ABSTRACT  The synthesis of cytoplasmic eukaryotic ribosomes is an extraordinarily energy-demanding cellular activity that occurs progressively from the nucleolus to the cytoplasm. In the nucleolus, precursor rRNAs associate with a myriad of trans-acting factors and some ribosomal proteins to form pre-ribosomal particles. These factors include snoRNPs, nucleases, ATPases, GTPases, RNA helicases, and a vast list of proteins with no predicted enzymatic activity. Their coordinate activity orchestrates in a spatiotemporal manner the modification and processing of precursor rRNAs, the rearrangement reactions required for the formation of productive RNA folding intermediates, the ordered assembly of the ribosomal proteins, and the export of pre-ribosomal particles to the cytoplasm; thus, providing speed, directionality and accuracy to the overall process of formation of translation-competent ribosomes. Here, we review a particular class of trans-acting factors known as "placeholders". Placeholder factors temporarily bind selected ribosomal sites until these have achieved a structural context that is appropriate for exchanging the placeholder with another site-specific binding factor. By this strategy, placeholders sterically prevent premature recruitment of subsequently binding factors, premature formation of structures, avoid possible folding traps, and act as molecular clocks that supervise the correct progression of pre-ribosomal particles into functional ribosomal subunits. We summarize the current understanding of those factors that delay the assembly of distinct ribosomal proteins or subsequently bind key sites in pre-ribosomal particles. We also discuss recurrent examples of RNA-protein and protein-protein mimicry between rRNAs and/or factors, which have clear functional implications for the ribosome biogenesis pathway.

INTRODUCTION  Ribosomes are complex ribonucleoprotein organelles that are responsible for protein synthesis. In all organisms, ribosomes are composed of two ribosomal subunits (r-subunits), the large one (LSU) being about twice the size of the small one (SSU) [1]. The production of ribosomes is an extraordinarily complicated cellular challenge. All organisms invest an important percentage of their resources to produce and subsequently assemble the individual constituents of the ribosomes, ribosomal RNAs (rRNAs) and ribosomal proteins (r-proteins), which must be represented in equimolecular amounts [2-4]. This process, known as the ribosome biogenesis pathway, is a highly coordinated process that, in addition to the rRNAs and r-proteins, involves RNA and protein trans-acting factors. Trans-acting factors transiently bind to pre-ribosomal particles in a distinctive spatiotemporal manner and have precise functions during (i) the transcription, processing and chemical modification...
of the precursor rRNAs (pre-rRNAs); (ii) the folding and rearrangements of the pre-rRNAs within the pre-ribosomal particles; (iii) the synthesis, dedicated chaperoning, nuclear import (only in eukaryotes), assembly and repositioning within pre-ribosomal particles of the different r-proteins; (iv) also in eukaryotes, the intranuclear transport, acquisition of export competence and the exit of pre-ribosomal particles to the cytoplasm, as well as the cytoplasmic maturation steps that newly synthesized ribosomes must undergo before entering translation.

In bacteria, only about a dozen of protein trans-acting factors have been reported to participate in ribosome biogenesis [4-6]. In archaea, about 50 small RNAs (i.e. modification guide small RNAs) and 40 protein trans-acting factors have been described [5, 7, 8]. In eukaryotes, however, ribosome biogenesis has clearly increased its complexity. Thus, in the yeast Saccharomyces cerevisiae, about 80 small nucleolar RNAs (snRNAs) and more than 250-300 protein trans-acting factors are currently known to participate in this process [9, 10]. In humans, about 300 snoRNAs and more than 600 protein factors have been shown so far to be required for the biogenesis of ribosomes [11, 12].

Ribosome synthesis is clearly a directional process [13]. Thus, most reactions occur irreversibly during the maturation of pre-ribosomal particles, among them, the pre-rRNA processing steps, the pre-rRNA folding, the snRNA-dependent modifications, and the stepwise exchange of some protein trans-acting factors (for a general scheme of the ribosome biogenesis process, see Figure S1). In eukaryotes, therefore, pre-ribosomal particles must travel across the nucleolus and, after the acquisition of export competence, exit to the cytoplasm. Different factors are part of a quality control machinery that allows export-competent pre-ribosomal particles to associate with transport factors, which mediate their interactions with the nuclear pore complexes and their transport to the cytoplasm [14]. Moreover, although it has been suggested that some translation might occur in the nucleus [15], it seems clear that, at least, nascent nuclear pre-ribosomal particles are incompetent for translation until the release of the last protein trans-acting factors in the cytoplasm and the assembly of the last r-proteins [16-18]. These events, the displacement of the last factors and the assembly of the last r-proteins in the cytoplasm, are apparently prerequisites to confer translational competence to r-subunits (i.e. [19-21]).

Several protein trans-acting factors have been reported that act as placeholders. The word “placeholder” has different meanings, among others it refers to someone who occupies a professional position on behalf of someone else. In Molecular Biology, a placeholder corresponds to a factor that temporarily binds a target until its replacement by a second factor, which binds to the same target normally with a higher affinity. Placeholder factors have been described to participate in different cellular processes, including chromatin remodelling and transcription (e.g. [22-24]). In this review, we outline the current knowledge about placeholder factors involved in the biogenesis of ribosomes, focusing primarily on those from the yeast S. cerevisiae, in which this process has been most extensively studied. These factors include trans-acting factors, such as Mrt4 or Rlp24, which are paralogous placeholders of distinct r-proteins, or others trans-acting factors, such as Arx1, Nog1, Nog2/Nug2 or Tsr1, whose replacement pairs are not r-proteins. We also examine the role of distinct adaptors and chaperones, such as Rrb1, Sqt1 or Yar1, which recognize domains on their r-protein partners that are normally involved in binding to rRNAs. This latter phenomenon also resembles that known as RNA mimicry, by which some factors, such as Fap7 or Syo1, interact with a specific r-protein through the establishment of a protein interface that imitates part of the rRNA-binding surface of these r-proteins. We emphasise how all these factors, whose functions during ribosome biogenesis expand in many cases beyond their placeholder activity, and strategies render properly assembled r-subunits competent for translation.

THE CLASSICAL VIEW: PARALOGUES OF RIBOSOMAL PROTEINS

In yeast, most r-proteins genes are duplicated and encode identical or nearly identical paralogous r-proteins, which, apparently in numerous cases, are functionally redundant [25]. Strikingly, few r-proteins have additional paralogues that share extensive identity and similarity to them (e.g. [26-28]). These paralogues, also known as ribosomal-like proteins, are not natural components of mature ribosomes and are unable to functionally replace their r-protein counterparts, even when overexpressed (e.g. [26, 29]), but interestingly, all of them have a role during ribosome biogenesis [28, 30-32]. This fact prompted S. J. Baserga to propose that each ribosomal-like protein could act as a placeholder for its paralogous r-protein on the pre-rRNA. A placeholder factor acts by preventing the premature assembly of its r-protein counterpart on its rRNA binding site, which, both proteins, considering their extensive homology, may share [31].

Ribosomal-like proteins include Imp3, Mrt4, Rlp7 and Rlp24, which display considerable sequence homology to r-proteins S9 (uS4 according to the recently proposed r-protein nomenclature [33]), P0 (uL10), L7 (uL30) and L24 (eL24), respectively. In addition, part of the Nob1 endonuclease exhibits significant homology to S26 (eS26) [17] (Figure S2). Moreover, several trans-acting factors have gained ancient RNA-binding motifs in their structures that resemble those present in distinct r-proteins; for instance, Rrp5 contains 12 tandem S1 (5S1) RNA-binding motifs in its N-terminal domain [34]; Snu13 is member of a family of K-turn binding proteins that also includes human r-proteins L7A (eL8) and S12 (eS12), and yeast r-protein L30 (eL30) [35]. In this section, we discuss the functional relationship of Mrt4, Rlp24, Rlp7 and Imp3 with their paralogous r-proteins. The implications of the homology between Nob1, Rrp5 or Snu13 and their respective r-protein counterparts will not be discussed. In these three cases, it remains to be determined whether the trans-acting factors could have a placeholder activity.
From all these examples, perhaps the best-studied, homology-sharing pair of proteins consists of Mrt4 and P0. Mrt4 is homologous to the N-terminal domain of P0 (Figure S2), which corresponds to the rRNA binding domain of the r-protein [36]. P0 has an additional C-terminal extension that is exposed to the solvent and interacts with the acidic P1 and P2 r-proteins and translation elongation factors [37]. A few years ago, we could show that yeast P0 and Mrt4 are unable to bind simultaneously to r-particles by analysing the presence of either protein in complexes purified using functional TAP-tagged Mrt4 or P0 as affinity baits, respectively [29]. This observation, together with the fact that a Mrt4-P0 chimera protein, containing as N-terminal domain the Mrt4 ORF, is able to partially complement the otherwise lethal absence of P0 [29], and that a truncated P0 r-protein lacking its C-terminal domain functionally resembles Mrt4 [38], strongly suggest that P0 and Mrt4 compete for the same rRNA site in r-particles, thus, successively occupying this site during LSU maturation [29]. A similar scenario has been reported for human Mrt4 and P0 [39]. In full agreement with this hypothesis, the cryo-electron microscopy (cryo-EM) reconstruction of two distinct yeast pre-60S r-particles, which carry Mrt4 but lack P0, has revealed that indeed in these particles Mrt4 unequivocally localizes to a position equivalent to the one of P0 in the P-stalk of the mature LSU (Figures 1A and 1C) [40, 41]. Strikingly, theoretical estimation of the free RNA binding energy of both proteins suggests that P0 might bind to its rRNA site a little tighter than Mrt4 [29], a fact whose biological significance will be further discussed.

The dynamics of the sequential exchange reaction of Mrt4 with P0 have been studied in vivo. Different evidence indicates that Mrt4 is a nucleo-cytoplasmic shuttling assembly factor, which associates with early to intermediate pre-60S r-particles and predominantly dissociates from late, cytoplasmic pre-60S r-particles [20, 45]. The replacement of Mrt4 by P0, thus, takes place mostly in the cytoplasm, although it could also occur in the nucleus [38]. This replacement is a prerequisite to recycle Mrt4 back to the nucleolus [20]. How exactly this reaction takes place mechanistically is still unknown. Moreover, the exchange does apparently not occur directly, but instead, requires the participation of Yvh1, which is another nucleo-cytoplasmic shuttling assembly factor, non-homologous to either Mrt4 or P0 that co-enriches with late/cytoplasmic pre-60S r-particles [42, 45, 46]. Interestingly, Yvh1-containing r-particles do neither contain Mrt4 nor P0 and equivalent results are obtained in reciprocal experiments [42, 45, 46]. However, whether Yvh1 competes with the rRNA-binding site of Mrt4 and P0 has not been addressed until very recently [42]. It has been shown that the stable association of Yvh1 with pre-60S r-particles depends on the r-protein L12 (uL11), which is the closest neighbour of P0 at the base of the P-stalk [44, 47]. However, still in the absence of L12, there is apparently no difference in the efficiency of Mrt4 re-importation to the nucleus ([48], and our unpublished results). In conclusion, despite the fact that yeast Mrt4 and Yvh1 are non-essential proteins under standard laboratory conditions, both factors may play important roles controlling the position and timing of the assembly of P0, simultaneously providing a surveillance

**Figure 1: Mrt4 and Mex67 act as placeholder factors for the P0 r-protein.** (A) Position of Mrt4 (red) in the early pre-60S r-particles purified with Nog2-TAP (PDB ID: 3JCT; [40]). (B) Mex67-binding sites at the P0 neighbourhood (green), identified by CRAC [42], have been highlighted in the late/cytoplasmic Nmd3-TAP pre-60S r-particle (PDB ID: SH4P; [43]). (C) Position of P0 (red) in the mature 60S r-subunit (PDB ID: 3U5I, 3USH; [44]). Particles are viewed from the subunit interface slightly turned to the left. For orientation, the positions of the 5S rRNA (yellow), the L9 r-protein (royal blue) and the above CRAC sites of Mex67 (green) have been highlighted in the three structures. The ITS2 foot has also labelled in A. Note that some of the CRAC sites of Mex67 overlap with Mrt4 and P0 in A and C, respectively. The rest of RNAs are coloured in pale blue and the rest of r-proteins and/or factors in light cornflower blue. Images were generated using the UCSF Chimera program (www.cgl.ucsf.edu/chimera).
point to ensure that only mature LSUs can engage in translation (see below; further discussed in [14, 17, 20]).

Interestingly, the Hurt laboratory has recently described that the nuclear-export factor Mex67 is another placeholder of the P0 r-protein [42], even though, Mex67 barely displays sequence homology with either Mrt4 or P0 (Figure S2). This group has identified that the heterodimeric Mex67•Mtr2 complex, which is involved in the export of late pre-60S r-particles [49, 50], binds in vitro at two distant positions on Yvh1-purified pre-60S r-particles; the first one overlaps with the rRNA-binding site within the 5.8S rRNA of the RNA helicase Mtr4/Dob1, which is a cofactor of the exosome complex responsible of the 3’ end maturation of 7S pre-rRNAs to 5.8S rRNAs ([51]; for a review, see [52]); strikingly, the second position overlaps with the binding site of Mrt4 and P0 in pre-60S r-particles and the mature LSU, respectively (Figure 1B) [42]. Remarkably, it could be shown that the Mex67•Mtr2 complex can hardly bind late pre-60S r-particles containing Mrt4 in vitro; this result is in agreement with a competition between Mex67•Mtr2 and Mrt4 for the same binding site if assuming only a minor contribution of the Mex67•Mtr2 rRNA-binding site at the 5.8S rRNA in these particles [42]. Moreover, the structural characterization of Yvh1-containing pre-60S r-particles by cryo-EM reveals that Yvh1 binds adjacent to L12, a position that is close to but apparently not mutually exclusive to those of Mex67•Mtr2, Mrt4 or P0 [42].

Taken together, the following model for the timing of P0 assembly has been proposed: (i) Mrt4 binds first at the incipient P-stalk site of nuclear pre-60S r-particles. (ii) Later, but still in the nucleus, the heterodimer Mex67•Mtr2 bound to Yvh1 replaces Mrt4 in the pre-60S r-particles; then, Mex67•Mtr2 acts as one of the RanGTP-independent factors involved in the export of pre-60S r-particles to the cytoplasm; (iii) once in the cytoplasm, the assembly of P0 occurs concomitantly to the release of Mex67•Mtr2 and Yvh1 from the base of the P-stalk in the cytoplasmic pre-60S r-particles. How can we reconcile this model with apparently contradictory findings indicating that the release of Mrt4 takes place mostly in the cytoplasm? The answer is not obvious, but the scenario clearly suggests the existence of alternative pathways to perform the same reaction. Moreover, as nuclear export of pre-60S r-particles is mediated by several redundant but cooperative systems (reviewed in [14]) and Yvh1 is a non-essential factor [45, 46], it is conceivable that Mrt4 may exchange in the cytoplasm, not only independently of Mex67•Mtr2, but also of Yvh1.

Rlp24 versus L24
Another well-studied paralogous pair comprised of a ribosomal-like protein and an r-protein is represented by the conserved eukaryotic trans-acting factor Rlp24 and the LSU r-protein L24. Rlp24 and L24 share the N-terminal domain, which for L24 corresponds to the region that binds to mature LSU (Figure S2). Yeast Rlp24 belongs to the category

Figure 2: Rlp24 functions as a placeholder factor for the L24 r-protein. (A) Position of Rlp24 (red) in the early pre-60S r-particles purified with Nog2-TAP (PDB ID: 3JCT; [40]). (B) Position of L24 r-protein (red) in the mature 60S r-subunit (PDB ID: 3U5I, 3U5H; [44]). Particles are viewed from the subunit interface. For orientation, the positions of the 5S rRNA (yellow) and the L23 r-protein (royal blue) have been highlighted. The ITS2 foot has also labelled in A. Note that the last ca. 50 amino acids from the C-terminal part of Rlp24 and ca. 20 amino acids from the C-terminal end of L24 could not be modelled in the respective structures. The rest of rRNAs are coloured in pale blue and the rest of r-proteins and/or factors in light cornflower blue.
of trans-acting factors known as B factors, which are required for the proper maturation of 27S pre-rRNAs within intermediate pre-60S r-particles [53]. L24 is a non-essential LSU r-protein, whose role in LSU biogenesis has so far not been properly characterized [28]. In any case, there is enough evidence to conclude that Rlp24 functions as a bona fide placeholder for L24. First, L24 is not present in pre-60S r-complexes purified using TAP-tagged Rlp24 as a bait [28]. Second, both proteins seem to recognize the same binding site on r-particles, as shown by cryo-EM studies of distinct nuclear pre-60S r-particles, which reveal that the density found at the location of L24 in these particles clearly corresponds to the N-terminal part of Rlp24 (Figure 2) [40, 41]. As a corollary of this, release of Rlp24 from pre-60S r-particles is a pre-requisite for assembly of L24.

The replacement of Rlp24 with L24 occurs in the cytoplasm following different steps: (i) Rlp24, most likely assisted by the WD-40 repeat protein Mak11, associates in the nucleolus with very early pre-60S r-particles [28, 54]. This reaction appears to be coupled to the recruitment of the GTPase Nog1 to pre-60S r-particles, which directly and specifically interacts with Rlp24 [28, 40]. (ii) Upon arrival in the cytoplasm, the AAA-ATPase Drg1, which forms hexamers in the presence of ATP, binds to the exported pre-60S r-particles and allows the specific dissociation of Rlp24. This dissociation step is a prerequisite for the subsequent cytoplasmic maturation steps of these pre-60S r-particles, including the release of other shuttling factors such as Nog1 and Bud20 and the recruitment of later-acting cytoplasmic factors such as Rei1 [55-58]. A monomer of Drg1 is composed of an N-terminal domain followed by two consecutive AAA-ATPase domains: D1 and D2 (reviewed in [59]). In vitro, Drg1 binds specifically and directly to the C-terminal domain of Rlp24, which is not conserved in L24 [60]; consistently, expression of a truncated version of Rlp24 lacking the last 53 amino acids (Rlp24ΔC) prevents the recruitment of Drg1 to pre-60S r-particles in vivo, and, as a consequence, Rlp24ΔC is not properly released from cytoplasmic pre-60S r-particles and final maturation of nascent LSUs cannot be completed [55]. In vitro, the C-terminal domain of Rlp24 also stimulates ATP hydrolysis in both AAA domains of Drg1 [60]; while ATP hydrolysis in the D2 domain triggers the dissociation of Rlp24 from pre-60S r-particles, ATP hydrolysis in the D1 domain is required for the subsequent release of Drg1 from Rlp24 and likely the dissociation of the Drg1 hexamer into monomers [60, 61]. Interestingly, it has been shown that Drg1 also directly binds the FG-repeat nucleoporin Nup116, and more importantly, that this interaction optimizes the release of Rlp24 from pre-60S r-particles, suggesting some coupling between the export of pre-60S r-particles and the initiation of their cytoplasmic maturation [60]. (iii) Finally, Rlp24 is recycled back to the nucleolus and L24 stably assembles into pre-60S r-particles. However, in clear contrast to P0, which is required for the efficient release of its placeholder Mrt4 from pre-60S r-particles [20], likely indirectly by its role in dissociating Vyh1 and the Mex67•Mrt2 complex from those particles [55], it appears that L24 does not contribute to the release of Rlp24 from pre-60S r-particles.

Thus, the complete absence of L24, by the double deletion of the RPL24A and RPL24B genes, does not lead to a failure in either the release or the subsequent nuclear recycling of Rlp24 (cited as unpublished results in [28]).

The cytoplasmic assembly of L24 appears to be coupled to the recruitment of the non-essential factor Rei1 to pre-60S r-particles [62]. Rei1 is highly homologous to Reh1, and, it has been shown that both factors have a partially redundant function during maturation of nascent LSUs [62, 63]. Rei1 directly interacts with the J-domain protein Jjj1, which recruits and activates the Hsp70-type ATPase Ssa1-Ssa2 [64-66]. Different authors have shown that one of the primary roles of Rei1 is the release and nuclear recycling of the heterodimeric Arx1•Alb1 complex from cytoplasmic pre-60S r-particles, although it is not known how this reaction mechanistically occurs [64, 66, 67]. Moreover, this activity has indeed been questioned and attributed to either Jjj1 and Ssa [68] or Reh1 [43], which both have been suggested to release simultaneously Rei1 and Arx1. The recent cryo-EM characterization of r-particles containing Rei1, Arx1 and Jjj1 or with Reh1, Arx1 and Alb1 at near-atomic resolution [68, 69] will allow the development of models that clearly will help to solve this question. These and others models derived from cryo-EM reconstruction analyses of selected pre-60S r-particles (e.g. see [70]), unambiguously show that Arx1 binds near the solvent-exposed side of the polypeptide exit tunnel (PET), suggesting that it could function as a placeholder for different nascent chain-associated factors, including methionine aminopeptidases (MetAPs) (discussed later). Interestingly, these analyses also reveal the global structure of Rei1 on r-particles, notably showing that its C-terminal segment penetrates into the PET and extends almost up to the peptidyl transferase centre (PTC) [68]. Strikingly, this segment is structurally homologous to the C-terminal extension of the GTPase Nog1 [40] and to the one of Reh1 [43], which could also similarly insert into the PET; thus, the binding of these three factors to pre-60S r-particles is mutually exclusive and, as it will be discussed later, confers directionality to the cytoplasmic LSU maturation. These findings therefore indicate that Nog1 is a placeholder factor for Rei1, and in turn, Rei1 a placeholder factor for Reh1. Alternatively, Rei1 and Reh1 may have redundant functions and Nog1 could function as placeholder for either factor (see below).

THE EXCEPTIONS

Not all ribosomal-like proteins act as placeholders of their respective paralogous r-proteins. Indeed, different studies on the trans-acting factors Rlp7 and Imp3 clearly contradict the intuitive hypothesis that these factors could compete with their counterpart r-proteins L7 and S9, respectively, for the same binding sites on the pre- or mature rRNAs.

Rlp7 versus L7

Our group, in collaboration with that of M. Fromont-Racine and A. Jacquier, reported a few years ago that Rlp7 and L7 could coexist in the same pre-60S r-particles; consistently, cross-linking and cDNA analysis (CRAC) experiments dem-
onstrated that the Rlp7 and L7 binding sites are actually distinct in pre- and/or mature rRNAs and distant enough from each other that they do not result in steric binding interference [71]. Similar findings were independently obtained by the laboratory of J. L. Woolford, Jr. [72]. Rlp7, which is 78 amino acids longer than L7, shares a considerable overall sequence and structure homology with L7, except in its N-terminal region, its internal loop and few other discrete regions (Figure S2). Rlp7 belongs to the group of proteins known as A3 assembly factors [10], which hierarchically and interdependently associate with early pre-60S r-particles and are globally required for optimal 5’ to 3’ exonucleolytic trimming of the 27SA3 pre-rRNA to the 27SBS pre-rRNA, a processing step that generates the 5’ end of mature 5.8S rRNA [10, 31, 52, 73, 74]. However, the rRNA-binding site of Rlp7, as those of the other A3 assembly factors, maps to positions in the ITS2 spacer instead of positions close to the 5’ end of 27SA3 pre-rRNA in the ITS1 spacer [71, 72, 75]. It has been suggested that the A3 assembly factors may play structural roles in chaperoning ITS2 within pre-60S r-particles, thus protecting 27S pre-rRNAs from rapid turnover and facilitating their correct processing [further discussed in [10, 73, 75], see also [51]].

Cryo-EM analyses have confirmed the rRNA-binding sites of Rlp7 and several other A3 factors. These sites cluster around ITS2, providing an explanation to the interdependent association of A3 factors with pre-60S r-particles [40, 41]. However, in the crystal structure of mature LSUUs, the globular domain of L7 binds domain II of 25S rRNA, as well as 5S rRNA, while its N-terminal extension interacts with the expansion segment ES7 of 25S rRNA (Figures 3A and 3B) [44, 47]. In agreement with our data [71], the L7 interactions already exist in pre-60S r-particles, as also revealed by cryo-EM analyses of selected pre-60S particles (Figures 3A and 3B) [40, 41]. Curiously, it has been shown that L7 is also required for 27SA3 pre-rRNA processing [76-78]. It seems that the assembly of L7, as that of any of its neighbouring r-proteins in RNA domain II, such as L4 (uL4), L6 (eL6), L14 (eL14), L16 (uL13), L18 (eL18), L20 (eL20), L32 (eL32) and L33 (eL33), allows the stabilisation of rRNA structures within pre-60S r-particles, which is a prerequisite for the stable association and function of A3 assembly factors ([77, 79], further discussed in [80]).

Likely, Rlp7 has evolved from the highly conserved L7 r-protein to use a similar recognition motif to bind and function at a different location during LSU maturation. However, it is unclear how proteins showing apparently very similar architectures could be specifically targeted to different places in pre-ribosomal complexes, instead of competing for the same RNA substrates. As a possibility, we can imagine a scenario in which distinct co-factors specifically recruit the trans-acting factor or the corresponding paralogous r-protein to the pre-rRNAs at their different RNA binding sites and help their stable association or assembly.

Figure 3: Rlp7 is not the placeholder factor for the assembly of L7 r-protein. (A) Position of Rlp7 (red) and L7 (purple) in the early pre-60S r-particles purified with Nog2-TAP (PDB ID: 3JCT; [40]). (B) Position of L7 r-protein (purple) in the mature 60S r-subunit (PDB ID: 3U5I, 3U5H; [44]). Particles are viewed from the solvent side. For orientation, the positions of the 5S rRNA (yellow), 5.8S rRNA (gold) and the L9 r-protein (royal blue) have been highlighted. The ITS2 foot has also labelled in A. Note that L7 is found at its final assembly position within Nog2-TAP pre-60S r-particles. The first ca. 20 amino acids of both proteins could not be modelled in either structure. The rest of rRNAs are coloured in pale blue and the rest of r-proteins and/or factors in light cornflower blue.
Imp3 versus S9

Imp3 is a paralogue of the SSU r-protein S9. The similarity of these two proteins extends all over their complete sequences (Figure S2), including their putative, conserved RNA-binding domains [32]. The functional role of Imp3 has been investigated in vitro and in vivo; Imp3 is an essential trans-acting factor required for SSU biogenesis, more specifically for the cleavage at the early sites A0, A1 and A2 in the 35S pre-rRNA within 90S pre-ribosomal particles [32]. It has also been shown that Imp3, together with the factors Mpp10 and Imp4, forms a stable sub-complex [32, 81], which co-transcriptionally associates with the 5'-ETS region of 35S pre-rRNA [82-84]. In vitro experiments have suggested that the association of this sub-complex with 90S pre-ribosomal particles is required to mediate and stabilize specific base-pair interactions of residues in 5' ETS with the hinge region on the 5' end of the U3 snoRNP [85, 86]; these base pairings are indeed critical for in vivo ribosome maturation (reviewed in [87]). In turn, S9 is an essential r-protein, which is also required for early pre-rRNA processing at the A0-A2 sites [88]. In yeast and humans, S9 is a primary binding r-protein that assembles likely co-transcriptionally to the body of the SSU [89, 90], more specifically at positions corresponding to helices H3, H12, H17, and expansion segment E56S of the 18S rRNA, while also interacting with other SSU r-proteins such as S2 (uS5), S4 (eS4), S24 (eS24) and S30 (eS30) (Figure 4) [44, 91]. The cryo-EM reconstruction of 90S pre-ribosomal particles from both S. cerevisiae and Chaetomium thermophilum, an ascomycete related to S. cerevisiae [92], has recently been obtained [93-95]. These reconstructions have allowed the identification of the position of many trans-acting factors from these particles, among them, Imp3 and about a dozen r-proteins from the SSU. Some of these r-proteins have consistently been identified as stable components of purified 90S pre-ribosomal particles [96-98]. In the reconstructions, Imp3 binds to Mpp10 and Imp4, and this latter approaches the 5' part of U3 snoRNP. In agreement with its early assembly, S9 was found among those SSU r-proteins identified in the 90S r-particles. Importantly, S9 appears to be bound to its final rRNA-binding site within the nascent 18S rRNA, adopting a mature-like conformation in the particles (Figure 4) [93, 94]. In conclusion, all these data indicate that Imp3, despite its similarity to S9, is not its placeholder factor in 90S pre-ribosomal particles.

NEW PLACEHOLDER FACTORS: THE MOST RECENT DATA

In the last ten years, the use of CRAC and cryo-EM methodologies has permitted gaining information concerning the rRNA-binding sites and the location of a considerable number of trans-acting factors, especially from yeast, within pre-ribosomal particles. These achievements, together with the information available on the structure and location of all r-proteins revealed by crystal structures of ribosomes or r-subunits of different eukaryotes, the compositional analysis of pre-ribosomal particles and, importantly, the extended body of genetic and biochemical data on the role of trans-acting factors and r-proteins, are providing clues into the mechanistic details of the ribosome assembly process for the first time at high resolution. Particularly relevant to the scope of this review has been the discovery
of many other examples of placeholder factors that mask particularly important ribosomal sites until a specific r-subunit maturation event has been accomplished. In this section, we enumerate several examples of placeholder factors that we consider to have a clear biological relevance, focusing on those that block the recruitment of essential translation factors or r-proteins to cytoplasmic pre-ribosomal intermediates, thus, ensuring that only mature r-subunits engage in protein synthesis.

**Tsr1 blocks binding of both the GTPase eIF5B and the ATPase Rno1 to late pre-40S ribosomal particles**

Tsr1 is an essential conserved trans-acting factor required for efficient nucleo-cytoplasmic transport of pre-40S r-particles and processing of 20S pre-rRNA to mature 18S rRNA [99, 100]. Tsr1 has been shown to be recruited to early pre-40S r-particles in the nucleolus and accompany them together with a few other trans-acting factors (Dim1, Enp1, Ltv1, Pno1/Dim2, Nob1, and Rio2) to the cytoplasm. There, Tsr1 rapidly dissociates and is recycled back to the nucleolus [100, 101]. Interestingly, Tsr1 is structurally related to translational GTPases such as EF-Tu or eIF5B/Fun12 in their GTP-bound form. However, Tsr1 neither is a GTP-binding protein nor has GTPase activity [102]. As expected from this structural similarity, cryo-EM and CRAC analyses confirmed that Tsr1 binds, albeit differently than standard translational GTPases such as EF-Tu or eIF5B/Fun12 in their GTP-bound form. However, Tsr1 is neither a GTP-binding protein nor has GTPase activity [102].

As expected from this structural similarity, cryo-EM and CRAC analyses confirmed that Tsr1 binds, albeit differently than standard translational GTPases such as EF-Tu or eIF5B/Fun12 in their GTP-bound form. However, Tsr1 is neither a GTP-binding protein nor has GTPase activity [102].

Moreover, the position of Tsr1 on pre-40S r-particles potentially impedes joining of these particles to LSUs and occludes part of the mRNA channel [21, 102].

In conclusion, the presence of Tsr1 on pre-40S r-particles is incompatible not only with maturation of SSU but also with translation. Thus, Tsr1 is a good example of a placeholder factor for a set of distinct factors that times key steps during SSU formation and function. How and when the dissociation of Tsr1 from pre-40S r-particles is triggered is still unknown.

It is worth mentioning that Tsr1 shares substantial sequence identity with another GTPase, Bms1 [99]. As Tsr1, Bms1 is an essential trans-acting factor involved in SSU biogenesis, but in contrast to Tsr1, Bms1 is required for pre-rRNA processing at the early sites A0, A1 and A2 [99, 105]. Bms1 is a stable component of 90S pre-ribosomal particles [96] that likely binds co-transcriptionally to the nascent pre-rRNA [83, 84, 106] and apparently efficiently dissociates following the formation of early nuclear pre-40S r-particles [100]. As mentioned above, Tsr1 seems to be recruited to these type of pre-40S r-particles. Interestingly, Bms1 has been unambiguously modeled into the cryo-EM structure of 90S pre-ribosomal particles [93-95]. In one of these articles, the authors have claimed that the binding site of Bms1 overlaps significantly with that of Tsr1 and suggested that Bms1 could likely work as placeholder for Tsr1 during the transition of 90S to pre-40S ribosomal particles [95]. However, whether or not release of Bms1 is linked to recruitment of Tsr1 is still unexplored.

**Molecular events involving Nmd3**

Nmd3 is an essential conserved factor that connects pre-60S r-particles to the Crm1/Xpo1 exportin by its nuclear export sequence (NES), thereby enabling the RanGTP-dependent export of late pre-60S r-particles from the nucleolus [107, 108]. Nmd3 exits to the cytoplasm associated with pre-60S r-particles where it is released and then recycled back [107]. It has been demonstrated that a truncated version of the Nmd3 protein lacking its last 100 amino acids, which includes its NES but not its nuclear localization sequence (NLS), is able to bind pre-60S r-particles that remain trapped in the nucleolus [107]. Dissociation of Nmd3 from cytoplasmic pre-60S r-particles is imperative for r-subunit joining and translation initiation [109]. Moreover, this reaction involves the activity of the cytoplasmic GTPase Lsg1/Kre35 and the assembly of L10 (uL16), which seems to stably lock into its final position on cytoplasmic pre-60S r-particles concomitantly to the removal of Nmd3 [110, 111]. In line with this model, distinct mutations in LSG1 or RPL10 or depletion of L10 cause a retention of Nmd3 on cytoplasmic pre-60S r-particles and are synthetically lethal with specific nmd3 mutants [111], while over-expression of wild-type Nmd3 or the presence of mutated Nmd3 versions with reduced affinity for pre-60S r-particles suppress the growth defect and the failure to recycle Nmd3 in lsg1 and/or rpl10 mutants [111, 112].

Cryo-EM analyses of purified mature LSUs harbouring in vitro-reconstituted MBP-tagged Nmd3 or of native Nmd3-purified pre-60S r-particles have shown that Nmd3 binds to the intersubunit face of pre-60S r-particles spanning from the L1 (uL1) stalk to the position where Tif6 binds (see later), going through the E- and P-sites (Figure 5C) and contacting helices H38 (also known as the A-site finger), H65 and H95, in addition to the sarcin/ricin loop (SRL) in 25S rRNA [43, 113, 114]. These findings are consistent with the Nmd3-binding sites detected by the CRAC method (Figure 5A) [115]. The association of Nmd3 to pre-60S r-particles seems to be very dynamic and, in unison with the L1-stalk, it could adopt several states [114]. Most, if not all, of these states are incompatible with the simultaneous presence of the Sdo1•Efl1 complex in the r-particles (also discussed later) [114, 116] or r-protein L40 (eL40), which, as L10, also assembles in the cytoplasm [117]. In agreement, purified pre-60S r-particles purified via TAP-tagged Nmd3 clearly lack some r-proteins, among them L10, L40, L12 (uL11) and L41 (eL41) [43]. Thus, due to the fact that mature LSUs contain r-proteins L10 and L40, it is expected that the position of Nmd3 in the in vitro reconstituted LSUs may not fully correspond with the one it adopt within native pre-60S r-particles [43, 114] (see also Figure 5C). Moreover, as a corollary of the steric clash between Nmd3 and Sdo1•Efl1, it could be deduced that Lsg1 might not bind to the GTPase-associated centre (GAC) on pre-60S r-particles; indeed, the Hurt laboratory has suggested that Lsg1 could contact pre-60S r-particles also at the interface face of LSU but close to helix H69 of 25S rRNA.
These suggestions have been later confirmed by cryo-EM analysis of native or reconstituted Nmd3-containing particles [43, 114]. These cryo-EM studies fully explain why r-subunit joining and translation are not possible as long as Nmd3 is not released from pre-60S r-particles. Moreover, they could also reveal that the C-terminal domain of Nmd3 adopts a structure that mimics that of translation elongation factor eIF5A and binds similarly as eIF5A to the E-site of pre-60S r-subunits [114]. Importantly, these studies also revealed that most of the Nmd3-binding sites overlap with those of the GTPase Nog2 [115], which have been determined by cryo-EM [40]. In agreement, purification of pre-60S r-particles indicates that Nog2 and Nmd3 are not simultaneously present on the same pre-60S r-particles (Figure 5).

Different experimental approaches indicate that the binding of Nog2 to early pre-60S r-particles precedes that of Nmd3 (e.g. [40]), and consistently, the depletion of Nog2 causes the premature binding of Nmd3 to these particles [115].

Altogether, these experiments indicate that Nog2 is the placeholder factor of Nmd3, which acts by blocking the premature recruitment of the latter and, therefore, providing the time frame necessary for pre-60S r-particles to acquire their export competence. Interestingly, this activity seems to be coupled to that of other trans-acting factors, such as the AAA-ATPase Rea1 and its substrate Rsa4, in a way where stable binding of Rea1 and Rsa4 to pre-60S r-
Based on the structural similarity, it has been suggested to each other, Tif6 and r-protein L10 [19, 119, 121, 122]. Domains, which resembles bacterial ribosome recycling factors, such as Tif6, Nmd3 also impedes premature association of pre-60S r-particles with SSUs.

**Efl1•Sdo1 probes critical functional sites on pre-60S r-particles during LSU maturation**

Efl1/Ria1 and Sdo1 (yeast SBDS orthologue) are quasi-essential trans-acting factors required for cytoplasmic LSU maturation [19, 119, 120]. Efl1 is a cytoplasmic GTPase composed of five structural domains; it is highly homologous to the translation elongation eEF2 factor, which is responsible for the translocation reaction of the ribosome following each round of polypeptide elongation [1, 19, 119]. In turn, Sdo1 is a very flexible protein, formed by three domains, which resembles bacterial ribosomal recycling factor RRF [116, 121]. Both proteins are functionally related to each other, Tif6 and r-protein L10 [19, 119, 121, 122]. Based on the structural similarity, it has been suggested that Efl1 interacts with pre-60S r-particles in a very similar manner as eEF2 does with 80S ribosomes; recruitment of Efl1 needs the presence of P0, the largest component of the ribosomal P0/P1/P2 stalk (uL10/P1/P2) [55] and the P-stalk base, the r-protein L12 (uL11), on the pre-60S r-particles [48]; then, Sdo1-stimulated GTP hydrolysis might trigger a sort of translocation reaction that facilitates the dissociation and recycling of Tif6 (see [116, 120-122], and references therein).

Recently, cryo-EM analyses of reconstituted pre-60S r-particles containing or lacking endogenous Tif6 from Dictyostelium discoideum and harbouring both human EFL1 and SBDS have allowed building models that deduce the precise interaction of these three factors with pre-60S r-particles (Figure 5). These models also provide a molecular scenario to understand how the release of Tif6 from these particles occurs [116]. In this possible scenario: (i) first, SBDS is recruited to Tif6-containing late cytoplasmic Tif6 from pre-60S particles; in the assembly of L10 and the P-stalk have already occurred. Tif6 is at its canonical position on the LSU interface bound to the C-terminal part of L23 (uL14) in the proximity of the SRL and the N-terminus of L24, thereby inhibiting r-subunit joining and, thus, preventing pre-60S r-particles from prematurely engaging in translation [47, 123]; the three domains of SBDS adopt a "closed conformation": domain I occupies the P-site of LSU and is in contact with a flexible loop of L10 (named P-site loop) [122], components of the PTC and the entrance of the PET; domain II mediates binding of SBDS to 25S rRNA, and domain III, which structurally resembles domain V of Efl1, contacts the SRL and the neighbouring base of the P-stalk [116]. (ii) Second, EFL1 in its GTP-bound form binds the nascent GAC from the pre-60S r-particles, while contacting both Tif6 and SBDS, and r-proteins P0 and L12. Interestingly, the binding of domain III of SBDS on pre-60S r-particles is mutually exclusive with that of domain V of EFL1, thus, it seems that, upon EFL1 recruitment, SBDS undergoes a switch to reposition itself in a "open conformation" where its domain II rotates 60° relative to its domain I and, especially, its domain III rotates 180° away from the base of the P-stalk [116]. (iii) These changes are supposed to accommodate EFL1 into the GAC leading to slight conformational adjustments that result in a more extensive binding of domain I of EFL1 to the SRL in the pre-60S r-particles; this new SRL-bound conformation overlaps with part of the Tif6-binding site and it is supposed to facilitate the release of Tif6 from these particles. (iv) The accommodated state of EFL1 also seems to stimulate the GTP hydrolysis of the factor, which causes another conformational change of EFL1 that destabilizes its interaction with pre-60S r-particles and that of SBDS; as a consequence, both SBDS and GDP-bound EFL1 dissociate from these Tif6-lacking pre-60S r-particles. Still, the exact role of GTP hydrolysis for Tif6 eviction remains to be elucidated [116].

Together, these findings indicate that Efl1 and Sdo1, as other above-mentioned examples, are factors with decisive roles in timing LSU maturation. Sdo1 examines the structure and function of important sites of LSUs, such as the PTC, the P-site and the entrance of the PET. Sdo1 seems to verify the assembly of L10. In turn, Efl1 proofreads the integrity of the GAC in LSUs, once the P-stalk has been properly assembled. Any delay or defect in the evaluation of all these sites would have as a consequence the inefficient release of Tif6 from pre-60S r-particles, thus, preventing translation by these apparently aberrant particles. Warren and co-workers assume that the Efl1•Sdo1-dependent maturation reaction might occur downstream of the assembly of L10 and the release of Nmd3 ([107, 108, 111]; for a review, see [17]). This scenario differs from the one previously and currently reported by the Johnson laboratory, claiming that the release of Tif6 occurs prior to [18, 55] or could be coupled with the Lsg1-mediated release of Nmd3 [114], respectively. Future experiments are clearly required to clarify these issues.

**Nog1, Re1 and Re1 are plugs that block the nascent polypeptide exit tunnel of LSUs**

As previously mentioned, cryo-EM has allowed to deduce that Nog1 is the placeholder of Re1 (for a review, see [124]). Notably, both Nog1 and Re1 enter the tunnel from the exit side with their C-terminal ends oriented to the PTC [40, 68] (Figure 6A and B). This N- to C-terminal orientation resembles that of the nascent polypeptide chain during translation. Nog1 is replaced by Re1 in the cytoplasm, however, the precise mechanism of this exchange is still elusive [55-58]. Another protein, SBDS (Sdo1), is able to bind the exit tunnel, but in this case, the interaction takes place only via a very short extension, involving tunnel insertion of the N-terminal end of Sdo1, nearby the PTC [116]. In a recent review, B. J. Greber has nicely modelled how SBDS and Re1 interact without overlapping in the tunnel in an arrangement where the N-terminal end of SBDS is positioned close to the C-terminal end of Re1, a circumstance that could not occur in the case of SBDS and Nog1 [124]. In this manner, the entire tunnel is filled, thus,
providing a situation that could allow the complete functional proofreading of the integrity of this ribosomal site in cytoplasmic pre-60S r-particles.

Recently, the C-terminal helix of Reh1, which is highly homologous to that of Rei1 both at the level of sequence and structure, has also been found inserted in the PET of cytoplasmic pre-60S r-particles. The orientation of the C-terminal tail of Reh1 inside the tunnel is similar to that of the C-terminal extension of Rei1 (Figure 6C) [43]. It seems that Reh1 binds downstream of Rei1 during the maturation of these particles, as suggested by the fact that Reh1-containing pre-60S r-particles lack the Arx1•Alb1 complex [63].

Nug1 and Dbp10: more overlapping interactions

The conformation of the nascent PTC is also verified by different factors during LSU maturation, which are expected to act sequentially on this site (Figure 5). First, distinct residues of the PTC are known to be subjected to pseudouridylation and 2'-O-methylation by specific snORPs (for an example, see [125]), or base methylation by the site-specific methylases Spb1 and Nop2 [126, 127]. These snORPs and methylases seem to associate with and act on early pre-60S r-particles [53, 128]. Second, also the trans-acting factor Nsa2 [129] has been shown to bind to rRNA near the immature PTC, most specifically to positions close to the base of helix H89 in 25S rRNA [130], where it interacts with Nog1 and Nog2 [40]. Interestingly, Nsa2 associates with pre-60S r-particles only after formation of 27S pre-rRNAs [53, 131] and this association is dependent on the presence of other LSU maturation factors, including Nop2, Dbp10 and Nog1, on pre-60S r-particles [53]. Third, it has been also shown that the RNA helicase Dbp10 binds around the base of helix H89 [118]. These sites partially overlap with those of Nsa2, Nmd3, and of GTPases Nug1 and Nog2 [115, 118, 130]. Indeed, Nug1 and Dbp10 functionally interact with each other [132]. Moreover, the association of Dbp10 with pre-60S r-particles is dependent on the presence of Nug1 [118].

All these findings, together with the above described binding sites of Nmd3, Nog1, Nog2 or Sdo1, as well as the assembly position of r-proteins such as L10, provides an idea of the, still to be unveiled, complex arrangement of sequential actions and interactions of factors during the maturation of strategically relevant, functional sites of r-subunits (for a review, see also [124]).

Arx1 versus translation-associated factors that bind the ribosome exit tunnel.

Arx1 is a non-essential trans-acting factor that has the capability to bind to FG-repeat nucleoporins, thereby, functioning as a RanGTP-independent export factor of pre-60S r-particles [133-135]. The human orthologue of yeast Arx1, EBP1, folds as a MetAP, an enzyme removing the N-terminal methionine from nascent polypeptides as they emerge from the exit tunnel of the ribosome [133]. Given the close homology between EB1 and Arx1, it was deduced that Arx1 also conserves the MetAP core fold. However, both EB1 and Arx1 lack MetAP activity, as the critical residues of the methionine-binding pocket are different [133]. The interpretation of the cryo-EM structures of either purified pre-60S r-particles containing Arx1 [70] or in vitro reconstituted 60S r-subunits complexed with Arx1 [69] is in full agreement with this deduction. In these mod-
els, Arx1 binds nearby to the outside part of the PET (Figures 6A and 6B) [69, 70] in a position that seems to overlap with the one of MetAPs [136]. Consistently with this, the rRNA residues contacted by Arx1, which were identified by CRAC analysis, are clustering at the outside of the ribosome exit tunnel [70]. Moreover, either the addition of a GFP-tag to r-proteins L25 (uL23) or L35 (uL29), which both surround the outside of the PET, or the depletion of L35 significantly reduce the binding of Arx1 to pre-60S r-particles [67, 137].

In conclusion, these findings indicate that Arx1 could act as a structural proofreader of the correct formation of the solvent-side part of the exit tunnel [138]. The inefficient recruitment of Arx1 to eventually aberrant pre-60S r-particles may impair export of these particles to the cytoplasm, leading to their transient accumulation in the nucleus and inducing their rapid turnover. Arx1 may also represent a placeholder for those ribosome-associated factors that bind the outside of the tunnel during translation, such as the ribosome-associated complex, the nascent polypeptide-associated complex, the chaperones Ssb1/Ssb2 or the signal recognition particle (for a review see, [139]). This activity could prevent the premature recruitment of all these factors. The presence of Arx1 at its binding site also impairs the association of pre-60S r-particles with the endoplasmic reticulum-translocon complex, which acts as a channel to deliver nascent proteins to the lumen of the endoplasmic reticulum (e.g. see [140]).

PARAPHERNALIA FOR THE NUCLEAR IMPORT AND ASSEMBLY OF RIBOSOMAL PROTEINS

Most newly synthesized r-proteins need to be transported to the nucleolus to reach their assembly sites in the pre-ribosomal particles. The specific interactions of the r-proteins with the general import factors, mostly β-karyopherins [141, 142], are normally mutually exclusive with their interactions with the rRNAs. This feature also applies for the interactions that selected r-proteins undergo with specific factors that help their import or assembly, also referred as dedicated chaperones and escortins [143-145]. In addition to help import or assembly, β-karyopherins, dedicated chaperones and escortins prevent the aggregation of r-proteins, which are prone to aggregate since they contain highly basic and intrinsically disordered extensions [146]; these factors also impede r-proteins to either be degraded [147] or inappropriately interact with other cellular RNAs prior to their assembly into pre-ribosomal particles [146].

In a recent report, it has been shown that most, if not all, NLSs of yeast r-proteins reside within long non-globular extensions of the proteins. These extensions thread across the surface of the r-subunits making extensive contacts with the rRNAs or penetrate into the interior of the r-subunits intertwining with and stabilizing rRNAs [141]. Kap123 is the RanGTP-dependent β-karyopherin in charge of recognising the NLSs of most r-proteins [142], although other β-karyopherins, such as Kap104, Kap108 or Kap121 [143, 148, 149], and even the importin-α Kap60 [150] have been reported to bind the NLSs of specific r-proteins. To our knowledge, no structural data of any r-protein bound to a karyopherin are available at atomic resolution, except for the recent determination of the crystal structure of Kap104 in complex with the PY-NLS of L4 (uL4) [149]; here, the residues of the PY-NLS of L4 engages the concave surface on the structure of Kap104, similarly as other importins recognize their selected NLSs [145].

So far, seven specific systems, composed of a dedicated chaperone or an escortin and an r-protein, have been reported in yeast, a list that may still be far from being completed: Acl4•L4, Bcp1•L23, Rbr1•L3, Sqtl•L10, Syo1•L5•L11, Tsr2•S26 and Yar1•S3 (for a review, see [151]). For most of them, the binding sites of the chaperone on the respective r-protein have been mapped, and for several of them structural information is also available. From all these studies, it could also be concluded that the mode of interaction of the respective r-proteins with their specific chaperone partners is highly similar and mutually exclusive with that observed for these r-proteins when assembled into the pre-ribosomal particles. Interestingly, most of these chaperones capture co-translational their targets by, in most cases, interacting with their very N-terminal ends, a property that minimizes the risk of r-protein aggregation [152].

Acl4 and L4

Acl4 is a non-essential tetratricopeptide repeat-like (TPR-like) factor, which has been identified as the specific dedicated chaperone of free L4 (uL4) [153, 154]. Acl4 constitutes one exception to the principle of co-translationally recruitment through the N-terminal end of the r-protein partner since, although it is true that Acl4 is recruited to nascent L4, it directly interacts with the long internal loop of the r-protein (amino acids 43-114) [149, 153, 154]. Additionally, the eukaryote-specific C-terminal extension of L4 harbours a PY-NLS, which binds specifically to another Acl4 molecule that is later replaced by the karyopherin Kap104 [149, 153, 154]. Importantly, both the internal loop and the C-terminal tail of L4 occupy strategic positions in mature LSUs [44, 47]; the internal loop penetrates into the core of the LSU in the direction to the PTC, forming a narrow constriction within the exit tunnel where it even forms some contacts with various residues of the nascent protein chains [155]; the C-terminal tail of L4 threads a long tour across the solvent-exposed interface of the LSU in direction of the P-stalk, approaching and making numerous contacts with other r-proteins, such as L18 (el18), L7 (ul30), L20 (el20) and L21 (el21) and rRNA sequences, including the expansion segments ES15 and ES7 of the 25S rRNA [44, 156]. Recently, the crystal structure of L4, excluding its C-terminal extension, in complex with Acl4 has been resolved at atomic resolution [149]. In this complex, the majority of the internal loop of L4 is bound by the concave surface of Acl4, an interaction that is totally mutually exclusive with that this region of L4 forms within the LSUs [149].

It has been shown that Acl4 actively travels as a trimeric Kap104•L4•Acl4 from the cytoplasm to the nucleolus where the assembly of the r-protein in early pre-60S r-
particles takes place [79, 152]. As for other specific chaperones, Acl4 does not significantly associate with pre-60S r-particles [153, 154], but is expected to facilitate the assembly of L4 into these particles; how this process mechanistically occurs is currently unclear.

**Rrb1 and L3**

Rrb1 is WD-repeat protein predicted to form a β-propeller structure and involved in LSU maturation [157, 158]. The function of Rrb1 in LSU biogenesis is linked to that of the r-protein L3 (uL3) [157, 158]. Indeed, the Kressler laboratory has nicely demonstrated that Rrb1 captures nascent L3 in a co-translational manner via its interaction with the first 15 amino acids of the r-protein [152]. Following this cytoplasmic recognition, Rrb1 then accompanies L3 to its assembly site on early nucleolar pre-60S r-particles [78, 152, 157]. Unfortunately, no structural data have so far been reported for this interaction, although it has been speculated to occur in a similar manner as that between Sqt1, another WD-repeat protein, and its specific partner, the r-protein L10 (see below) [151]. Despite this issue, it is clear that the interaction of L3 with Rrb1 is mutually exclusive with the ribosomal interaction of L3. In this sense, the N-terminal end of L3, which is recognized by Rrb1, penetrates deep into the core of the LSU where it reaches towards the PTC, nested between helices H90 and H92 of 25S rRNA [44, 159].

**Sqt1 and L10**

The essential WD-repeat protein Sqt1 is a dedicated chaperone that has been proposed to facilitate the assembly of L10 (uL16) into cytoplasmic pre-60S r-particles [110, 152, 161]. Two decades ago, Sqt1 was identified as a high-copy suppressor of selected rpl10 mutants [161]. Additionally, it has been reported that Sqt1 interacts with L10 by the two-hybrid system [161], by co-immunoprecipitation [110, 152] or by an in vitro binding assay [152]. Sqt1 recognizes the N-terminal part of L10 [110, 152, 162] and, as described for Acl4 and Rrb1, the chaperone is already recruited to the nascent L10 as it is translated from its mRNA [152]. The complex between the WD-repeat propeller-like domain of Sqt1 and the N-terminal part of L10 has been co-crystallized and structurally resolved to atomic resolution [152]. This analysis shows how the very basic N-terminal end of L10, which forms an α-helix, is accommodated by the negatively charged top surface of the propeller [152]. Importantly, this study also revealed that the conformation of the corresponding N-terminal residues of L10 bound to

![Image](https://via.placeholder.com/150)

**Figure 7:** The helix H84 in the 60S r-subunit, the symportin Syo1 and the p53 regulator MDM2 share the same binding site on L11 r-protein. (A) Structure of the 5S RNP as is assembled in the mature 60S r-subunit bound to 25S rRNA (nucleotides 2651-2750 comprising helices H83, H84, H85 and H86). (B) Interaction of a specific region of Syo1, called Syo1-HS (amino acids 328-384), with L11 in the context of the 5S RNP. (C) Structure of the 5S RNP bound to a distinct fragment of MDM2 (amino acids 293-334). The fragment of 25S rRNA is coloured in yellow, that of Syo1 in light gold, and that of MDM2 in dark gold; 5S rRNA in highlighted in red, L5 in cyan and L11 in blue. PDB ID: The 5S RNP bound to 25S rRNA was taken from 3U5I and 3U5H [44]; Syo1-HS fragment was extracted from 5AFF [163] after superimposing the structure shown here with that present in the PDB file 4GMN [144]; MDM2 fragment was taken from 4XXP [164] after superimposing the structure shown in this file with that of L11 shown in A.
Sqt1 is not compatible, hence mutually exclusive, with the positioning of these residues, which interact with helix H89 of 25S rRNA, in the mature LSU. This information, together with all the genetic and biochemical data available for L10 and Sqt1, as well as those factors functionally related to L10, such as Ndm3, Sdo1 or Efl1, is very relevant to unravel the molecular dynamics of the delivery of L10 from Sqt1 to cytoplasmic pre-60S r-particles, which is still not fully understood (see [152] for further discussion of this issue).

**Syo1 and L5 and L11**

The symporter Syo1 is an exceptional factor since it is the only described case of a chaperone dedicated to the synchronous co-import of two conserved r-proteins to the nucle(o)lus: L5 (uL18) and L11 (uL5), which are the two protein components of the 5S rRNP structure of LSUs [144]. The crystal structure of *C. thermophilum* Syo1 complexes reconstituted with L5, L11 and/or 5S rRNA have been reported [144, 163]; Syo1 is an open α-solenoid comprised of four consecutive ARM-repeats followed by six HEAT-repeats; notably, the acidic loop, connecting the two first ARM-repeats; HEAT repeats 1 to 3 (for details, see [163]).

The intensive work performed on Syo1, together with the significant amount of genetic, biochemical and structural data available on the formation of the 5S rRNP complex, has allowed to establish the following model for the assembly of this complex into early pre-60S r-particles: (i) first, Syo1 captures nascent L5, in this manner, preventing L5 to misfold and aggregate [152]. Binding of L11 to Syo1 also apparently occurs in the cytoplasm but not in a cotranslational manner [144, 154]. (ii) The trimeric Syo1•L5•L11 complex is then recognized, via the N-terminal PY-NLS of Syo1, by the importin Kap104, and escorted to the site of assembly in the nucle(o)lus [144]. (iii) Upon nuclear arrival, RanGTP promotes the release of Kap104 and, concomitantly, the 5S rRNA likely binds to the trimeric complex [144]. Structural data suggest that binding of 5S rRNA induces a change in the conformation of both r-proteins within the Syo1•L5•L11 complex [163]. (iv) Now, the pre-5S rRNP is likely ready for incorporation into early pre-60S r-particles [41, 165], a process that is facilitated by the assembly factors Rfp2 and Rrs1 [166].

**Elctors of r-proteins: Tsr2 for S26, and Bcp1 for L23**

Another factor involved in the correct assembly of a specific r-protein is Tsr2. This factor was first identified as a non-essential protein required for 20S pre-rRNA maturation, and hence for SSU biogenesis [170]. More specifically, Tsr2 has been described to be required for cytoplasmic processing of 20S pre-rRNA to mature 18S rRNA [143]. This role of Tsr2 is clearly linked to its specific association with the r-protein S26 (eS26) [143, 170]. Indeed, the Panse laboratory has shown that S26 binds directly to Tsr2 and is also required for cytoplasmic processing of 20S pre-rRNA [143]. In clear contrast to other dedicated chaperones, Tsr2 only interacts with S26 once both proteins have been independently imported to the nucleus by selected karyopherins, such as Kap123 [143]. Biochemical evidence suggests that Tsr2 has the capacity to extract S26 from its karyopherin by a RanGTP-independent mechanism [143].

**Escortins of r-proteins**

Several recent studies suggest that Tsr2 and S26 form a stable complex that is essential for the correct assembly of the mature LSU [170]. This complex is formed at or near the SSU biogenesis initiation site and is thought to mediate the correct association of S26 with the SSU biogenesis initiation site [170].

**Structural and functional analyses**

Structural and functional analyses strongly suggest that the Rfp2•Rrs1 complex can bind the Syo1•5S rRNA•L5•L11 complex, by this way recruiting it to nascent pre-ribosomal particles [167-169]; however, Syo1 is never found associated to pre-ribosomal particles, thus, the interaction of Rfp2•Rrs1 complex with 5S RNP in the context of pre-60S r-particles must somehow induce the recycling of the symptporter [167, 169]. Interestingly, the recruitment of 5S RNP to particles includes the docking of L11 to helix H84 of 25S rRNA, while keeping the rest of the RNP in an almost 180° rotated configuration that is different from the one adopted in mature 60S r-subunits [41; 167, 169]. Since the Rfp2•Rrs1 complex (and Rsa4) interacts with the 5S RNP in this initial conformation but is unable to do it in the rotated, final conformation in mature LSUs, it has been suggested that the Rfp2•Rrs1 complex defines the timing for assembly of 5S RNP into distinct pre-60S pre-ribosomal particles, thereby exerting a quality control surveillance for this important step during the formation of LSUs [130, 169].
clear-cytoplasmic import. In the nucleus, Bcp1 appears to guide the assembly of L23 in early pre-60S r-particles. Similarly to what has been previously reported for Tsr2, the exchange of L23 from its karyopherin to Bcp1 is also likely RanGTP-independent [173]. Unfortunately, as for Tsr2, neither structural data nor information on the binding site of Bcp1 on L23 has so far been reported. These issues are of special interest to understand how L23 is delivered from Bcp1 to pre-60S r-particles and to get insight into its relation with trans-acting factors such as Tif6, whose binding site mainly involves L23 [47, 116, 123].

Yar1 and S3

The last member of this as yet likely incomplete inventory of factors is the ankyrin-repeat factor Yar1, which directly interacts with the free r-protein S3 {uS3} [174]. S3 consists of two well-defined globular domains, one N-terminal and another C-terminal, preceded and followed by unstructured terminal extensions, respectively [175]. As expertly envisioned by D. Lycan about a decade ago [174], Yar1 works as a molecular chaperone to keep S3 protected from in vivo aggregation [176]. Indeed, as described for many of the above dedicated chaperones, Yar1 directly interacts with free S3 by capturing its N-terminal unstructured domain in a co-translational manner [152]. Then, Yar1 accompanies S3 from the cytoplasm to the nucleus where it seems to assist the proper assembly of S3 into late pre-40S r-particles [176, 177]. The detailed characterization done for Yar1 has suggested the following scenario for the stable assembly of S3: (i) Yar1 co-translationally binds the N-terminal domain of S3, particularly the N-terminal α-helix of S3, by a concave pocket that is formed by its four ankyrin repeats, while its fourth ankyrin-repeat mediates contacts with the C-terminal domain of S3 [152, 178, 179]. (ii) Importantly, it has been shown that cytoplasmic S3 dimerizes through its C-terminal globular domain in vitro and in vivo [178]. Curiously, in vivo, only one S3 N-terminal domain of the dimer is associated with Yar1, while the second one is bound by the importin-α Kap60/Srp1 [150]. Kap60 binds the very N-terminal monopartite NLS of S3, most likely through its major binding site for cargos, and then to importin-α Kap95 through its orthodoxal IBB domain [150]; the S3 NLS is located contiguous to the Yar1-binding site, however, it has been established that the binding of Yar1 and Kap60 to one N-terminal domain of S3 is mutually exclusive, suggesting that one Kap60 molecule rapidly replaces only one Yar1 protein in the Yar1•S32 tetrameric complex [150]. (iii) This asymmetric Kap95•Kap60•S3•S3•Yar1 complex is actively imported into the nucleus, where rapid RanGTP-dependent dissociation of the importer system presumably occurs. Other importins have been proposed to be redundantly able to mediate the import of S3, including Kap123 [150]. (iv) How exactly the transition from Yar1-bound S3 to pre-40S r-particle-bound S3 occurs is unclear; it is worth to mention that these two states are incompatible with each other, since the N-terminal domain of S3, which binds Kap60 and Yar1, is in contact, with several neighbouring r-proteins, such as S10 (eS10), S20 (uS10) and S29 (uS14), and specific rRNA residues of the 18S rRNA helix H41 within mature SSUs [44, 91, 141, 178]. Moreover, the N-terminal domain of S3 in the S3•Yar1 complex is rotated ca. 180° relative to the C-terminal domain compared to its configuration in mature SSUs, and therefore, away from its contact surfaces in SSUs. Additionally, the dimerization of S3 masks the SSU rRNA-binding site of the C-terminal domain [179]. Initial assembly of S3 within pre-40S r-particles is suspected to occur through the C-terminal domain. This fact implies that the second copy of S3 from the tetramer is released as soon as the assembly of one molecule of S3 initiates and likely assembles in parallel within another orphan pre-40S r-particle. Concomitantly, the remaining Yar1 molecule dissociates and is replaced by the trans-acting factor Ltv1, which seems to have higher affinity for S3 [179, 180]. Ltv1 and Yar1 have partially overlapping binding sites within the N-terminal domain of S3, as demonstrated in vivo [179]. This result is consistent with the reported cryo-EM position of Ltv1 on pre-40S r-particles [21], and the sites of interaction of Ltv1, described by the CRAC methodology, within helices H41 and H16 of the 18S rRNA, which are located at the head and the shoulder of the SSU, respectively [103]. Importantly, different evidence indicates that, although S3 is present in late pre-40S r-particles containing Ltv1, it is still not bound in its final conformation [21, 101, 177, 181]. Thus, it can be extracted from these particles in the presence of a high salt concentration as a complex with Ltv1 and another 40S assembly factor, Enp1 [101]. Presumably, in pre-40S r-particles, S3 is associated via its C-terminal domain at a position close to its final binding site [180]. However, its N-terminal domain is likely still in the rotated conformation it adopts when bound to Yar1, while it probably interacts similarly to the central region of Ltv1 [179, 182]. (v) The stable incorporation of S3 must occur concomitantly to the release of Enp1 and Ltv1 from cytoplasmic pre-40S r-particles, which is dependent on the phosphorylation of at least Ltv1 at selected serine residues by the kinase Hrr25 (human casein kinase1 δ or 1ε) [101, 179, 180]. It has been postulated that the release of Ltv1 from pre-40S r-particles allows the N-terminal domain of S3 to assemble into its mature binding site within the context of S10, S20 and S29 and 18S rRNA residues of helix H41 [179, 180]. Indeed, the interaction of the C-terminal part of Ltv1 with pre-40S r-particles seems to be incompatible with the positioning of S3 and the presence of S10 in these particles [21, 103, 177]. The C-terminal domain of S3 also changes its conformation during the cytoplasmic maturation of SSUs so that it acquires its final position by stably interacting with r-proteins Asc1, which is the yeast orthologue of mammal RACK1, and S17 (eS17) [for experimental evidence, see references [21, 177]]. More recently, refined cryo-EM analysis of late/cytoplasmic pre-40S r-particles confirmed that S3 is still not bound at its mature site in these precursor particles, however, this study has questioned this particular model, involving the repositioning of the globular N- and C-terminal domains of S3 [183].
Nevertheless, given the intrinsic dynamic nature of the maturation pathway of pre-ribosomal subunits, it could also be feasible that these precursors had already undergone many of the conformational changes, involving S3 and Ltv1 that have been suggested to occur. Moreover, whether these structural rearrangements end with the release of Enp1 and Ltv1 also needs further clarification.

PLACEHOLDING BY MOLECULAR MIMICRY

The term "Protein-RNA mimicry" applies to the capability of a protein (or a protein domain) to imitate the structure of a distinct domain of an RNA that normally binds to a different RNA or protein. The interaction of the latter molecule with either its natural partner or the mimicking protein is normally used as a control step for the correct function of the biological process where this molecule participates (for a review, see [190]). Few cases of molecular mimicry have been reported related to the ribosome; the most classical one groups those translation factors that mimic tRNAs and that bind to the ribosome similarly as tRNAs do [190]. The pathway of ribosome assembly has also taken advantage from using the protein-RNA mimicry concept. One of these examples, as already mentioned above, represents the Syo1-HS domain that imitates the 25S rRNA helix H84, which seems to control the timing of assembly of L11 into pre-60S r-particles [163]. Strikingly, the same mimicry principle has been exploited for the activation of p53 during the mammalian nucleolar stress response, which is triggered by sequestering the E3 ubiquitin ligase MDM2 through its complex with 5S RNP (reviewed in [191]). Thus, recent structural work has shown that, similarly to Syo1, a particular domain of MDM2 mimics the 25S rRNA binding to L11 so that the interaction of 5S rRNP to MDM2 or to the LSU are also mutually exclusive (Figures 7A and 7C) [164]. In this section, we review on other biological relevant examples of mimicry on ribosome assembly (see also, [192]).

Fap7 and S14

Fap7 is a conserved essential ATPase required for SSU formation, more specifically for the cytoplasmic maturation of 20S pre-rRNA to 18S rRNA [170, 193]. This function is

Table 1. Examples of placeholding situations during the ribosome biogenesis pathway.

| Factor         | Counterpart                      | Reference |
|----------------|----------------------------------|-----------|
| Mrt4           | Mex67, P0 (uL10)                 | [20, 45, 46] |
| Rlp24          | L24 (eL24)                       | [28]      |
| Trans-acting factors |                                |           |
| Arx1           | EBP1, RAC, NAC, Ssb1/2, Sec61 complex | [69, 70] |
| Bms1           | Tsr1 (?)                         | [95, 99]  |
| Dbp10          | Nmd3                             | [118]     |
| Dim2           | Krr1 (?)                         | [189]     |
| Efl1           | EF-2 (Eft1/Eft2), Tif6           | [19, 116] |
| Nmd3           | elf5A                            | [43, 114] |
| Nog1           | Re1                              | [40]      |
| Nog2/Nog2      | Nmd3                             | [40, 115] |
| Nsa2           | Nmd3                             | [40]      |
| Nug1           | Nmd3                             | [118]     |
| Rei1           | Rhl1                             | [43, 68]  |
| Sdo1           | Nmd3                             | [114, 116]|
| Tsr1           | elf5B, Rlo1                      | [21, 102] |
| Dedicated chaperones and escortins |                |           |
| Acl4           | L4 (uL4)                         | [149, 153, 154] |
| Bcp1           | L23 (uL14)                       | [173]     |
| Fap7           | S14 (uS11)                       | [171, 184, 185] |
| Rrb1           | L3 (uL3)                         | [152, 157, 158] |
| Sgt1           | L10 (uL16)                       | [152, 162] |
| Syo1           | L5 (uL18), L11 (uL5)             | [144, 163] |
| Tsr2           | S26 (eS26)                       | [143, 170, 171] |
| Yar1           | S3 (uS3)                         | [150, 152, 174, 178, 179] |
| Box H/ACA snoRNP assembly factors |          |           |
| Naf1           | Gar1                             | [188]     |
| Shq1           | H/ACA snoRNA                     | [186, 187] |

A question mark indicates that the placeholder correspondence or the mutually exclusive interaction is suspected and has not been experimentally addressed.
linked to that of r-protein S14 (uS11) and coordinated with that of other late SSU assembly factors (namely, Dim1, Enp1, Nob1, Pno1 and Tsr1); indeed, mutations in the eu-karyote-specific C-terminal tail of S14 block processing of 2OS pre-rRNA to mature 18S rRNA similarly as the depletion of Fap7 [194]; moreover, those late SSU assembly factors persisted in 80S-like particles in the absence of Fap7 [104].

It has been shown that Fap7 directly and stably interacts with S14 in vitro [193, 195] and in vivo [184, 195] but only weakly or transiently with pre-40S r-particles [104, 171, 193, 195]. The structure of the Fap7•S14 heterodimer has been solved. In this complex, the interaction of Fap7 with S14 blocks the RNA binding surface of S14 [184, 185], hence, the binding of Fap7 to S14 clearly competes with the positioning of S14 in its final conformation in mature or almost mature SSUs. Interestingly, the structural studies indicate that indeed, Fap7 acts as an RNA mimic, using protein side chains to reproduce specific contacts of the 18S rRNA with S14 [185]. Physiologically, this interaction which is regulated through the ATP binding and hydrolysis activities of Fap7 [for further details, see [184, 185]], suggests that Fap7 acts as a dedicated and enzymatic chaperone for S14. In this way, Fap7 might protect S14 from aggregation and/or nonspecific interaction with other RNAs and regulate the correct timing of S14 assembly into pre-40S r-particles. In agreement with this, recombinant S14 from E. coli showed poor solubility unless it is co-expressed with Fap7 [185]; moreover, depletion of Fap7 causes a strong decrease in the in vivo protein levels of S14 in S. cerevisiae [171]. However, there is so far no evidence for co-translational capturing of S14 by Fap7 (discussed in [151]).

Although different scenarios have been proposed, the mode of S14 release from Fap7 and the mechanism of its concomitant incorporation into pre-40S r-subunits are still unclear [171, 184, 185]. Interestingly, the Panse laboratory has recently shown that the assembly of S14 and S26 into pre-40S r-particles is interdependent [171]. Fap7 promotes the formation of a ternary complex containing both S14 and S26 [171], which are neighbouring proteins that directly interact in the mature SSU [44, 91]. In the Fap7-containing complex, S26 and S14 might interact with each other similarly as they do in the context of the mature SSU [171]. Thus, it was concluded that Fap7 is an example of a factor enabling nucleation a module of two r-proteins, which then assemble en bloc into relatively early pre-40S r-particles [171].

**Shq1, Naf1 and the formation of H/ACA snoRNPs**

Box H/ACA ribonucleoparticles (snoRNPs) are a family of small RNA-protein complexes conserved in archaea and eukaryotes that convert uridine into pseudouridine at specific sites within rRNAs and snRNAs, mediate early pre-rRNA processing reactions and r-subunit assembly and maintain telomerase stability (reviewed in [87, 196]). It has been proposed that all mature H/ACA snoRNPs are composed of a distinct box H/ACA snoRNA, which selectively base pairs with its target RNA, and two sets of four conserved core proteins, one per each hairpin motif of the bipartite structure of the snoRNA: the pseudouridinase Cbf5 (NAP57 in rodents and dyskerin in humans), Nop10, Nhp2 (L7Ae in archaea) and Gar1 [87, 196, 197]. Although these complexes can self-assemble in vitro [for a reference, see [198]], H/ACA snoRNP formation requires multiples trans-acting factors in vivo [197]. Among these factors, two conserved and essential proteins, Shq1 and Naf1, are specifically required for the stable accumulation of all box H/ACA snoRNAs, without being part of the mature particles in either the nucleolus or the Cajal bodies (e.g. [199-201, reviewed in [197]).

It has been shown that Shq1 functions in an early step of H/ACA snoRNP biogenesis [202, 203]. Shq1 apparently binds newly synthesized Cbf5 acting as a chaperone that prevents its misfolding, aggregation and degradation. Additionally, Shq1 prevents non-specific interactions of nascent Cbf5 with other RNAs before its association with box H/ACA snoRNAs [203]. The Shq1•Cbf5 interaction mainly occurs through the central and Shq1-specific C-terminal (SSD) domains of Shq1 and the RNA-binding interface of Cbf5 [203]. Indeed, it has been shown that Shq1 acts as a RNA mimic; specific residues of Shq1 occupy the same position on nascent Cbf5 as selected RNA residues of an H/ACA snoRNA do within mature H/ACA snoRNPs [186, 187]. As a corollary, the binding of Cbf5 to either Shq1 or an H/ACA snoRNA is obligatorily mutually exclusive. The subsequent release of Shq1 by a specific chaperone complex, R2TP, allows the progression of H/ACA snoRNP biogenesis. Moreover, it has been proposed that another assembly factor, known as Naf1, brings Cbf5, Nop10 and Nhp2 to nascent box H/ACA RNAs at their site of transcription [196, 197].

Naf1 was identified as a nuclear factor able to interact with both the C-terminal domain of RNA polymerase II and nascent H/ACA snoRNAs [200]. At this time, a certain structural homology between Naf1 and the core domain of Gar1 was predicted [200], thus, being suggestive of a placeholder activity of Naf1 for Gar1. In agreement with this hypothesis, human NAF1 and GAR1 bind NAP57 (human Cbf5) competitively and in a mutually exclusive manner [204]. Moreover, crystallography demonstrated the structural homology between yeast Naf1 and the protein domain that in archaeal Gar1 binds Cbf5 [188]. Importantly, Naf1-containing nascent H/ACA snoRNPs seem to be fully inactive regarding pseudouridinase activity; only the later replacement of Naf1 by Gar1 during H/ACA snoRNP biogenesis permits the formation of an active snoRNP [198, 204]. This latter result strongly suggests that the physiological function of Naf1, as the one of a placeholder factor, is keeping the emerging RNA-based machine inactive in order to prevent undesired effects on non-cognate substrates.

**FUTURE PERSPECTIVES AND CONCLUSIONS**

In this review, we have described a relatively large number of trans-acting assembly factors that work as placeholder factors during the ribosome biogenesis process. The placeholder hypothesis, which was initially suggested as the
functional corollary of the presence of a few assembly factors showing a high degree of similarity to distinct r-proteins over their entire length [31], has been confirmed for more trans-acting factors than initially envisaged. This pleasant surprise has come as the experimental consequence of the combination of powerful biochemical, cell biological and genetic studies with the structural characterization of pre-ribosomal particles or reconstituted r-subunits by X-ray crystallography and cryo-EM from sub-to near-atomic resolution (e.g. [40]). This new mode to approach the pathway of ribosome biogenesis is providing for the first time clues on how trans-acting factors bind and operate to accommodate other factors or r-proteins during the maturation reactions of the nascent r-subunits (for a review, see [124]).

The phenotypic analyses of loss-of-function mutant variants of placeholder factors has proven to be very useful for revealing their functional relevance during the eukaryotic ribosome assembly process and have provided insights on why these factors have been positively selected during evolution. We have discussed several functions for placeholder factors: (i) Some placeholder factors (e.g. Nog2 or Mrt4) clearly control the position and timing of association or assembly of their counterparts. In the absence of these placeholder factors, the respective counterparts are prematurely recruited to the nascent pre-ribosomal particles, impeding important events that only the placeholder factors are able to exert. We can envisage a scenario where these placeholder factors are guarding key sites in pre-mature r-particles from the binding of other factors or r-proteins. The premature recruitment of these later-associating proteins could even generate kinetically trapped assembly intermediates that impede downstream maturation steps. However, in other cases (e.g. Rlp24), although the function of the placeholder factor has been shown essential for the correct progression of the maturation of the r-subunit, it is still unclear whether a direct functional relationship exists between the placeholder factor and its counterpart r-protein. (ii) Many of the strategies involving placeholder factors during ribosome biogenesis ensure that the nascent r-particles follow a step-dependent and ordered process of assembly. These steps are unidirectional and therefore irreversible. For example, nuclear pre-60S r-particles are unable to acquire export competence until Nog2 is replaced by Nmd3; cytoplasmic maturation of pre-60S r-particles does not properly proceed if Nog1 is not efficiently released and replaced by Rei1; Tsr1 delays association of either eIF5B or Rio1 with cytoplasmic pre-40S r-particles. (iii) Specific dedicated chaperones and/or escortins carry out mutually exclusive interactions with other factors, rRNA or r-proteins. As discussed herein, these factors assist the import and assembly of distinct r-proteins while preventing their intrinsic tendency to aggregate, their degradation and/or their non-specific association to non-cognate substrates. In some cases, these chaperones act as rRNA mimics when they exert their placeholders activity. By mimicking, chaperones and escortins are even able to guarantee the proper folding of their substrates. As also discussed, these factors are even able to promote the interdependent assembly of more than one r-protein at the same time ensuring stoichiometry. (iv) A considerable group of placeholder factors might have critical roles in structural proofreading, as previously discussed by A. W. Johnson [138]. A mechanism based on structural proofreading implies that the binding of a distinct factor or r-proteins depends on the proper generation of a specific site only after completion of selected upstream assembly steps. This strategy is used during export and cytoplasmic maturation of r-subunits, provides tools and mechanisms to detect assembly errors and ensures the specific cytoplasmic assembly of the last r-proteins. Equally important are strategies based on functional proofreading (discussed in [55, 122]) where key functional centres in r-subunits are inspected by a considerable number of factors, such as Arxl, which examines the solvent-exposed exit side of the PET, Nog1, Rei1, Reh1 and Sdo1, which probe the length and integrity of the PET, Nmd3, which examines the E- and P-sites of nascent LSUs, Efl1, which together with Sdo1 inspects the integrity of the P-site and the GAC regions of nascent LSUs, the P-stalk protecting Mrt4, etc. This functional proofreading strategy ensures that only properly assembled nascent r-particles undergo final maturation while simultaneously preventing premature translation by masking the active sites of the ribosome.

There is plenty more work ahead to fully understand the exact function of most, if not all, placeholder factors and the exact relationship with their counterparts during ribosome assembly. Further work is required to acquire more data on the binding sites and complete 3D maps at high resolution of r-particles containing many of the placeholder factors so far known. These aspects are essential to obtain precise information on the location of the placeholder factors relative to the pre-rRNAs, other factors and r-proteins. For many of them, their structures are still unresolved (e.g. Drg1, Mex67, Nog2). This also applies to some of the dedicated chaperones and escortins so far identified (e.g. Bcp1, Rrb1, Tsr2). Most importantly, the exact sequence of the mechanistic events that lead to the exchange of a placeholder by its counterpart also remains to be dissected for many of the examples described herein.

Finally, we would like to remark that mutations in several placeholder factors have been linked to human diseases. (i) These include the Shwachman-Diamond Syndrome, where about 90% of the patients have mutations in the SBDS gene. These mutant variants have been shown to be defective in the release of Tif6 from cytoplasmic pre-60S r-particles (see, [116, 205], and reference therein). (ii) Moreover, mutations in the RPL10 gene have been identified in patients of T-cell acute lymphoblastic leukaemia and they also seem to impair the efficient release of Tif6 and Nmd3 from cytoplasmic pre-60S r-particles (see [206], and references therein). (iii) The Syndrome 5q- seems to be caused by a RPS14 haploinsufficiency (reviewed in [207]), thus, it is reasonable to imagine that certain loss-of-function mutations in FAP7 could be identified in the future as linked to this disease, in a similar manner as mutations in RPS26 or its escortin TSR2 have been linked to the Diamond-Blackfan
Anemia (discussed in [151]). (iv) Some mutations in dyskerin (human Cbf5) related to X-linked dyskeratosis congenita lead to the destabilization of the interaction of the mutant variant of dyskerin with its placeholder SHQ1 [187]. (v) Finally, mutually exclusive binding of MDM2 and 25S rRNA to the 5S RNP is the basis of p53 activation and signalling in numerous pathophysiological situations (discussed in [191]). In conclusion, both the academic and the biomedical fields could benefit of the surely attractive and productive research on placeholder situations during ribosome biogenesis in the coming years.

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**SUPPLEMENTAL MATERIAL**

All supplemental data for this article are available online at www.microbialcell.com.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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**REFERENCES**

1. Schmeing TM, Ramakrishnan V (2009). What recent ribosome structures have revealed about the mechanism of translation. Nature 461(7268): 1234-1242.

2. Warner JR (1999). The economics of ribosome biosynthesis in yeast. Trends Biochem Sci 24(11): 437-440.

3. Nomura N (1999). Regulation of ribosome biosynthesis in *Escherichia coli* and *Saccharomyces cerevisiae*: diversity and common principles. J Bacteriol 181(22): 6857-6864.

4. Wilson DN, Nierhaus KH (2007). The weird and wonderful world of bacterial ribosome regulation. Crit Rev Biochem Mol Biol 42(3): 187-219.

5. Hage AE, Tollervey D (2004). A surfeit of factors: why is ribosome assembly so much more complicated in eukaryotes than bacteria? RNA Biol 1(1): 10-15.

6. Shajani Z, Sykes MT, Williamson JR (2011). Assembly of bacterial ribosomes. Annu Rev Biochem 80: 501-526.

7. Omer AD, Zago M, Chang A, Dennis PP (2006). Probing the structure and function of an archaean C/D-box methylation guide sRNA. RNA 12(9): 1708-1720.

8. Ebersberger I, Simm S, Leisegang MS, Schmitzberger P, Mirus O, von Haeseler A, Bohnsack MT, Schleff E (2013). The evolution of the ribosome biogenesis pathway from a yeast perspective. Nucleic Acids Res 42(3): 1509-1523.

9. Piekna-Przybyska D, Decatur WA, Fournier MJ (2007). New bioinformatic tools for analysis of nucleotide modifications in eukaryotic rRNA. RNA 13(3): 305-312.

10. Woolford JL, Jr., Baserga SJ (2013). Ribosome biogenesis in the yeast *Saccharomyces cerevisiae*. Genetics 195(3): 643-681.

11. Tafforeau L, Zorbas C, Langhedries JL, Mullineux ST, Stamatopoulou V, Mullier R, Wacheil L, Lafontaine DL (2013). The complexity of human ribosome biogenesis revealed by systematic nucleolar screening of Pre-rRNA processing factors. Mol Cell 51(4): 539-551.

12. Lestrade L, Weber MJ (2006). snoRNA-LBME-db, a comprehensive database of human H/ACA and C/D box snoRNAs. Nucleic Acids Res 34(Database issue): D158-D162.

13. Kressler D, Hurt E, Bassler J (2010). Driving ribosome assembly. Biochim Biophys Acta 1803(6): 673-683.

14. Nerurkar P, Altvater M, Gerhardy S, Schütz S, Fischer U, Weirich C, Panse VG (2015). Eukaryotic Ribosome Assembly and Nuclear Export. Int Rev Cell Mol Biol 319: 107-140.

15. Iborra FJ, Jackson DA, Cook PR (2001). Coupled transcription and translation within nuclei of mammalian cells. Science 293(5532): 1193-1194.

16. Karbstein K (2013). Quality control mechanisms during ribosome maturation. Trends Cell Biol 23(5): 242-250.

17. de la Cruz J, Karbstein K, Woolford JL, Jr. (2015). Functions of ribosomal proteins in assembly of eukaryotic ribosomes in vivo. Annu Rev Biochem 84: 93-129.

18. Panse VG, Johnson AW (2010). Maturation of eukaryotic ribosomes: acquisition of functionality. Trends Biochem Sci 35(5): 260-266.

19. Senger B, Lafontaine DL, Graindorge JS, Gadal O, Camasses A, Sanni A, Garnier JM, Breitenbach M, Hurt E, Fasiolo F (2001). The nucleolar Tif6p and Efl1p are required for a late cytoplasmic step of ribosome synthesis. Mol Cell 8(6): 1363-1373.

20. Rodríguez-Mateos M, García-Gómez JJ, Francisco-Velilla R, Remacha M, de la Cruz J, Ballesta JPG (2009). Role and dynamics of the ribosomal protein P0 and its related trans-acting factor Mrt4 during ribosome assembly in *Saccharomyces cerevisiae*. Nucleic Acids Res 37(22): 7519-7532.

21. Strunk BS, Loucks CR, Su M, Vashisth H, Cheng S, Schilling J, Brooks CL, III., Karbstein K, Skiniotis G (2011). Ribosome assembly factors prevent premature translation initiation by 40S assembly intermediates. Science 333(6048): 1449-1453.
22. Alves-Rodrigues I, Ferreira PG, Moldón A, Vivancos AP, Hidalgo E, Guigó R, Ayté J. (2016). Spatiotemporal Control of Forkhead Binding to DNA Regulates the Meiotic Gene Expression Program. Cell Rep 14(4): 885-895.

23. Dunleavy EM, Almouzni G, Karpen GH. (2011). H3.3 is deposited at centromeres in S phase as a placeholder for newly assembled CENP-A in G1 phase. Nucleus 2(2): 146-157.

24. Blum R. (2014). Activation of muscle enhancers by MyoD and epigenetic modifiers. J Cell Biochem 115(11): 1855-1867.

25. Warner JR. (2001). Nascent ribosomes. Cell 107(2): 133-136.

26. Zuk D, Belk JP, Jacobson A. (1999). Temperature-sensitive mutations in the Saccharomyces cerevisiae MTR4, GCR5, SLA2 and THS1 genes result in defects in mRNA turnover. Genetics 153(1): 35-47.

27. Lalo D, Mariotte S, Thuriaux P. (1993). Two distinct yeast proteins are related to the mammalian ribosomal polypeptide L7. Yeast 9(10): 1085-1091.

28. Saveanu C, Namane A, Gleizes PE, Lebreton A, Rousselle JC, Cruz J, Santos C, Remacha M, Ballesta JPG. (2003). Sequential protein association with nascent 60S ribosomal particles. Mol Cell Biol 23(13): 4449-4460.

29. Rodríguez-Mateos M, Abia D, García-Gómez JJ, Morreale A, de la Cruz J, Santos C, Remacha M, Ballesta JGP. (2009). The amino terminal domain from Mrt4 protein can functionally replace the RNA binding domain of the ribosomal P0 protein. Nucleic Acids Res 37(11): 3514-3521.

30. Harnpicharchai P, Jakovljevic J, Horsey E, Miles T, Roman J, Rout M, Meagher D, Inai B, Guo Y, Brame CJ, Shabanowitz J, Hunt DF, Woolford JL, Jr. (2001). Composition and functional characterization of yeast 60S ribosome assembly intermediates. Mol Cell 8(3): 505-515.

31. Dunbar DA, Dragon F, Lee SJ, Baserga SJ. (1999). Imp3p and Imp4p, two specific components of the U3 small nucleolar ribonucleoprotein that are essential for 18S rRNA processing. Mol Cell Biol 19(18): 5441-5452.

32. Lee SJ, Baserga SJ. (1999). Imp3p and Imp4p, two specific components of the U3 small nucleolar ribonucleoprotein that are essential for 18S rRNA processing. Mol Cell Biol 19(18): 5441-5452.

33. Ban N, Beckmann R, Cate JH, Dinman JD, Dragon F, Ellis SR, Gao N, Wu S, Li N, Chen Y, Kan K, Zheng L, Lei J, Woolford JL, Jr., Gao N (2017). Structural snapshot of cytoplasmic pre-60S ribosomal particles bound by Nmd3, Lsg1, Tif6 and Reh1. Nat Struct Mol Biol 24(3): 214-220.

34. 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.

35. Kemmler S, Ochcipinti L, Veiuis M, Panse VG. (2009). Yvh1 is required for a late maturation step in the 60S biogenesis pathway. J Cell Biol 186(5): 863-880.

36. Lo KY, Li Z, Wang F, Marquette EM, Johnson AW. (2009). Ribosome stalk assembly requires the dual-specificity phosphatase Yvh1 for the exchange of Mrt4 with P0. J Cell Biol 186(6): 849-862.

37. 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.

38. Wawiórka L, Molestak E, Szajwaj M, Michalec-Wawiórka B, Boguszewska A, Borkiewicz L, Liudkovska V, Kufel J, Tchórzewski M. (2015). Functional analysis of the u111 protein impact on translational machinery. Cell Cycle 15(8): 1060-1072.

39. Yao W, Lutzmann M, Hurt E. (2008). A versatile interaction platform on the Mex67-Mtr2 receptor creates an overlap between mRNA and ribosome export. EMBO J 27(1): 6-16.

40. Yao W, Roser D, Kohler A, Bradatsch B, Bassler J, Hurt E (2007). Nuclear export of ribosomal 60S subunits by the general mRNA export export factor to the nascent 60S subunit. Mol Cell 26(1): 51-62.

41. Thoms M, Thomson E, Bassler J, Griesel M, Griesel H. (2015). The Exosome Is Recruited to RNA Substrates through Specific Adapter Proteins. Cell 162(5): 1029-1038.

42. Fernández-Pevida A, Kressler D, de la Cruz J. (2015). Processing of preribosomal RNA in Saccharomyces cerevisiae. Wiley Interdiscip Rev RNA 6(2): 191-209.

43. Talkish J, Zhang J, Jakovljevic J, Horsey EW, Woolford JL, Jr., Ma C, Lei J, Yuan Y, Li Z, Jakovljevic J, Ma C, Lei J, Dong MQ, Woolford JL, Jr., Gao N (2016). Diverse roles of assembly factors revealed by structures of late nuclear pre-60S ribosomes. Nature 534(7605): 133-137.

44. Leidig C, Thoms M, Holdermann I, Bradatsch B, Berninghausen O, Bange G, Sinning J, Hurt E, Beckmann R (2014). 60S ribosome biogenesis requires rotation of the 5S ribonucleoprotein particle. Nat Commun 5: 3491.

45. Sarkar A, Pech M, Thoms M, Beckmann R, Hurt E (2016). Ribosome-stalk biogenesis is coupled with recruitment of nuclear-export factor to the nascent 60S subunit. Nat Struct Mol Biol 23(12): 1074-1082.

46. Ma C, Wu S, Li N, Chen Y, Kan K, Zeng L, Lei J, Woolford JL, Jr., Gao N (2017). Structural snapshot of cytoplasmic pre-60S ribosomal particles bound by Nmd3, Lsg1, Tif6 and Reh1. Nat Struct Mol Biol 24(3): 214-220.

47. 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.

48. Michalec B, Krokowski D, Grela P, Wawiorka L, Sawa-Makarska J, Grankowski N, Tchorzewski M. (2010). Subcellular localization of ribosomal P0-like protein MRT4 is determined by its N-terminal domain. Int J Biochem Cell Biol 42(5): 736-748.

49. Yao W, Lutzmann M, Hurt E. (2008). A versatile interaction platform on the Mex67-Mtr2 receptor creates an overlap between mRNA and ribosome export. EMBO J 27(1): 6-16.

50. Yao W, Roser D, Kohler A, Bradatsch B, Bassler J, Hurt E (2007). Nuclear export of ribosomal 60S subunits by the general mRNA export receptor Mex67-Mtr2. Mol Cell 26(1): 51-62.

51. Thoms M, Thomson E, Bassler J, Griesel M, Griesel H. (2015). The Exosome Is Recruited to RNA Substrates through Specific Adapter Proteins. Cell 162(5): 1029-1038.

52. Fernández-Pevida A, Kressler D, de la Cruz J. (2015). Processing of preribosomal RNA in Saccharomyces cerevisiae. Wiley Interdiscip Rev RNA 6(2): 191-209.

53. Talkish J, Zhang J, Jakovljevic J, Horsey EW, Woolford JL, Jr. (2012). Hierarchical recruitment into nascent ribosomes of assembly factors required for 275B pre-rRNA processing in Saccharomyces cerevisiae. Nucleic Acids Res 40(17): 8646-8661.

54. Saveanu C, Rousselle JC, Lenormand P, Namane A, Jacquier A, Fromont-Racine M. (2007). The p21-activated protein kinase inhibitor Skb15 and its budding yeast homologue are 60S ribosome assembly factors. Mol Cell Biol 27(8): 2897-2909.

55. Lo KY, Li Z, Bussiere C, Bresson S, Marquette EM, Johnson AW (2010). Defining the pathway of cytoplasmic maturation of the 60S ribosomal subunit. Mol Cell 39(2): 196-208.
Cell Biol Hsp40 chaperone Jjj1 is required for the nucleo-cytoplasmic recycling of nucleocytoplasmic pre-60S factors. Reh1 and Rei1 in cytoplasmic 60S subunit maturation.

60S ribosomal subunit-associated factor Arx1 depends on Rei1 in ribosome biogenesis. J Biol Chem 289(7): 3913-3922.

 Insertion of the biogenesis factor Rei1 probes the ribosomal tunnel during 60S maturation. Cell Biol 199(5): 771-782.

The drug diazaborine blocks ribosome biogenesis by inhibiting the AAA-ATPase Drg1. J Biol Chem 289(7): 3913-3922.

Saccharomyces cerevisiae ribosomal subunit-associated factor Arx1 depends on Rei1 in ribosome biogenesis. Proc Natl Acad Sci USA 104(5): 1558-1563.

60S ribosomal subunit export machinery. Mol Cell Biol 27(19): 6581-6592.

60S ribosomal subunit with nuclear export factor Arx1 bound at the exit tunnel. Nat Struct Mol Biol 21(12): 1228-1233.

The conserved Bud20 zinc finger protein is a new component of the ribosomal 60S subunit export machinery. Mol Cell Biol 32(24): 4989-4912.

The power of AAA-ATPases on the pre-60S ribosome maturation--molecular machines that strip pre-ribosomal particles. Biochim Biophys Acta 1823(1): 92-100.

Nuclear recycling of the pre-60S ribosomal subunit during late nuclear stages of 60S ribosomal subunit assembly. DNA 22(1): 61-70.

F. J. Espinar-Marchena et al. (2017) Placeholders in ribosome biogenesis

Distinct sites on pre-60S ribosomal particles. Nucleic Acids Res 41(20): 9461-9470.

Identification of the binding site of Rlp7 on assembling 60S ribosomal subunits in Saccharomyces cerevisiae. RNA 19(12): 1639-1647.

Saccharomyces cerevisiae 60S ribosomal subunits: role of factors required for 27S pre-rRNA processing. EMBO J 30(19): 4020-4032.

A cluster of ribosome synthesis factors regulate pre-rRNA folding and 3.5S rRNA maturation by the Rat1 exonuclease. EMBO J 30(19): 4006-4019.

Functional redundancy of yeast ribosomal proteins L7 and L8 function in concert with six A subunits to propagate assembly of domains I and II of 25S rRNA in yeast 60S ribosomal subunits. RNA 18(10): 1805-1822.

The 90S preribosome is a multimodular structure that is depleted of large ribosomal subunit proteins. PLoS One 4(12): e8249.

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.

Principal components of 60S ribosomal subunit assembly emerging from recent studies in yeast. Biochem J 474(2): 195-214.

Components of an interdependent unit within the SSU processome regulate and mediate its activity. Mol Cell Biol 22(20): 7258-7267.

The 905 pre-rRNA is a multimodular structure that is assembled through a hierarchical mechanism. Mol Cell Biol 27(15): 5414-5429.

Elucidation of the assembly events required for the recruitment of Utp20, Imp4 and Bms1 onto nascent pre-ribosomes. Nucleic Acids Res 39(18): 8105-8121.

Stage-specific assembly events of the 6-MDa small-subunit processome initiate eukaryotic ribosome biogenesis. Nat Struct Mol Biol 22(11): 920-923.

The 905 pre-rRNA duplexes, possibly to recruit the small subunit processome to the pre-rRNA. Proc Natl Acad Sci USA 101(43): 15301-15306.

Imp3 unfolds stem structures in pre-rRNA and U3 snoRNA to form a duplex essential for small subunit processing. RNA 19(10): 1372-1383.
87. Watkins NJ, Bohnsack MT (2012). The box C/D and H/ACA snoRNPs: key players in the modification, processing and the dynamic folding of ribosomal RNA. Wiley Interdiscip Rev RNA 3(3): 397-414.

88. Ferreira-Cerca S, Pöll G, Gielesz PE, Tschochner H, Milkerit P (2005). Roles of eukaryotic ribosomal proteins in maturation and transport of pre-18S rRNA and ribosome function. Mol Cell 20(2): 263-275.

89. Ferreira-Cerca S, Pöll G, Kuhn H, Neudeer A, Jakob S, Tschochner H, Milkerit P (2007). Analysis of the in vivo assembly pathway of eukaryotic 40S ribosomal proteins. Mol Cell 28(3): 446-457.

90. O'Donouhe MF, Choesmel V, Faubladier M, Fichant G, Gielesz PE (2010). Functional dichotomy of ribosomal proteins during the synthesis of mammalian 40S ribosomal subunits. J Cell Biol 190(5): 853-866.

91. Rabl J, Leibundgut M, Aitaide SF, Haag A, Ban N (2011). Crystal structure of the eukaryotic 40S ribosomal subunit in complex with initiation factor 1. Science 331(6018): 730-736.

92. Amoorsy M, Sanges P, Flemming D, van Noort V, Kunze R, Devos DP, Arumugam M, Bork P, Hurt E (2011). Insight into structure and assembly of the nuclear pore complex by utilizing the genome of a eukaryotic thermophile. Cell 146(2): 277-289.

93. Chaker-Margot M, Barandun J, Hunziker M, Klinge S (2016). Architecture of the yeast small subunit processome. Science 355(6321).

94. Kornprobst M, Turk M, Kellner N, Cheng J, Flemming D, Kos-Braun F, J. Espinar-Marchena (2008). Architecture of the yeast small subunit processome. EMBO J 27(10): 3405-3415.

95. Sun Q, Zhu X, Qi J, An W, Lan P, Tan D, Chen R, Wang B, Zheng S, Chen S, Ye K (2016). Stepwise and dynamic assembly of the earliest precursors of small ribosomal subunits in yeast. Genes Dev 30(6): 718-732.

96. Zhang L, Wu C, Cai G, Chen Y, Ye K (2020). Nmd3p is a Crm1p-dependent adapter protein for nuclear export of the large ribosomal subunit. J Cell Biol 151(5): 1057-1066.

97. Gadal O, Strauss D, Kessl J, Trumpower B, Tollervey D, Hurt E (2001). Nuclear export of 60S ribosomal subunits requires Xpo1p and requires a nuclear export sequence-containing factor, Nmd3p, that associates with the large subunit protein RpL10p. Mol Cell Biol 21(10): 3405-3415.

98. Bernstein KA, Gallagher JE, Mitchell BM, Granneman S, Baserga SJ (2002). The small-subunit processome is a ribosome assembly intermediate. Eukaryot Cell 1(1): 1619-1626.

99. Bergeron D, J. Espinar-Marchena, et al. (2017). Placeholders in ribosome biogenesis.
biogenesis of the 60S subunit of the ribosome in Saccharomyces cerevisiae. Mol Genet Genomics 266(3): 454-462.

120. Menne TF, Goyenbeche B, Sanchez-Puig N, Wong CC, Tonkin LM, Ancliff PJ, Brost RL, Costanzo M, Boone C, Warren AJ (2007). The Schwaemm-Dobian-Diamond syndrome protein mediates translational activation of ribosomes in yeast. Nat Genet 39(4): 486-495.

121. Finch AJ, Hilkeno C, Basse N, Drynan LF, Goyenbeche B, Menne TF, Gonzalez Fernandez A, Simpson P, D’Santos CS, Arends MJ, Donadieu J, Bellanne-Chantelot C, Costanzo M, Boone C, McKenzie AN, Freund SM, Warren AJ (2011). Uncoupling of GTP hydrolysis from elf6 release on the ribosome causes Schwaemm-Dobian-Diamond syndrome. Genes Dev 25(9): 917-929.

122. Bussiere C, Hashem Y, Arora S, Frank J, Johnson AW (2012). Integrity of the P-site is probed during maturation of the 60S ribosomal subunit. J Cell Biol 197(6): 747-759.

123. Gartmann M, Blau M, Armache JP, Mielke T, Topf M, Beckmann R (2010). Mechanism of elf6-mediated inhibition of ribosomal subunit joining. J Biol Chem 285(20): 14848-14851.

124. Greber BJ (2016). Mechanistic insight into eukaryotic 60S ribosomal subunit biogenesis by cryo-electron microscopy. RNA 22(11): 1643-1662.

125. Baxter-Roshek JL, Petrov AN, Dinman JD (2007). Optimization of ribosome structure and function by rRNA base modification. Plos One 2(1): e174.

126. Bonnerot C, Pintard L, Lutfalla G (2006). Integrity of the P-site is probed during maturation of the 60S ribosomal subunit. J Cell Biol 170(6): 747-759.

127. Sharma S, Yang J, Watzinger P, Kötter P, Entian KD (2013). Methylation of a conserved rRNA position in yeast. The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. Nat Struct Mol Biol 16(6): 589-597.

128. Bussiere C, Hashem Y, Arora S, Frank J, Johnson AW (2012). Integrity of the P-site is probed during maturation of the 60S ribosomal subunit. J Cell Biol 197(6): 747-759.

129. Lebreton A, Saveanu C, Decourty L, Jacquier A, Fromont-Racine M (2006). Mechanism of eIF6-mediated inhibition of ribosomal subunit joining. J Biol Chem 281(37): 27099-27108.

130. Bassler J, Paternoga H, Holdermann I, Thoms M, Granneman S, Barrio-Garcia C, Nyarko A, Lee W, Stier G, Clark SA, Schraivogel D, Kallas M, Beckmann R, Tollreny D, Barbare E, Sinning I, Hurt E (2015). A network of assembly factors is involved in remodeling rRNA elements during preribosome maturation. J Cell Biol 207(4): 481-498.

131. Lebreron A, Rousselle JC, Lenormand P, Namane A, Jacquier A, Fromont-Racine M, Saveanu C (2008). 60S ribosomal subunit assembly dynamics defined by semi-quantitative mass spectrometry of purified complexes. Nucleic Acids Res 36(15): 4988-4999.

132. Bassler J, Kallas M, Hurt E (2006). The NUG1 GTFPase reveals and N-terminal RNA-binding domain that is essential for association with 60 S pre-ribosomal particles. J Biol Chem 281(34): 24737-24744.

133. Bradatsch B, Katahira J, Kowalski E, Bange G, Yao W, Sekimoto T, Baumgartel V, Boese G, Basler J, Wild K, Peters R, Yoneda Y, Sinning I, Hurt E (2007). Arx1 functions as an unorthodox nuclear export receptor for the 60S preribosomal subunit. Mol Cell 27(5): 767-779.

134. Hung NJ, Lo KY, Patel SS, Helmke K, Johnson AW (2008). Arx1 is a nuclear export receptor for the 60S ribosomal subunit in yeast. Mol Biol Cell 19(2): 735-744.

135. Yao Y, Demoinet E, Saveanu C, Lenormand P, Jacquier A, Fromont-Racine M (2010). Ec1m is a new pre-ribosomal factor involved in pre-60S particle export. RNA 16(5): 1007-1017.

136. Nyathi Y, Pool MR (2015). Analysis of the interplay of protein biogenesis factors at the ribosome exit site reveals new role for NAC. J Cell Biol 210(2): 287-301.

137. Babiano R, de la Cruz J (2010). Ribosomal protein L35 is required for 2758 pre-rRNA processing in Saccharomyces cerevisiae. Nucleic Acids Res 38(15): 5177-5192.

138. Johnson AW, Lund E, Dahlberg JE (2002). Nuclear export of ribosomal subunits. Trends Biochem Sci 27(11): 580-585.

139. Kramer G, Boehringer D, Ban N, Buka B (2009). The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. Nat Struct Mol Biol 16(6): 589-597.

140. Gerhardt S, Menet AM, Pena C, Petkowksi JJ, Pasve VG (2014). Assembly and nuclear export of pre-ribosomal particles in budding yeast. Chromosoma 123(4): 327-344.

141. Melnikov S, Ben-Shem A, Yusupova G, Yusupov M (2015). Insights into the origin of the nuclear localization signals in conserved ribosomal proteins. Nat Commun 6: 7382.

142. Rout MP, Blobel G, Altchison JD (1997). A distinct nuclear import pathway used by ribosomal proteins. Cell 89(5): 715-725.

143. Schütz S, Fischer U, Altavet M, Nerurkar P, Pena C, Gerber M, Chang Y, Caesar S, Schubert OT, Schlenstedt G, Pasve VG (2014). A RanGTP-independent mechanism allows ribosomal protein nuclear import for ribosome assembly. eLife 3: e03473.

144. Kressler D, Bange G, Ogawa Y, Stjepanovic G, Bradschat B, Pratte D, Amlacher S, Drauss G, Yoneda Y, Katalahar J, Sinning I, Hurt E (2012). Synchronizing nuclear import of ribosomal proteins with ribosome assembly. Science 338(6107): 666-671.

145. Bange G, Murat G, Sinning I, Hurt E, Kressler D (2013). New twist to nuclear import: When two travel together. Commun Integr Biol 6(4): e24792.

146. Jäkel S, Mingot JM, Schwarzmaier P, Hartmann E, Görlich D (2002). Importins fulfil a dual function as nuclear import receptors and cytoplasmic chaperones for exposed basic domains. EMBO J 21(3): 377-386.

147. Sung MK, Porras-Yakushi TR, Reitsma JM, Huber FM, Sweederski MI, Hoelz A, Hess S, Deshaies RJ (2016). A conserved quality-control pathway that mediates degradation of unassembled ribosomal proteins. eLife 5: e19105.

148. Sydorsky Y, Dilworth DJ, Yi EC, Goodlett DR, Wozniak RW, Altchison JD (2003). Intersection of the Kap123p-mediated nuclear import and ribosome export pathways. Mol Cell Biol 23(6): 2042-2054.

149. Huber FM, Hoelz A (2017). Molecular basis for protection of ribosomal protein L4 from cellular degradation. Nat Commun 8: 14354.

150. Mitterer V, Gantenbein N, Birner-Gruenberger R, Murat G, Bergler H, Kressler D, Pertschy B (2016). Nuclear import of dimerized ribosomal protein Rps3 in complex with its chaperone Yar1. Sci Rep 6: 36714.

151. Pellet B, Mitterer V, Kressler D, Pertschy B (2015). Hold on to your friends: Dedicated chaperones of ribosomal proteins: Dedicated chaperones mediate the safe transfer of ribosomal proteins to their site of pre-ribosome incorporation. Bioessays 39(1): 1-12.

152. Pausch P, Singh U, Ahmed YL, Pellet B, Murat G, Allteger F, Stier G, Thoms M, Hurt E, Sinning I, Barge G, Kressler D (2015). Co-translational capturing of nascent ribosomal proteins by their dedicated chaperones. Nat Commun 6: 7494.

153. Stelter P, Huber FM, 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. Mol Biol Cell 58(5): 854-862.
154. Pillet B, García-Gómez JJ, Pausch P, Falquet L, Bange G, de la Cruz J, Kressler D (2015). The dedicated chaperone Acl4 escorts ribosomal protein Rpl4 to its nuclear pre-60S assembly site. PLoS Genet 11(10): e1005565.

155. Zhang Y, Wolfe T, Rosset S (2013). Interaction of nascent chains with the ribosomal tunnel proteins Rpl4, Rpl17, and Rpl99 of Saccharomyces cerevisiae. J Biol Chem 288(47): 33697-33707.

156. Gamalinda M, Woolford JL, Jr. (2001). Characterization of Saccharomyces cerevisiae Npa2p (Urb1p), Nop8p, and Rsa3p involved in early steps of 60S ribosomal subunit biogenesis. Mol Cell Biol 21(4): 1260-1271.

157. Isingler DP, Dick FA, Denke E, Trumpower BL (1997). SQTL1, which encodes an essential WD domain protein of Saccharomyces cerevisiae, suppresses dominant-negative mutations of the ribosomal protein gene QSR1. Mol Cell Biol 17(9): 5146-5155.

158. Frénais F, Legrand P, Fribourg S (2008). Ribosomal protein L3 functions as a ‘rocker switch’ to aid in coordinating of large subunit-associated functions in eukaryotes and Archaea. Nucleic Acids Res 36(19): 6175-6186.

159. Meskauskas A, Dinman JD (2008). Ribosomal protein L3 functions as a ‘rocker switch’ to aid in coordinating of large subunit-associated functions in eukaryotes and Archaea. Nucleic Acids Res 36(19): 6175-6186.

160. Rosado IV, Dez C, Lebaron S, Caizergues-Ferrer M, Henry Y, de la Cruz J (2007). Characterization of Saccharomyces cerevisiae Npa2p (Urb1p) reveals a low-molecular-mass complex containing Dpb6p, Npa1p (Urb1p), Nop8p, and Rsa3p involved in early steps of 60S ribosomal subunit biogenesis. Mol Cell Biol 27(14): 1207-1221.

161. Zheng J, Lang Y, Zhang Q, Cui D, Sun H, Jiang L, Chen Z, Zhang R, Gao Y, Tian W, Wu W, Tang J (2015). Structure of human MDM2 complexed with RPL11 reveals the molecular basis of p53 activation. Genes Dev 29(14): 1524-1534.

162. Dechampsse A-M, Koroleva O, Léger-Silvestre I, Gas N, Canadien V (1999). Assembly of 5S ribosomal RNA is required at a specific step of the pre-rRNA processing pathway. J Cell Biol 145(7): 1369-1380.

163. Zhang J, Harnpicharnchai P, Tollervey D, Leulliot N (2014). Deletion of L4 domains reveals insights into the importance of ribosomal protein extensions in eukaryotic ribosome assembly. RNA 20(11): 1725-1731.

164. Zheng J, Lang Y, Zhang Q, Cui D, Sun H, Jiang L, Chen Z, Zhang R, Gao Y, Tian W, Wu W, Tang J (2015). Structure of human MDM2 complexed with RPL11 reveals the molecular basis of p53 activation. Genes Dev 29(14): 1524-1534.

165. Iouk TL, Aitchison JD, Maguire S, Wozniak RW (2007). Bcp1 is the Nuclear Chaperone of the 60S ribosomal protein Rpl32 in Saccharomyces cerevisiae. J Biol Chem 292(2): 585-596.

166. Zhang J, Harnpicharnchai P, Jakovljevic J, Tang L, Guo Y, Oeffinger Gao Y, Tian W, Wu W, Tang J (2015). Bcp1 is the Nuclear Chaperone of the 60S ribosomal protein Rpl32 in Saccharomyces cerevisiae. J Biol Chem 292(2): 585-596.

167. Loar JW, Seiser RM, Sondberg AE, Sagner HJ, Illias N, Zobel-Thropp F, Craig EA, Lycan DE (2004). Genetic and biochemical interactions among Yar1, Ltv1 and Rps3 define novel links between environmental stress and ribosome biogenesis in Saccharomyces cerevisiae. Genetcs 168(4): 1877-1889.

168. Graifer D, Malygin A, Zarkov DO, Karpova G (2014). Eukaryotic ribosomal protein S3: A constituent of translational machinery and an extraribosomal player in various cellular processes. Biochimie 99: 8-18.

169. Koch B, Mitterer V, Niederhauser J, Stanborough T, Murat G, Rechberger G, Bergler H, Kressler D, Perschky B (2012). Yar1 protects the ribosomal protein Rps3 from aggregation. J Biol Chem 287(26): 21806-21815.

170. Hector RD, Burlacu E, Altkén S, Bihan TL, Tujtel M, Zaplatina A, Cook AG, Granneman S (2014). Snapshots of pre-rRNA structural flexibility reveal eukaryotic 40S assembly dynamics at nucleotide resolution. Nucleic Acids Res 42(19): 12138-12154.

171. Holzer S, Ban N, Klinge S (2013). Crystal structure of the yeast ribosomal protein rps3 in complex with its chaperone Yar1. J Mol Biol 425(22): 4154-4160.

172. Mitterer V, Murat G, Rety S, Blaud M, Delbos L, Stanborough T, Bergler H, Leulliot N, Kressler D, Perschky B (2016). Sequential domain assembly of ribosomal protein S3 drives 40S subunit maturation. Nat Commun 7: 10336.

173. Ghalei H, Schaub FX, Doherty JR, Noguchi Y, Roush WR, Cleveland JC, Tollervey D, Leulliot N (2014). Structural Heterogeneity in Pre-40S Ribosomes. Structure 22(17): e1000213.

174. Merwin JR, Bogar LB, Poggi SB, Fitch RM, Johns on AW, Lycan DE (2014). Genetic analysis of the ribosome biogenesis factor Ltv1 of Saccharomyces cerevisiae. Genetics 198(3): 1071-1085.

175. Johnson MC, Ghalei H, Doxtader KA, Karbersthen K, Stroope ME (2017). Structural Heterogeneity in Pre-40S Ribosomes. Structure 25(2): 329-340.

176. Hellmich UA, Weis BL, Liuotkuv A, Wurm JP, Kaiser M, Christ NA, Hantke K, Ketterm P, Entman KD, Schlieff E, Wohnert J (2013). Essential ribosomal assembly factor Fap7 regulates a hierarchy of RNA-protein interactions during small ribosomal subunit biogenesis. Proc Natl Acad Sci USA 110(38): 15253-15258.

177. Loch J, Blaud M, Rety S, Lebaron S, Deschamps P, Bareille J, Jombart J, Robert-Paganin J, Delbos L, Chardon F, Zhang E, Charenton C, Tolleryw D, Leulliot N (2014). RNA mimicry by the fap7 adenylate kinase in ribosome biogenesis. PLoS Biol 12(5): e1001860.

178. Li S, Duan J, Li D, Ma S, Ye K (2011). Structure of the Shq1-Cdfl5-Nop10-Gar1 complex and implications for HACA RNP biogenesis and dykeratosis congenita. EMBO J 30(24): 5010-5020.
187. Walbott H, Machado-Pinilla R, Liger D, Blaud M, Réty S, Grozdanov PN, Godin K, van Tilbeurgh H, Varani G, Meier UT, Leulliot N (2011). The H/ACA RNP assembly factor SHQ1 functions as an RNA mimic. *Genes Dev* 25(22): 2398-2408.

188. Leulliot N, Godin K, Hoareau-Avella C, Quevillon-Cheurl S, Varani G, Henry Y, Van Tilbeurgh H (2007). The box H/ACA RNP assembly factor Naf1p contains a domain homologous to Gar1p mediating its interaction with Cbf5p. *J Mol Biol* 371(5): 1338-1353.

189. Vanrobays E, Gelugne JP, Caizergues-Ferrer M, Lafontaine DL (2004). Dim2p, a KH-domain protein required for small ribosomal subunit synthesis. *RNA* 10(4): 645-656.

190. Nissen P, Kjeldgaard M, Nyborg J (2000). Macromolecular mimicry. *EMBO J* 19(4): 489-495.

191. Pelava A, Schneider C, Watkins NJ (2016). The importance of ribosome production, and the 5S RNP-MDM2 pathway, in health and disease. *Biochem Soc Trans* 44(4): 1086-1090.

192. Lafontaine DL (2015). Noncoding RNAs in eukaryotic ribosome biogenesis and function. *Nat Struct Mol Biol* 22(1): 11-19.

193. Granneman S, Nandineni MR, Baserga SJ (2005). The putative NTPase Fap7 mediates cytoplasmic 20S pre-rRNA processing through a direct interaction with Rps14. *Mol Cell Biol* 25(23): 10352-10364.

194. Jakovljevic J, de Mayolo PA, Miles TD, Nguyen TM, Léger-Silvestre I, Gas N, Woorlford JL Jr. (2004). The carboxy-terminal extension of yeast ribosomal protein S14 is necessary for maturation of 43S preribosomes. *Mol Cell* 14(3): 331-342.

195. Zhang J, Bai D, Ma X, Guan J, Zheng X (2014). hCINAP is a novel regulator of ribosomal protein-HDM2-p53 pathway by controlling NEDDylation of ribosomal protein S14. *Oncogene* 33(2): 246-254.

196. Yu YT, Meier UT (2014). RNA-guided isomerization of uridine to pseudouridine-pseudouridylation. *RNA Biol* 11(12): 1483-1494.

197. Massenet S, Bertrand E, Verheggen C (2016). Assembly and trafficking of box C/D and H/ACA snoRNPs. *RNA Biol*.