteins and mature rRNAs that are fully processed and modified, but without *trans*-acting assembly factors, and under nonphysiological conditions (high Mg^{2+} and/or high temperature). In contrast, assembly in vivo occurs cotranscriptionally in the 5′-to-3′ direction; thus, local folding of nascent rRNA limits the population of folding intermediates and binding events. This effect, together with processing of rRNA spacers and functions of assembly factors, may bias the landscape of RNA folding and r-protein binding in vivo such that assembly can proceed more efficiently and accurately. Nevertheless, it has become clear, as described below, that common principles governing assembly have been retained from prokaryotes to eukaryotes.

INCORPORATION OF R-PROTEINS INTO NASCENT PRERIBOSOMAL PARTICLES: THE ROLE OF R-PROTEIN IMPORTERS AND CHAPERONES

How are newly synthesized r-proteins imported into the nucleus prior to their association with nascent rRNA? By what means are these abundant, highly basic RNA-binding proteins properly folded into stable, soluble forms and prevented from inappropriately interacting with other cellular RNAs before they assemble into preribosomes?

Although most r-proteins are small enough to passively enter the nucleus, due to the high cellular demand of ribosomes, their nuclear import is facilitated by transporters, which recognize their nuclear localization signals (NLSs) (64). Most nascent NLS-containing yeast r-proteins are imported into the nucleus bound mainly to the β-karyopherin Kap123, a HEAT (Huntingtin, elongation factor 3, protein phosphatase 2A, and target of rapamycin 1) repeat–containing protein (65). Kap123 is a nonessential protein; thus, other β-karyopherins (e.g., Kap108, Kap121) have a redundant role in r-protein import (65). Similarly, r-proteins from mammalian cells also use β-karyopherin as importers (e.g., 66). In the nucleus, the cargo r-proteins are released due to the presence of Ran-GTP. How exactly β-karyopherins recognize their cargo r-proteins remains unclear, given that no structural data of any r-protein in a complex with a β-karyopherin are available at atomic resolution.

In addition to this general mechanism of nuclear import, specific r-proteins require exclusive systems to be imported to the nucleus. In yeast, the best-characterized system consists of the nonessential symportin Syo1, which associates with the β-karyopherin Kap104; this complex helps simultaneously import r-proteins L5 and L11 (67). The crystal structure of *Chaetomium thermophilum* Syo1 in complex with the N-terminal extension of L5 has been resolved to atomic resolution (67). In the nucleus, the Syo1–L5–L11 complex is released from Kap104, as a result of its interaction with Ran-GTP. This action is concomitant with the binding of 5S pre-rRNA. Apparently, association of L5 with Syo1 or 5S rRNA is mutually exclusive, given that the 5S rRNA–binding site of L5 is also localized in the N-terminal extension of L5 (67). In the nucleus, the 5S rRNA–L5–L11 complex (known as 5S RNP) interacts with two assembly factors, Rpf2 and Rrs1 (33), which recruit the 5S RNP to early preribosomal particles. However, it is unclear how Rpf2 and Rrs1 replace Syo1 and how exactly these factors promote stable 5S RNP incorporation into the preribosomal particles.

Several other examples of yeast factors that promote efficient recruitment or assembly of specific r-proteins into preribosomal particles have been reported. The WD repeat–containing protein Rrb1 directly and specifically interacts with free L3 (68, 69). It is thought that Rrb1 binds L3 in the cytoplasm and delivers it to nascent pre-60S ribosomes, where L3 stably assembles (discussed in Reference 70). In contrast to Rpf2 and Rrs1, Rrb1 only very weakly associates with

preribosomal particles (68, 69). Two other WD repeat–containing proteins, Rrp7 and Sqt1, have been genetically linked to distinct r-proteins (71–73). The phenotypic defects of loss-of-function *rrp7* mutants are partially suppressed by overexpression of r-protein S27 (71), whereas Sqt1 is a high-copy suppressor of dominant-negative C-terminal truncated mutants of r-protein L10 (73). Sqt1 function is likely similar to that of Rrb1: It stably binds free L10 through the N-terminal part of L10 but weakly binds preribosomal particles (72). Strikingly, assembly of L10 is likely cytoplasmic; therefore, Sqt1 does not act as a nucleocytoplasmic transporter but rather as an L10-specific chaperone. Consistent with this function, L10 is highly unstable in vivo in the absence of functional Sqt1 (72).

Another specific transporter that also works as a chaperone is Yar1, a nonessential ankyrin-repeat protein that directly interacts with free r-protein S3 and functions as a molecular chaperone to keep S3 soluble in vivo (74). The Yar1–S3 complex is imported into the nucleus in a manner dependent on the presence of a functional NLS in the N-terminal domain of S3. Once there, Yar1 seems to assist the proper assembly of S3 into pre-40S ribosomes, but it only weakly interacts with pre 40S r-particles (74). The structure of the Yar1–S3 complex was also recently solved at molecular resolution (75). In this structure, Yar1 binds predominantly to the N-terminal domain of S3 but leaves the C-terminal domain free for interaction. A model for the assembly of S3 (75) includes a scenario in which the *trans*-acting factors Ltv1 and Enp1 might have an important role (32). Interactions between the C-terminal domain of S3 and Ltv1, as well as between Yar1 and Ltv1, are thought to provide a docking point for S3 delivery to pre-40S ribosomal particles (r-particles). Release of Yar1 after initial binding of S3 to pre-40S ribosomes can proceed without any energy input in vitro (76). The release of Yar1 would make the N-terminal domain available for interactions with the neighboring r-proteins in nuclear pre-40S r-particles (75).

General chaperones also assist r-protein assembly. The ribosome-associated chaperone complexes NAC (nascent polypeptide–associated complex) and SSB-RAC (stress 70 B-ribosome-associated complex) bind and functionally cooperate to prevent misfolding and aggregation of most r-proteins and several *trans*-acting assembly factors, perhaps even escorting them to the places where they are incorporated into preribosomal particles (77, 78). S31 and L40, r-proteins that are synthesized as ubiquitin-fusion precursor proteins, escape the role of these chaperone complexes, suggesting that their ubiquitin moiety promotes the folding of the nascent respective r-proteins. In agreement with this finding is the demonstration that ubiquitin contributes to the efficient synthesis of both S31 and L40 r-proteins (79; J.d.l.C., unpublished data). Nonetheless, in yeast ubiquitin is rapidly processed and released; thus, in contrast to other chaperones (e.g., Yar1, Rrb1), ubiquitin likely does not accompany S31 and L40 r-proteins into the preribosomal particles where they assemble. Interestingly, in *Giardia lamblia*, the ubiquitin-like protein fused to S27A (S31 in yeast) is present in the mature ribosome (80). This finding is unexpected because in yeast, mutations that block the cleavage of the ubiquitin in S31 are lethal or severely slow growth (79). No structural analyses of ribosomes from G. *lamblia* are available, so we do not yet know why this ubiquitin-like protein is tolerated in mature ribosomes.

ASSEMBLY OF 40S RIBOSOMAL SUBUNITS

Both pre-rRNA processing and binding of r-proteins to pre-18S rRNA occur largely cotranscriptionally in the nucleolus (**Figure 2*a***). Thus, studies of rRNA processing have long served as a proxy for studies of 40S r-subunit assembly. Analyses of rRNA processing in wild-type yeast strains, as well as in many of those depleted individually for *trans*-acting assembly factors, have revealed that in yeast there are four rRNA cleavage steps on the pathway to producing mature 18S rRNA—two each on the 5′ and 3′ ends, which first cleave nearby and then at the mature site

(**Figure 2*b***) (81). This finding suggests that at least four SSU assembly intermediates exist, one prior to each of the cleavage steps. Nevertheless, in wild-type cells only one pre-40S intermediate accumulates perceptibly; it contains an 18S rRNA precursor referred to as 20S pre-rRNA. This intermediate, formed in the nucleus, contains the mature 5′ end, and its 3′ end is the A_2 site; thus, it contains ~200 extra nucleotides at the 3′ end (**Figure 2*b***). A second intermediate, 23S pre-rRNA, is also found in many yeast strains. This intermediate contains an unprocessed 5′ end but has been cleaved at the 3′ end, downstream of the typical site, at site A_3 (81).

Independent sedimentation studies of r-particles have provided evidence for three broad classes of SSU assembly intermediates in wild-type cells. Two early intermediates sediment at ~90S and contain 23S or 35S pre-rRNAs (82–85). In addition, there is a late intermediate, which forms in the nucleus but is located in the cytoplasm at steady state. This late intermediate sediments at 43S and contains 20S pre-rRNA. Finally, the fourth observed intermediate, containing 23S rRNA, sediments at ~60S (85). Due to its similar size to the other intermediates and its presumed RNA content, this fourth intermediate may be related to a fifth one observed in cells in which helicase Dhr1/Ecm16 is mutated (86). Alternatively, the Dhr1-related intermediate may be a dead end observed only in the absence of Dhr1. Because most nascent rRNAs are cotranscriptionally cleaved at site A_2 (87–89), we do not know whether the presence of 35S pre-rRNA, or even 23S pre-rRNA, is relevant, as these may be degraded before they are mature. Unfortunately, with the exception of the late cytoplasmic 43S assembly intermediate, none of the other assembly intermediates has been purified to sufficient homogeneity to enable either biochemical or structural analyses. Consequently, our knowledge about the complement of assembly factors or r-proteins bound to each consecutive intermediate is tentative. Nevertheless, systematic analyses have shown that r-proteins of the SSU assemble in a bipartite manner and that individual r-proteins fall into three to four broad classes (**Table 1**) (**Figure 3*a*,*b***) (27, 28).

Generally, r-proteins that bind to the body of the SSU (5′ domain) appear to bind early during transcription (**Figure 3*a*,*b***). Their deficiency blocks assembly and processing at the early cleavage sites (A_0 and A_1) at the 5′ end of 18S rRNA (27, 28). Because the downstream processing events at A_2 and D appear to depend on the prior cleavage at site A_1 (and site A_2 for D-site cleavage), these are blocked as well. Next, the r-proteins that form the head domain of SSU assemble, and their deficiency impairs mainly cleavage at site A_2 (and, as a result, D-site cleavage), which separates the rRNAs destined for the SSU and LSU (**Figure 3*a*,*b***). Because processing at site A_2 also occurs predominantly cotranscriptionally (87–89), assembly of this group of r-proteins, which make up 70% of all SSU r-proteins, must also occur cotranscriptionally. Consistent with this idea are the findings that 21 of 22 r-proteins from the SSU that can be analyzed systematically (28) do precipitate significant amounts of 20S pre-rRNA, and only S11 and S13, which bind to the platform, precipitate substantial amounts of 35S rRNA (28). S26 is the only protein that does not efficiently precipitate 20S pre-rRNA, suggesting that it does not bind until 18S rRNA is mature (see the next two sections). Interestingly, the interactions between r-proteins and 35S pre-rRNA are particularly salt labile, suggesting that these r-proteins that bind early pre-rRNAs acquire additional interactions during assembly because of conformational rearrangements of pre-rRNAs, stepwise acquisition of RNA–protein contacts, or the cementing of these contacts by addition of neighboring proteins. A small subset of head-binding r-proteins (S3, S15, S18, and S19) is also specifically required for export of the nascent subunit (28). Finally, another subset of r-proteins (S10, S20, S26, S29, S31, and Asc1) may assemble in the cytoplasm and is required only for the cytoplasmic processing of 20S pre-rRNA. With the exception of Asc1, these proteins have in common a location near or at the mRNA-binding channel (40).

Excitingly, the order in which these r-proteins assemble to yeast ribosomes in vivo seems to parallel the assembly of their bacterial counterparts in vitro (43, 90–92), indicating that the

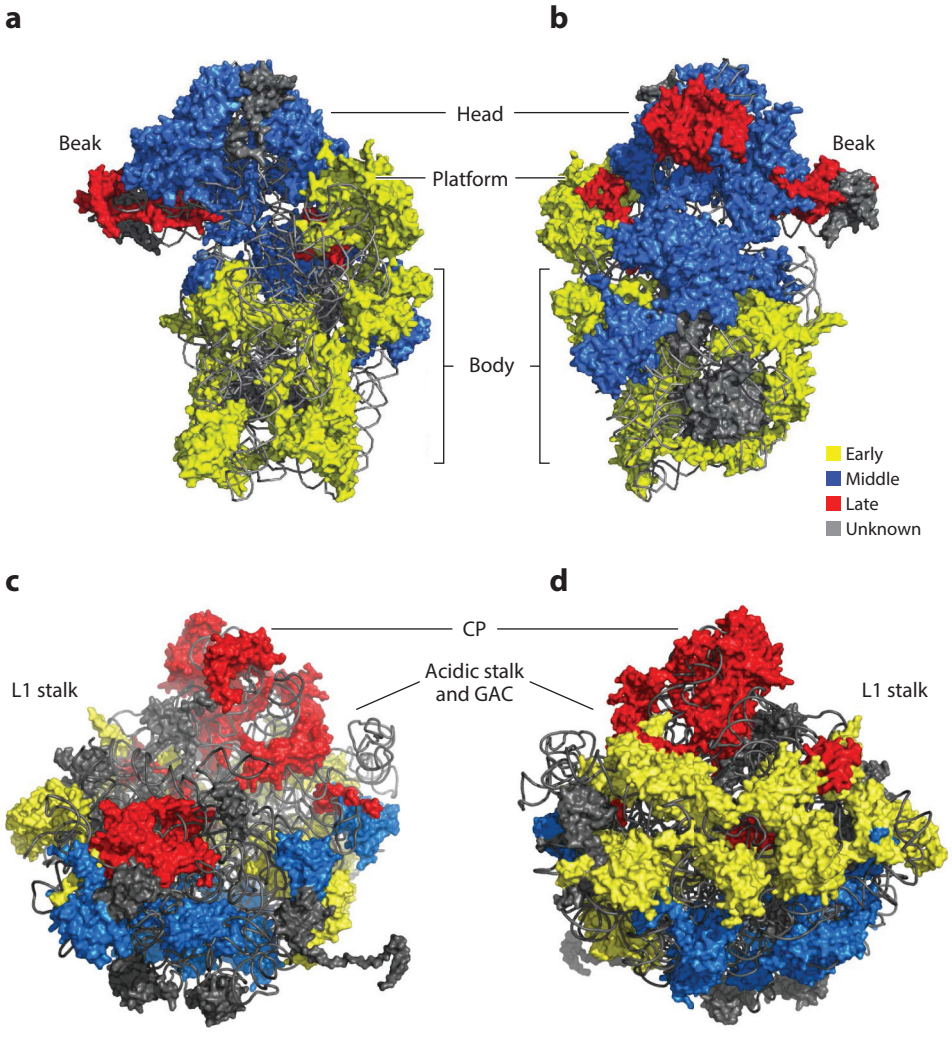


Figure 3

Correlation of function and location of the small subunit (SSU) and large subunit (LSU) r-proteins of *Saccharomyces cerevisiae*. Early-acting (*yellow*), middle-acting (*blue*), and late-acting (*red*) r-proteins are mapped onto the crystal structure. (*a*,*c*) The subunit interface of the SSU and LSU, respectively. (*b*,*d*) The solvent-exposed surface of the SSU and LSU, respectively. Ribosomal RNA (rRNA) and r-proteins are shown in cartoon and surface representation, respectively. Abbreviations: CP, central protuberance; GAC, GTPase-activation center. The crystal structure is adapted from Protein Data bank codes 3U5D and 3U5E.

assembly is dictated largely by the biophysical features of the RNA–protein interactions. This finding also validates decades of in vitro studies of bacterial assembly. This confirmation will be especially important in the future because reconstituted systems for r-protein binding to rRNA are not yet available in yeast, and the details of RNA–protein interactions and their acquisition are currently difficult to study in vivo. Note that practically the same principles of SSU assembly as those described herein for yeast have also been reported in mammalian cells (93).

MODULATION OF 40S R-PROTEIN BINDING BY ASSEMBLY FACTORS

An emerging theme in ribosome assembly is that there are multiple instances of *trans*-acting assembly factors that delay the incorporation of r-proteins by sterically blocking their site of assembly. Two such examples are S10 and S26, two of the three last r-proteins to be incorporated into the nascent SSU (57). Premature binding of S10 to the beak structure is blocked by Ltv1, and binding of S26 to the platform is blocked by Pno1/Dim2 (57). Release of Ltv1 from pre-40S subunits requires its phosphorylation by the kinase Hrr25 (76). How Pno1 is released remains unknown. Interestingly, S10 and S26 are located at the entrance and exit, respectively, of the mRNA-binding channel, suggesting that blocking the binding of these two r-proteins and then regulating the release of the factor (at least in the case of S10) prevent the recruitment of mRNAs to the nascent 40S r-subunit. A comparison between this process and bacterial SSU assembly both in vivo and in vitro also suggests that the platform and beak structure are intrinsically slow to form and initially misfolded. Thus, Ltv1 and Pno1 appear to exploit several already-existing features. First, structural and biochemical analyses of successive in vitro assembly intermediates indicate that the beak and platform regions are the last to assemble r-proteins (91). Second, an in vitro analysis of rRNA folding has shown that these structures are the slowest to fold (94). Third, both the beak structure and the platform region are initially misfolded and then refolded during the heat-dependent activation step (95). Finally, and intriguingly, just as Ltv1 modulates the incorporation of S10 and S3 in eukaryotes (57), RimM modulates the assembly of the bacterial ortholog of S3 (also known as S3), by regulating folding of 16S rRNA (96). Other examples of an assembly factor delaying r-protein incorporation are discussed in the section titled Roles of R-Protein Paralogs: The Placeholder Hypothesis, below.

CYTOPLASMIC STEPS OF 40S RIBOSOMAL SUBUNIT MATURATION

The cytoplasmic steps of 40S r-subunit assembly include the incorporation of several r-proteins, as described above; formation of the 3′ end of mature 18S rRNA; and dissociation of the remaining assembly factors. These events are integrated into a translation-like cycle that serves to "test-drive" nascent ribosomes to ensure that they are competent in key functionalities of the SSU (**Figure 4*a***) (97, 98).

As maturing 40S r-subunits emerge from the nucleus, they contain 20S pre-rRNA and most r-proteins. S20, S29, and S31 are incorporated around the time of export, and at least S20 and S29 appear to be only partially occupied in a purified cytoplasmic intermediate (57). S10, S26, and Asc1 are completely absent, and S3 and S14 appear not to be positioned at their final location (32, 55). This assembly intermediate also contains seven stably bound assembly factors: Enp1 and Ltv1, bound to the beak structure; Rio2, Tsr1, and Dim1, located at the subunit interface; and Nob1 and Pno1 on the platform (57). Phosphorylation of the assembly factor Ltv1 initiates the cytoplasmic maturation cascade by releasing Ltv1 from the beak (76). Because these two assembly factors hold S3 in a premature conformation and block the binding of S10 (57), their release is expected to lead to final assembly of the mRNA entry channel at the beak structure. Release of Ltv1 is also required for eIF5B-dependent joining of the LSU to initiate the translation-like cycle (76).

The next isolated intermediate is an 80S-like complex that contains pre-40S subunits bound to mature 60S subunits (97, 98). Rio2 dissociates from this intermediate, and the levels of S10 and Asc1 are akin to those found in mature ribosomes (97). The exact order of subsequent events remains unclear, but Nob1-dependent 18S rRNA maturation occurs within 80S-like ribosomes (99) and is somehow regulated by the kinase Rio1 (100–102). Interestingly, although Rio1 cosediments with and copurifies 40S-sized complexes (100, 101), overexpression of dominant-negative forms leads to the accumulation of 80S-like ribosomes (101). Furthermore, Dim1 and Tsr1 must dissociate

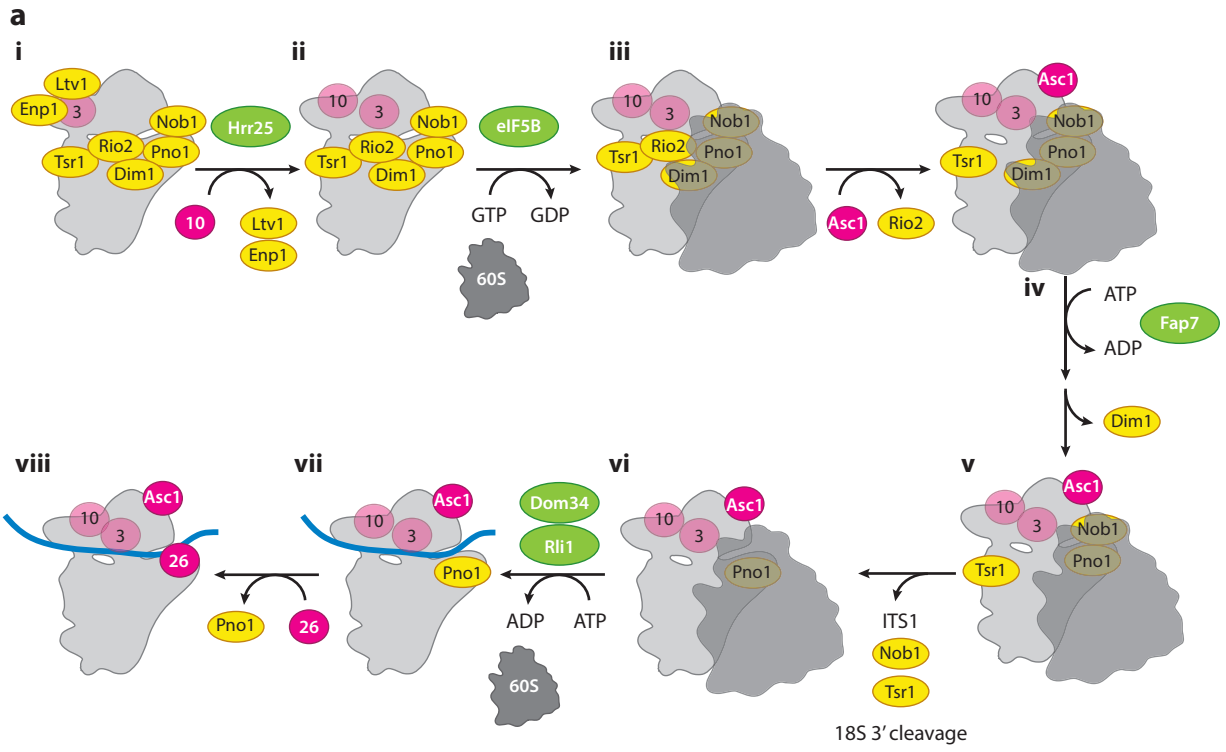
from 80S-like ribosomes. Because Tsr1 and the termination factor Rli1 share a binding site, Tsr1 dissociation allows for the dissociation of 80S-like ribosomes (97). The newly liberated 40S subunits are now mature, except for the presence of Pno1 and the absence of S26 (28, 97). Note that a recent contrasting study suggested that S26 might be recruited to early nucleolar ribosomes (103). Clearly, this translation-like cycle has the potential to test the subunit's ability to bind 60S subunits and to position and activate the translation factors eIF5B and Rli1. Because Rli1 has a cofactor, Dom34, that is also involved in this process and binds the decoding center, this cycle might also test the integrity of this site. Nevertheless, we do not yet know how the successful function of these translation factors is linked to progress in the assembly cascade, including the incorporation of the remaining r-proteins.

ASSEMBLY OF 60S RIBOSOMAL SUBUNITS

Assembly of 60S subunits has been most simply defined by the six successive steps of processing of pre-rRNAs within pre-60S particles (**Figure 2*b***). More recently, assembly factors that are present in these 66S preribosomes during defined intervals of biogenesis have provided additional landmarks for LSU maturation (25, 104).

Whereas assembly of the SSU may occur largely cotranscriptionally, only initial stages of LSU assembly are cotranscriptional. Production of 60S r-subunits is initiated by cleavage of nascent pre-rRNA at the A_2 site. This cleavage occurs once transcription by RNA polymerase I has proceeded ~1–1.5 kb 3′ of that site (**Figure 2*a***) (87–89). Upon completion of transcription, the first detectable precursor particle that is specific for LSU assembly is formed; it contains $27SA_2$ pre-rRNA as well as most of the r-proteins and approximately one-third of the 75 assembly factors dedicated to making the LSU (105). Nevertheless, these r-proteins are bound rather weakly and thus have not yet fully formed all of their native interactions with rRNA. As assembly proceeds, association between these r-proteins and preribosomes is strengthened (25), and downstream steps in pre-rRNA processing occur. In addition, early-acting assembly factors are released, and late-acting assembly factors join nascent ribosomes, then dissociate upon carrying out their respective functions. As described later in this section, an examination of the effects of depleting LSU r-proteins revealed that assembly of 60S subunits proceeds in a hierarchical fashion, neighborhood by neighborhood.

Initially, investigators studied the roles of a few individual r-proteins in LSU biogenesis by depleting them via shutoff of conditional promoter fusions, then assaying effects on cell growth, levels of free r-subunits and polysomes, pre-rRNA processing, and nuclear export of pre-rRNPs (e.g., 26, 31, 33, 34, 106–112). More recently, the roles of most 60S r-proteins have been systematically examined, with assays extended to measuring changes in protein constituents of purified preribosomal particles and, in a few cases, interrogation of effects on pre-rRNP structure (25, 29, 30, 104, 113). Because most 60S r-proteins associate with the earliest pre-60S particles and contact multiple different intertwined domains of rRNA secondary structure in mature subunits, one might have predicted that depletion of each r-protein would globally affect the earliest stages of assembly. Thus, it was surprising to discover distinct classes of pre-rRNA processing phenotypes upon depletion of 60S r-proteins: Depletion of any of 12 60S r-proteins (L3, L4, L6, L7, L8, L13, L16, L18, L20, L32, L33, and L36) impairs the earliest steps of $27SA_2$ pre-rRNA processing, as evidenced by the accumulation of $27SA_2$ or $27SA_3$ pre-rRNAs and diminished amounts of all downstream pre-rRNAs (for references, see **Table 1**). Depletion of 11 other LSU r-proteins (L9, L17, L19, L23, L25, L26, L27, L31, L34, L35, and L37) causes accumulation of significant amounts of the next processing intermediate, 27SB pre-rRNA (**Table 1**). Seven r-proteins (L2, L5, L11, L21, L28, L39, and L43) are required for processing of 7S pre-rRNA or 6S pre-rRNA


a
i
ii
iii
iv
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Ltv1
Enp1
3
Nob1
Rio2
Pno1
Tsr1
Dim1
10
Hrr25
Ltv1
Enp1
eIF5B
GTP
GDP
60S
Asc1
Rio2
ATP
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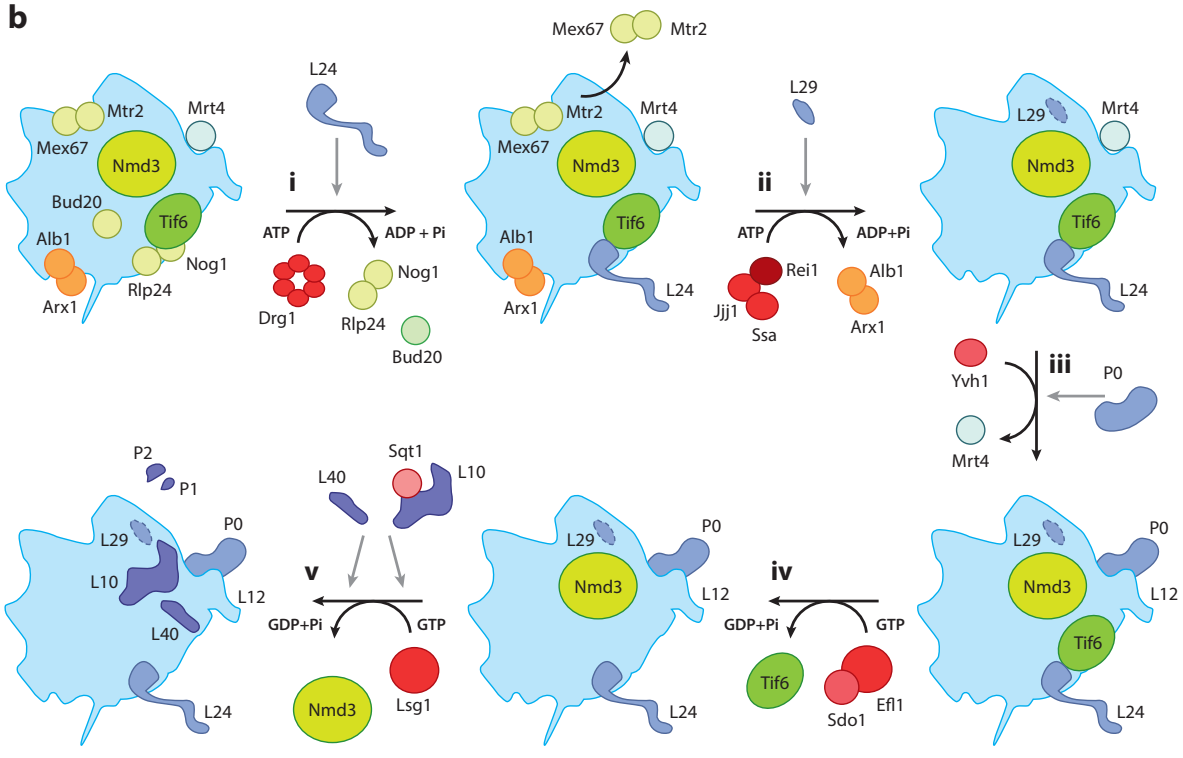
Fap7
Dim1
Dom34
Rli1
ADP
ATP
ITS1
Nob1
Tsr1
18S 3′ cleavage
26
Pno1


b
Mex67
Mtr2
Mrt4
Nmd3
Bud20
Tif6
Alb1
Nog1
Arx1
Rlp24
L24
i
ATP
ADP + Pi
Drg1
Nog1
Rlp24
Bud20
L29
ii
ATP
ADP+Pi
Rei1
Jjj1
Ssa
Alb1
Arx1
iii
Yvh1
Mrt4
P0
P2
P1
L10
L12
L40
Sqt1
v
GDP+Pi
GTP
Lsg1
iv
Tif6
Sdo1
Efl1

just before nuclear export of preribosomes. Depletion of some of the latter proteins also has moderate effects on the processing of 27SB pre-rRNA (**Table 1**). Finally, depletion of LSU r-proteins that assemble with pre-60S intermediates predominantly in the cytoplasm (e.g., L10, L29, L40, P0, P1, and P2) has little or no direct effect on pre-rRNA processing (**Table 1**).

Strikingly, these classes of pre-RNA processing phenotypes correlate with the location of the corresponding r-proteins within mature 60S r-subunits (**Figure 3*c*,*d***) (104). The r-proteins that are necessary for the early steps of 27SA pre-rRNA processing are located on the solvent-exposed surface of LSU, bound primarily to domains I and II of 25S rRNA. The group of r-proteins necessary for the middle steps of pre-rRNA processing clusters in a neighborhood around the exit of the polypeptide exit tunnel (PET), defined by domains I and III of 25S rRNA and 5.8S rRNA. r-Proteins that function in 7S pre-rRNA processing are located on the intersubunit surface, and those required for late nuclear and cytoplasmic steps of maturation cluster around the CP, where 5S rRNA lies between domains II and V of rRNA (**Figure 3*c*,*d***). These results suggest that assembly of LSUs may proceed in a hierarchical fashion, beginning with the solvent-exposed surface, followed by the PET, the intersubunit interface, and finally the CP. Importantly, bacterial LSUs assemble with a similar hierarchy (61), suggesting that the principles governing assembly of LSUs are evolutionarily conserved, despite the added complexities of ribosome biogenesis in eukaryotes.

An examination of the effects of these depletions on association of other LSU r-proteins or assembly factors confirmed that there is an assembly hierarchy in which early-acting r-proteins are necessary for stable association of middle-acting r-proteins with pre-rRNPs. Middle-acting r-proteins in turn are required for assembly of late-acting r-proteins (25, 26, 31, 104, 106, 108). A similar hierarchical dependence upon r-proteins for association of assembly factors with preribosomes was observed. In general, the most affected proteins in these depletion strains are r-proteins and assembly factors located in close proximity to the depleted r-protein. For example, depletion

Figure 4

(*a*) Cytoplasmic steps of 40S incorporation. (*i*) A 40S assembly intermediate containing seven stably bound assembly factors accumulates in the cytoplasm at steady state. Phosphorylation of Ltv1 by the kinase Hrr25 releases Ltv1 and Enp1, allowing for (*ii*) repositioning of S3 and incorporation of S10 at the messenger RNA (mRNA) entry channel (76). (*iii*) Release of Ltv1 allows for eIF5B-dependent joining of the 60S subunit to form 80S-like ribosomes. (*iv*) Before Fap7 acts on 80S-like ribosomes, Rio2 is released, and independently, Asc1 joins. (*v*) Dim1 is released (J. Trepreau & K. Karbstein, unpublished data) before (*vi*) Nob1-dependent 18S formation and Tsr1 release. (*vii*) Tsr1 release allows for binding of Dom34/Rli1 to dissociate 80S-like ribosomes. (*viii*) Exchange of Pno1 for S26 occurs in polysomes (97). The nascent 40S subunit is shown in light gray, the mature 60S subunit in dark gray, r-proteins in magenta, stably bound assembly factors in yellow, transiently bound assembly factors/translation factors in green, and mRNA in blue. S3 and S10 are in lighter shades to indicate their location on the solvent side of the molecule. (*b*) Cytoplasmic maturation of pre-60S ribosomal particles. Pre-60S ribosomal particles that arrive in the cytoplasm contain only a few stably bound export adaptors (Arx1–Alb1, Mex67–Mtr2, Nmd3, Bud20) and assembly factors (Mrt4, Nog1, Rlp24, Tif6) that are sequentially released to enable assembly of the remaining r-proteins. Note that the exact order of some steps (e.g., release of Mex67–Mtr2, assembly of L29) has still not been properly addressed. (*i*) The first step is the release of Rlp24, Nog1, and Bud20 by the ATPase Drg1, which then permits the assembly of L24 and the recruitment of Rei1. (*ii*) Rei1, together with the J protein Jjj1 and the HSP70 ATPase Ssa, enables the release of Arx1–Alb1, located near the polypeptide exit tunnel. Thus, this functional ribosomal site is inactive until the release of Arx1–Alb1. (*iii*) Then, or in parallel, Yvh1 is required for the removal of Mrt4, which is replaced in the pre-60S particles by the stalk r-protein P0. The stalk is required for recruitment of translation elongation factors (eEFs); thus, pre-60S particles lacking P0 are inactive. (*iv*) Pre-60S particles containing P0 are able to recruit the GTPase Efl1, which is closely related to eEF2. Efl1, together with Sdo1, facilitates the release of Tif6 from pre-60S ribosomal subunits (r-subunits). Tif6 inhibits r-subunit joining, thus preventing pre-60S particles from prematurely engaging in translation. (*v*) The release of Tif6 leads to activation of the GTPase Lsg1 to release the export adaptor Nmd3. Assembly of L40 and L10, aided by the chaperone Sqt1, is also required for the release of Nmd3. Nmd3 binds to the joining surface of the 60S r-subunit, thus also impeding joining with 40S r-subunits. Release of Nmd3 allows the 60S r-subunits to gain translation competence. Finally, acidic r-proteins P1 and P2 assemble to the r stalk at the moment when the mature 60S r-subunit is joined to the 40S r-subunit and committed to translation.

of early-acting L8 bound to domain I of 25S rRNA significantly decreases binding of the adjacent r-proteins L13, L16, and L36, as well as binding of six assembly factors that cross-link to nearby rRNA sequences (31). Like L8, L13, and L16, these assembly factors are specifically required for processing of $27SA_3$ pre-rRNA (34).

An analysis of the synthesis and turnover of intermediates that accumulate in the absence of r-proteins revealed that pre-60S rRNPs become more stable as assembly progresses (104). Depletion of any one of the early-acting r-proteins leads to rapid turnover of pre-rRNAs. In contrast, when middle-acting r-proteins are depleted, pre-rRNAs turn over less rapidly than in the early class of mutants. Finally, depletion of late-acting r-proteins has a very small effect on pre-rRNA turnover. The increasing stability of pre-rRNPs as they assemble likely reflects the establishment of greater numbers of contacts between the r-proteins and rRNA.

Determining how these neighborhoods of the LSU are sequentially assembled, including how pre-rRNA processing is coupled with remodeling of RNP domains within the LSU, remains an important challenge. On the basis of results obtained to date, we present a working model for the pathway of LSU assembly in the following subsections.

Initiating assembly. The earliest steps of LSU assembly likely involve bringing together and stabilizing rRNP domains containing both the 5′ and 3′ ends of $27SA_2$ pre-rRNA, aided by binding of r-proteins such as L3 to both rRNA domains. In mature subunits, L3 is positioned close to both the 5′ end of 5.8S rRNA and the 3′ end of 25S rRNA, and it makes the greatest number of contacts with domain VI at the 3′ end of 25S rRNA (e.g., 45). Thus, L3 may be located in preribosomes near the 5′ and 3′ ends of $27SA_2$ pre-rRNA. Furthermore, depletion of L3 has the greatest effect of any LSU r-protein on assembly of pre-60S particles; it causes the earliest block in pre-rRNA processing, leading to a mild accumulation of the $27SA_2$ pre-rRNA and rapid turnover of nascent particles (104, 112). A set of assembly factors functionally related to L3 (Npa1/Urb1, Npa2/Urb2, Dbp6, Dpb7, Dbp9, Rsa3, and Nop8) and Rrp5 may participate in this initial compaction of the nascent particles (114, 115). Each factor, like L3, is important for the processing of $27SA_2$ pre-rRNA, and each exhibits genetic interactions with *rpl3* mutations (115). Several other observations support this model. Sequences near the 3′ end of yeast pre-rRNA are important for the initiation of processing at site A_3 near the 5′ end of $27SA_2$ pre-rRNA (116). In bacteria, sequences flanking each end of 23S rRNA form a helix that is necessary for production of the mature rRNA (117). L3 binds to these ends of 23S rRNA and is required to initiate in vitro assembly of the bacterial LSU (2, 18). Furthermore, in bacteria, domains I and II at the 5′ end of 23S rRNA plus domain VI at the 3′ end are assembled first, before domains III, IV, and V (118, 119). Thus, in both prokaryotes and eukaryotes, formation of an initial, compact, pre-LSU intermediate may be an important step for launching assembly of LSUs.

Coupling early steps of large subunit assembly and preribosomal RNA processing with middle steps. During ribosome assembly, stabilization of initial encounter complexes between r-proteins and rRNA appears to occur in a sequential neighborhood-by-neighborhood fashion, in concert with the binding and function of assembly factors, and may be coupled with pre-rRNA processing. A mutually interdependent association between r-proteins and assembly factors and domains I and II of the rRNA (see **Table 1** for these r-proteins) likely allows the formation of an assembly intermediate that is stable enough to carry out the first step of pre-rRNA processing within pre-60S r-particles, namely removal of the remainder of ITS1 from $27SA_2$ pre-rRNA (34, 120). Formation of the "bow tie" structure of 5.8S rRNA, by base-pairing of the 5′ and 3′ ends of domain I rRNA, may be essential for the preparation of a functional substrate for removal of ITS1. Moreover, proper folding of ITS2 and the proximal stem may be a prerequisite for

removal of ITS1 (120–122), indicating tight coupling between the steps for processing of these two spacer sequences. Finally, proper folding of domains I and II may also create a platform for stable formation of downstream rRNA tertiary structures; an inspection of mature LSU rRNA in both prokaryotes and eukaryotes revealed helices in domain II that project toward domains IV and V (50, 104).

Coupling of middle steps of assembly and preribosomal RNA processing. The next step in assembly, strengthening association of middle-acting r-proteins with domains I and III, appears to trigger cleavage of the C_2 site in the ITS2 spacer. These r-proteins are necessary to recruit the last two assembly factors required for C_2 cleavage, Nsa2 and Nog2/Nug2. Unlike the other middle-acting assembly factors that bind to early-assembly intermediates, Nsa2 and Nog2 assemble immediately prior to C_2 cleavage (123, 124). How they depend on these r-proteins is not clear; their loading may require long-range interactions within pre-60S particles, given that Nog2 binds to helices in domains II, IV, and V on the subunit interface of the pre-60S r-particles (125), whereas the middle-acting r-proteins bind domains I and III on the opposite side of the subunit.

The middle-acting r-proteins are also required for the stable assembly of r-proteins L2, L39, and L43, which are located on the subunit interface adjacent to the PTC (25). L2 and L43, along with Nog2, are necessary for processing of 7S pre-rRNA (25). How the stable assembly of these r-proteins is coupled with this step of pre-rRNA processing is unclear, although the effect may be somewhat proximal, given that these r-proteins lie adjacent to the foot structure containing the ITS2 sequences removed in this step, but on different sides of the 60S subunit (56).

Coupling the late nuclear steps of large subunit assembly with nuclear export. The last step of LSU biogenesis prior to nuclear export is completion of formation of the CP, which contains 5S rRNA bound to r-proteins L5 and L11. This 5S RNP is delivered into early pre-60S r-particles by assembly factors Rpf2 and Rrs1 (see the section titled Incorporation of r-Proteins into Nascent Preribosomal Particles: The Role of r-Protein Importers and Chaperones, above) (33) but initially is rotated 180° from its final position in the mature LSU (56). Thus, a late step in LSU maturation appears to be rotation of the CP, as well as remodeling of adjacent rRNA helices in the 25S rRNA. L21, which lies at the base of the CP, seems to play at least an indirect role in both CP rotation and nuclear export of pre-60S particles by enabling release of assembly factors Rsa4, Nog2, Rpf2, and Rrs1 (25). L21 may do so by helping structure the adjacent neighborhood containing these factors so that they can be removed by the AAA ATPase Rea1 (126). Release of Rpf2 and Rrs1 bound to the 5S RNP, and of Rsa4, which is present between the CP and the remainder of the pre-60S structure, may be required for rotation of the 5S RNP into its mature position.

Reorganization of the CP containing 5S RNP, and release of Nog2, might be coupled to nuclear export of nascent LSUs. Nog2 and the export factor Nmd3 occupy overlapping positions on the pre-60S particles (125); consequently, release of Nog2 enables binding of Nmd3. In addition, the export factor Mex67 is thought to bind to the 5S RNP in pre-60S r-particles at this point, perhaps as a result of reorientation of the CP (127).

CYTOPLASMIC STEPS OF 60S RIBOSOMAL SUBUNIT ASSEMBLY

As for the SSU, the final steps of LSU maturation occur in the cytoplasm, where 5.8S rRNA is generated from 6S pre-rRNA; eight r-proteins, L10, L24, L29, L40, L42, P0, P1, and P2, join the LSU; and seven assembly factors are removed (**Figure 4*b***). These eight r-proteins are specifically enriched in late and cytoplasmic pre-60S r-particles (25), and none of them are directly necessary for any steps of pre-rRNA processing, even conversion of 6S pre-rRNA to 5.8S rRNA (e.g., 29, 106, 128). Furthermore, five of these r-proteins, L24, L29, L41, P1, and P2, are not essential for

growth and thus for assembly of translation-competent LSUs (**Table 1**). All but L24 are located on the subunit interface, adjacent to the CP, the PTC, and the GTPase-activating center (GAC). In the absence of L29 and L40 or upon depletion of L10, the LSU does not efficiently join to the SSUs (106, 129, 130). Thus, the last steps of LSU assembly are reserved for completion of active sites. Doing so may provide yet another mechanism to prevent inactive, nascent LSUs from interacting prematurely with SSUs. Importantly, the strategy to assemble functional sites of ribosomes last resembles that previously shown for the maturation of bacterial LSUs as well as for yeast SSUs (57, 118, 119).

As is the case for the SSU, premature assembly of r-proteins L10, L24, and P0 into the LSU is sterically blocked by assembly factors bound to late cytoplasmic preribosomes. Removal of these assembly factors is coupled with binding of the corresponding r-proteins, as part of an ordered pathway of cytoplasmic maturation of the LSU (**Figure 4*b***) (35, 131). This pathway is carried out by a series of GTPases and ATPases that are recruited to and activated by factors present in late cytoplasmic pre-60S particles, triggering release of their respective target proteins and, in some cases, association of the corresponding r-protein. Each step in this pathway appears to be coupled to the next downstream remodeling event.

The first factor that functions in cytoplasmic maturation of pre-60S particles is the AAA ATPase Drg1, which is recruited and activated by the assembly factor Rlp24, a paralog of r-protein L24 (132). Drg1 catalyzes the removal of Rlp24, after which L24 joins the cytoplasmic pre-60S subunits (132, 133). This step enables binding of Rei1, which together with the HSP70 ATPase Ssa1 and its cofactor Jjj1 releases the export adaptor Arx1 and its partner Alb1 (35, 134, 135). In parallel, r-protein L12 recruits the assembly factor Yvh1, which displaces Mrt4, the paralog of r-protein P0, from its rRNA-binding site in the GAC (35, 136, 137). This reaction enables the irreversible binding of P0 (128). In contrast, the P1 and P2 r-proteins, which together with P0 form the stalk structure (reviewed in Reference 138), cycle on and off of mature 60S subunits (139). Assembly of the P stalk is necessary for binding and activation of the GTPase Efl1 (35).

It is during these last steps of subunit maturation that the LSU undergoes functional proofreading to test the assembly of the tRNA P site adjacent to the PTC and the GAC. It is thought that the flexible loop of L10, which is positioned adjacent to the P site in translating ribosomes, detects whether or not assembly of the PTC occurs properly and, if so, transmits signals to activate Efl1 and Sdo1, thereby releasing Tif6 (140). This process then triggers release of Nmd3 by Lsg1. In addition, Efl1 binds to and is activated by the P stalk. Thus, Efl1 might couple proofreading of the functionality of the LSU with removal of factors that prevent nascent LSUs from interacting with 40S subunits. Thereafter, newly made LSUs can enter the pool of functioning subunits (reviewed in Reference 131). Intriguingly, Efl1 is structurally related to translation factor eEF2, suggesting that it carries out a step resembling the translocation function of eEF2 (141).

It is also tempting to speculate that the translation-like cycle that functions in 40S subunit assembly (97, 98) might similarly be used to proofread nascent 60S subunits. eIF5B, the key functional player, might be as unable to distinguish between mature 60S and pre-60S ribosomes as it is unable to distinguish between 40S and pre-40S ribosomes.

ROLES OF R-PROTEIN PARALOGS: THE PLACEHOLDER HYPOTHESIS

Several r-proteins have paralogs that are present in preribosomes and function in ribosome assembly. These include r-proteins L7, L24, and P0, which show significant homology to LSU assembly factors Rlp7, Rlp24, and Mrt4, respectively (133, 142–144). In addition, r-proteins S1, S9, and S26 exhibit partial homology to SSU assembly factors Rrp5, Imp3, and Nob1, respectively

(145, 146). These observations prompted the hypothesis that these assembly factors might function as placeholders for their paralog r-proteins by binding to the same rRNA structures in preribosomes that the r-proteins bind to in mature ribosomes (143). Alternatively, these assembly factors might simply use ancient RNA-binding motifs, but may have evolved enough to bind to distinct sites, thus not serving as placeholders to delay r-protein incorporation.

The placeholder hypothesis has been experimentally addressed for some of these paralog pairs in the yeast *S. cerevisiae* and demonstrated to be true for some cases but unlikely for others. The best-characterized pair of paralogs is Mrt4 and P0. Mrt4 is a nonessential 60S r-subunit biogenesis factor (142) that is released into the cytoplasm and replaced with P0 through an unknown mechanism. Mrt4 shows significant similarity throughout its entire primary sequence to the N-terminal domain of P0. This portion of P0 is responsible for its binding to the GAC of 25S rRNA, whereas the exclusive C-terminal domain of P0 is involved in binding to translation factors and r-proteins P1 and P2 (128, 144). Several findings support the idea that Mrt4 and P0 compete for the same site in the rRNA: (*a*) These two proteins are not present at the same time in pre-60S or mature 60S r-subunits (144), (*b*) a chimera Mrt4–P0 protein in which the first 121 amino acids from P0 were replaced by the equivalent 137 amino acids of the N-terminal region from Mrt4 can partially rescue the lethality resulting from the absence of P0 (144), and (*c*) cryo-EM of nuclear pre-60S r-particles reveals that Mrt4 localizes to the same site as P0 in mature ribosomes (56). This pathway for P0 assembly is conserved among eukaryotes (35), suggesting that it provides evolutionary advantages, even though Mrt4 is not essential. Mrt4 may provide a surveillance point to control the production of functional LSUs (131) and to impede the binding of translation factors to nuclear pre-60S particles. Furthermore, Mrt4 may delay the final steps in folding of the GAC domain, until P0 is stably assembled.

As described above, the AAA ATPase Drg1 specifically dissociates Rlp24 from cytoplasmic pre-60S particles (132), but whether or not this event is coupled to assembly of its paralog r-protein L24 remains unclear. In contrast to the Mrt4–P0 pair, L24 is not required for the release of Rlp24 from cytoplasmic pre-60S ribosomes, as demonstrated by the fact that the predominantly nucleolar localization of Rlp24 is not affected in an *rpl24* null mutant (133). Although there is not yet clear evidence that Rlp24 functions as a placeholder for L24 within pre-60S particles, a cryo-EM study of nuclear pre-60S particles showed that the density found at the location of L24 fits with the N-terminal part of Rlp24 (56). If we take into account that Rlp24 is enriched in nuclear pre-60S particles and that L24 assembles in the cytoplasm, it seems likely that Rlp24 shares the same rRNA-binding site with L24. Analogous to the exchange of Mrt4 with P0 is that the replacement of Rlp24 with L24 may provide a checkpoint to order the next step in maturation of the LSU.

The placeholder hypothesis may also be true for r-proteins from the SSU, although direct proof is lacking. Imp3 is an assembly factor present in 90S preribosomal particles; its entire primary sequence is similar to that of r-protein S9 (145). Similarly, Rrp5 is similar over most of its sequence to the bacterial S1 protein, whose terminal four S1 domains are most similar to the terminal four S1 domains of Rrp5 (146) and which binds near the platform of the SSU (40, 43). The C-terminal part of the endonuclease Nob1 shares some discrete similarity with r-proteins S25 and S26. In yeast, the binding site of S26 overlaps with that of Pno1/Dim2, which is a cofactor of Nob1 (57). Pno1 prevents premature assembly of S26, which normally occurs in the cytoplasm after formation of 18S rRNA (57, 90, 147). Thus, in this case, it appears likely that Nob1 simply borrowed a functional domain already existent in ribosomes, but acquired a distinct rRNA-binding site.

In at least one case, a paralog does not function as a placeholder. Rlp7 shares ~30% sequence identity with r-protein L7 (143, 148–150); however, Rlp7 is not the placeholder for L7 during LSU assembly. Rlp7 and L7 can coexist in the same preribosomal particles (151), and they bind

RNA sites within preribosomal particles that are distant from each other (151, 152). The Rlp7 rRNA-binding site, identified by cross-linking and cryo-EM, maps to ITS2 and is adjacent to the binding sites of two other assembly factors, Nop15 and Cic1, which function at the same step in LSU biogenesis (56, 120, 151, 152). In contrast, L7 binds to positions in helix ES7^{L}b in domain II of mature 25S rRNA and to a long region of mature 5S rRNA over helices H2, H4, and H5 (151). The strong conservation between Rlp7 and L7 suggests that both proteins likely use similar RNA recognition motifs. However, it is not known how these two similar proteins, instead of competing for the same RNA substrates, are specifically targeted to different regions of the rRNA.

R-PROTEINS AND DISEASE

The synthesis of ribosomes utilizes much of a growing and dividing cell's resources (153), and in turn, the efficient and accurate function of ribosomes affects cellular health, as indicated by the antiproliferative effect of antibiotics targeting the ribosome and its fidelity. Thus, ribosome biogenesis is regulated by numerous pathways that sense cellular stress, and complex cellular pathways have evolved to detect and respond to defects in ribosome biogenesis and/or function. For example, target of rapamycin (TOR), the central regulator of cell growth, and the oncoproteins p53 and MYC regulate ribosome biogenesis, and their activity is regulated by r-proteins (9, 154–158). Consequently, it is not surprising that mutations that disrupt ribosome synthesis or the pathways regulating assembly lead to diseases affecting cellular growth.

Emerging technologies have enabled the discovery of a growing list of mutations in r-protein or ribosome assembly factor genes that cause rare inherited disorders in humans, so-called ribosomopathies (14, 15, 159, 160). Although these diseases share common etiologies reflecting defects in ribosome production, they most often include very heterogeneous pathologies with distinct clinical phenotypes. The most common of these, Diamond–Blackfan anemia (DBA), results from mutations in any of 11 r-proteins from either subunit (**Table 1**). Two puzzling questions have emerged from studies of ribosomopathies. First, why are these diseases often tissue specific? Second, why is loss of ribosome function, which leads to cell death, associated with a large increase in the probability of developing cancer, a disease associated with increased translational capacity?

Given that most r-proteins are essential for assembly and/or function of their respective subunits, the simple expectation was that loss of function of almost any r-protein might be lethal to an organism, due to the failure to provide sufficient ribosomes to sustain growth and proliferation of its cells. Possible explanations for mutations in r-proteins often resulting in tissue-specific phenotypes include the following.

1. Subsets of cell type–specific mRNAs that are translated less efficiently than most other mRNAs, due to the presence of certain sequences or RNA structures, could be biased against if the pool of translating ribosomes is globally diminished even slightly.
2. Alternatively, heterogeneity of ribosome composition (reviewed in Reference 161) could account for these disease phenotypes. Mutations in a tissue-specific r-protein or assembly factor could compromise ribosome assembly or function in a subset of cells, or affect ribosomes dedicated to the translation of a class of mRNAs that are important for the function of specific cells.
3. Depletion of r-proteins leading to abortive assembly results in enlarged pools of unassembled r-proteins, which activate p53, leading to cell-cycle arrest and apoptosis. Such nucleolar stress responses might be tissue specific.

To explain how ribosomopathies resulting in congenital hypoproliferative diseases can cause hyperproliferative diseases such as cancer (159) later in life, investigators have speculated that the

appearance of secondary mutations that overcome the problem of reduced ribosome biogenesis may be sufficient to drive cancer. These secondary mutations would be able to restore the normal levels of ribosomes but without "repairing" the effect of the primary mutation linked to the ribosomopathy. Therefore, ribosomes would still be defective and may alter translation homeostasis in a way that perturbs the regulation of certain genes, such as oncogenes and tumor suppressors, and promotes cellular transformation (162). A recent study found that growth-suppressor mutants of a distinct *rpl10* mutation in yeast mimic the effects most commonly identified in acute lymphoblastic T cell leukemia (163). The suppressor mutation, which maps in the *trans*-acting factor Nmd3, suppresses the growth and ribosome biogenesis defects of the *rpl10* mutant but does not restore the structural and functional defects of the mutant ribosomes (163).

An additional possibility is that loss of function of r-proteins compromises the immune system in these patients so that its ability to clear cancerous cells is compromised. This hypothesis seems reasonable because both red blood cells and immune cells derive from the same pool of stem cells. Nevertheless, such compromise, if it exists, must be minimal, as these patients can be successfully vaccinated. Finally, it is also possible that partial loss of ribosome activity has differential effects on the translation of growth-supporting versus growth-suppressing proteins, as already shown for other modifications to the translational machinery (164, 165).

So far, mutations in only a subset of r-proteins have been demonstrated to cause these diseases. Is there something in common among them that might explain the DBA phenotypes? Interestingly, ~50% of DBA patients carry mutations in r-proteins that act late in assembly of the SSUs (93), probably because assembly intermediates lacking early binding proteins would not escape the nucleus and might not even allow assembly of secondary or tertiary binding proteins. In contrast, if late-binding proteins are missing, the largely matured cytoplasmic assembly intermediates might be able to carry out most functions of translation, yet would be sufficiently defective that a subset of cellular mRNA would not be properly translated.

SUMMARY POINTS

1. General or specialized chaperones solubilize r-proteins, facilitate their nuclear import, and help them integrate into ribosomes.
2. Most r-proteins associate with nascent ribosomes cotranscriptionally and become more stably associated as assembly proceeds, likely reflecting more robust protein–RNA interactions.
3. The SSU is assembled largely cotranscriptionally, but only the initial stages of LSU assembly are cotranscriptional. Subsequent pre-rRNA processing and remodeling occur posttranscriptionally.
4. Subdomains of both subunits are assembled hierarchically. The body of the SSU is assembled first, followed by the head, and the mRNA-binding channel is formed last. The solvent-exposed surface of the LSU is assembled first, followed by the neighborhood surrounding the PET, then the intersubunit surface, and finally the CP. Thus, the active sites of each subunit are assembled late in the pathway, perhaps to prevent inactive nascent subunits from prematurely entering the pool of active ribosomes.
5. Late construction of active sites may in part result from assembly factors delaying incorporation of certain r-proteins by sterically blocking their binding sites.
6. Nascent subunits undergo quality-control checks for proper assembly of functional sites.

FUTURE ISSUES

1. The functions of r-proteins need to be explored using mutants other than depletions, for example, to investigate possibilities that r-proteins have multiple separable functions.
2. What are the roles of eukaryote-specific r-proteins and eukaryote-specific features such as extensions? Did the eukaryote-specific extensions coevolve with the expansion sequences, and if so, what advantage does this coevolution convey?
3. What roles do posttranslational modifications play in r-protein assembly and function?
4. Are there multiple alternative pathways of assembly? If so, what roles do r-proteins play in these pathways?
5. What are the mechanisms by which pre-rRNA folding, processing, and binding to r-proteins are coupled, and how does coupling regulate assembly?
6. Are there cell type–specific r-proteins, and if so, do they participate in the assembly of cell type–specific ribosomes?
7. How do different mutant alleles of r-proteins lead to disease? Exactly how are pathways regulating and regulated by ribosomes disrupted by these mutations?

DISCLOSURE STATEMENT

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