Structural insights into assembly of the ribosomal nascent polypeptide exit tunnel

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The nascent polypeptide exit tunnel (NPET) is a major functional center of 60S ribosomal subunits. However, little is known about how the NPET is constructed during ribosome assembly. We utilized molecular genetics, biochemistry, and cryo-electron microscopy (cryo-EM) to investigate the functions of two NPET-associated proteins, ribosomal protein uL4 and assembly factor Nog1, in NPET assembly. Structures of mutant pre-ribosomes lacking the tunnel domain of uL4 reveal a misassembled NPET, including an aberrantly flexible ribosomal RNA helix 74, resulting in at least three different blocks in 60S assembly. Structures of pre-ribosomes lacking the C-terminal extension of Nog1 demonstrate that this extension scaffolds the tunnel domain of uL4 in the NPET to help maintain stability in the core of pre-60S subunits. Our data reveal that uL4 and Nog1 work together in the maturation of ribosomal RNA helix 74, which is required to ensure proper construction of the NPET and 60S ribosomal subunits.
Translation of the genetic code and catalysis of peptide bond formation to synthesize proteins is carried out by ribosomes. Assembly of ribosomes requires the transcription, folding, modification, and processing of ribosomal RNA (rRNA), and stable association of ribosomal proteins (RPs) facilitated by trans-acting assembly factors (AFs). In eukaryotes, this process takes place in three separate subcellular compartments beginning in the nucleolus, continuing in the nucleoplasm, and concluding in the cytoplasm. To ensure their proper function, these complex ribonucleoprotein particles must undergo accurate assembly of their functional centers including the decoding center (DC) in the small subunit (40S) and the peptidyltransferase center (PTC) and nascent polypeptide exit tunnel (NPET) in the large subunit (60S). The NPET is the 90 angstrom-long conduit through which all nascent polypeptide chains travel from the PTC to the exterior of the 60S subunit. It has become increasingly clear that the NPET functions as a sensor of both specific sequences in nascent polypeptides and small molecule effectors, including antibiotics, to modulate the activity of the PTC and regulate the synthesis of specific subsets of proteins. The physicochemical properties of the NPET have also been shown to actively affect protein folding. Thus, we are particularly interested to learn how cells ensure a properly constructed NPET.

The crystal structure of mature yeast ribosomes first revealed that the eukaryotic NPET is composed of rRNA from five out of six domains of 25S rRNA, and the RPs uL4 (rpL4), uL22 (rpL17), and eL39 (rpL39). Some of the most conserved elements are the constriction sites formed by the internal loops of uL4 and uL22. In eukaryotes, the bilobal tunnel domain (TD) at the tip of the uL4 internal loop forms two constriction sites as opposed to one found in bacteria. The internal loops of both uL4 and uL22 have each been shown to be important for the assembly and function of ribosomes in both prokaryotes and eukaryotes. Additionally, eL39 further distinguishes eukaryotic from prokaryotic NPETs and can help explain differences between translation modes observed between the two.

Recent cryo-electron microscopy (cryo-EM) of native yeast pre-60S subunits has provided numerous structural snapshots of assembly intermediates, including glimpses of ordered stages of NPET construction (Fig. 1a, b). In early nucleolar pre-60S particles (Nsa1 state 2), a precursor to the tunnel is visible, with the alpha helix of AF Rpf1 sterically hindering incorporation of 25S rRNA domain II into the NPET (Fig. 1a). At this stage, uL4 is present in preribosomes but the TD of uL4 is not visible and thus is likely to be in a flexible state. Next, in Nsa1 state C particles, Rpf1 has been released, the amino-terminal domain of AF Rpf1 contacts portions of 25S rRNA domain I that will become part of the tunnel, and the uL4 TD has stabilized and becomes visible. A more complete tunnel-like structure forms in later nucleolar intermediates. In Nsa1 state E particles, Rpf1 has been released and the CTD of AF Spb1 traverses the upper portion of the tunnel, contacting the TD of uL4 at the first constriction site, possibly functioning to further stabilize uL4 in the NPET. As pro-60S particles transition from the nucleolus to the nucleoplasm, several AFs are released and others join pre-60S subunits. In the ensuing Nog2 state 1 particles, the CTD of AF Nog1 can be seen occupying almost the full length of the tunnel, contacting the uL4 TD, and eL39 is recruited to the tunnel exit. Upon release of Spb1, insertion of the Nog1 CTD may serve to maintain stability of the uL4 TD. Once preribosomes are exported to the cytoplasm (Arx1 particles), Nog1 is released by the AAA-ATPase Drg1, and replaced in the NPET by the CTD of AF Re1. Re1 is then removed and replaced in the NPET by its homologue Reh1 (Nmd3 particles), which is released upon completion of 60S subunit assembly. The CTDs of both Re1i and Reh1 also contact the TD of uL4. Thus, remarkably, the NPET is sequentially occupied by carboxy- and amino-terminal extensions of AFs from early nucleolar stages of 60S subunit assembly until final steps in the cytoplasm.

Despite this wealth of structural information, it remains unclear precisely how the NPET is constructed, what are the specific roles of ribosomal proteins and assembly factors in NPET assembly, and how NPET formation fits into the hierarchy of 60S subunit biogenesis. To investigate these questions, we focused on uL4 and Nog1, which contact each other inside the NPET during late nucleolar and nucleoplasmic (middle) stages of assembly (Fig. 1).

We used biochemical and molecular genetic analyses, together with cryo-EM, to investigate the effects on ribosome assembly when we deleted the TD of uL4 or the Nog1 CTD. We found that pre-60S particles lacking the TD of uL4 harbor a misassembled NPET that lacked the densities of eL39 and the Nog1 CTD and were blocked or stalled in at least three different stages of 60S subunit assembly. These blocks begin with rRNA helix H74 (H74), which lies in close proximity to the TD of uL4, being improperly assembled. Consequently, adjacent helices H75, H76, and H68, are also displaced. We also found that the Nog1 CTD functions to stabilize the uL4 TD and ensure proper maturation of H74. Thus, truncation of the Nog1 CTD causes an assembly defect similar, but not identical to that of deleting the uL4 TD. Our work suggests that the quality of the NPET is monitored throughout assembly of the 60S subunit, and errors in its construction can be propagated through rRNA.

**Results**

**The uL4 tunnel domain is necessary for 60S assembly.** Previously, the tunnel domain (TD) of uL4 was shown to be necessary for 60S subunit assembly. However, its precise function during ribosome biogenesis remains unclear. Because the bilobal TD of uL4 (amino acids 63–87) forms constriction sites in the NPET and has more recently been shown to interact with a number of tunnel-probing ribosome assembly factors, we wanted to investigate its specific role in 60S subunit assembly in greater detail. To this end, we have characterized multiple strains expressing different mutations or full deletions of the uL4 TD. We focused on the effects of the rpl4Δ63–87 mutation because it removes both constriction sites from the NPET (Fig. 2a, b). To assay effects of the rpl4Δ63–87 mutation, we expressed the mutant uL4 protein from a plasmid in a strain conditional for expression of endogenous wild-type uL4 (GAL-RPL4). This strain grows at wild-type rates on galactose-medium but fails to grow on glucose-medium where only the mutant protein is expressed.

In order to determine which stages of the 60S subunit assembly pathway are affected in the rpl4Δ63–87 mutant, we assayed pre-rRNA processing using primer extension and northern blotting. Compared to the wild-type strain, the rpl4Δ63–87 mutant accumulated both 27SB and 7S pre-rRNAs, which normally undergo processing during late nucleolar and nucleoplasmic stages of 60S assembly (Supplementary Fig. 1a). To confirm that these middle steps of 60S subunit assembly are affected in the rpl4Δ63–87 mutant, we assayed localization of pre-60S subunits using uL23-eGFP (rpL25), a reporter of both mature and pre-60S subunits, and Nop1-mRFP, a nucleolar marker. Relative to the wild-type strain, we observed accumulation of pre-60S subunits in the nucleolus and nucleoplasm in the rpl4Δ63–87 mutant (Supplementary Fig. 1b). These results demonstrate that the uL4 TD is necessary for late nucleolar and nucleoplasmic stages of 60S subunit assembly prior to pre-60S export from the nucleoplasm.
The tunnel domain of uL4 stabilizes rRNA. Because our assays thus far suggest a defect during late nucleolar/nucleoplasmic stages of 60S assembly, we used the late nucleolar/nucleoplasmic acting AF Nog2 as bait to affinity-purify particles from this stage of assembly. We performed cryo-EM on these Nog2-associated pre-60S particles, which enabled observation of both small and dramatic changes in rRNA and protein conformation that would otherwise be undetectable by other methods.

Previously, cryo-EM structures of wild-type Nog2-associated particles revealed three distinct consecutive assembly intermediates during middle stages of 60S subunit assembly: Nog2 states 1, 2, and 3. Nog2 state 1 particles contain a prerotated 5S RNP, Nog2 state 2 contains a rotated 5S RNP along with AFs Sda1, the Rix1 complex, and Rei1,24 while Nog2 state 3 contains a rotated, near mature 5S RNP. The CTD of Spb1 (dark green) can be seen toward the entrance of the NPET. In Nog2 particles (PDB: 3ICT), a nearly mature NPET, now containing eL39 (green), can be visualized with the CTD of Nog1 (dark purple) stretching almost the entire length of the NPET. The Arx1 (PDB: 5APN) and Nmd3 (PDB: 5H4P) particles reveal no further NPET structure make up the NPET. In Nog2 state 1, the NPET contains mostly domains I (red) and II (orange). A small portion of domain IV (green) can also be seen and domain V (blue) has not yet folded into the NPET. In Nog2 state 2, domain III (yellow) has folded into a stable conformation to join the NPET. In Nog2 state 1, domain V has stably folded into the NPET while a portion of domain II has also folded into a near mature state. Minor rearrangements in NPET rRNA can be observed in Arx1, Nmd3, and mature particles (PDB: 4V88). For clarity, only NPET-relevant portions of proteins and rRNA are shown.

Fig. 1 Ordered stages of NPET assembly. a Cryo-EM structures of preribosomes revealed that the NPET undergoes distinct stages of assembly. The Nsa1 state 2 particle (PDB: 6COF) contains Rfp1 (sky blue) associated with an NPET precursor. The uL4 (cyan) and uL22 (bright orange) internal loops can be seen, but the tunnel domain (TD) of uL4 is not yet visible. The Nsa1 state C particle (PDB: 6EMI) displays Rfp1 (teal green) in an incomplete NPET. The TD of uL4 (cyan) is now visible at this stage. The Nsa1 state E particle (PDB: 6ELZ) contains a more complete NPET. The CTD of Spb1 (dark green) can be seen toward the entrance of the NPET. In Nog2 particles (PDB: 3ICT), a nearly mature NPET, now containing eL39 (green), can be visualized with the CTD of Nog1 (dark purple) stretching almost the entire length of the NPET. The Arx1 (PDB: 5APN) and Nmd3 (PDB: 5H4P) particles reveal no further NPET RNA maturation but show that the CTDs of Rei1 (dark pink) and Reh1 (brown), respectively, now occupy the NPET. b Five domains of rRNA secondary structure make up the NPET. In Nsa1 state C, the NPET contains mostly domains I (red) and II (orange). A small portion of domain IV (green) can also be seen and domain V (blue) has not yet folded into the NPET. In Nog2 state 1, domain III (yellow) has folded into a stable conformation to join the NPET. In Nog2 state 1, domain V has stably folded into the NPET while a portion of domain II has also folded into a near mature state. Minor rearrangements in NPET RNA can be observed in Arx1, Nmd3, and mature particles (PDB: 4V88). For clarity, only NPET-relevant portions of proteins and rRNA are shown.
We next looked at effects of the rpl4Δ63–87 mutation on the structure of pre-rRNA in Nog2 particles. Although class R2 exhibits wild-type rRNA conformations, significant shifts in rRNA helices 68–69 and 74–79 could be seen in class R1 (Fig. 3a, b). Further analysis of the atomic model of class R1 revealed how a misassembled NPET could cause these rRNA conformational changes (Fig. 3c). These aberrant shifts begin with a loss of the interaction between the TD of uL4, the Nog1 CTD, and the linker between rRNA helices 73 and 74 (Fig. 3d).

Recent cryo-EM structures further suggest the importance of this interaction. H74 is displaced ~4 Å relative to the wild-type conformation, indicating that this portion the NPET is improperly assembled (Fig. 3e). The conformational change of H74 affects the adjacent H75, which can be seen shifted up to ~33 Å from its native position in Nog2 state 1 (Fig. 3e). In this aberrant position, H75 clashes with H68 and displaces it up to ~62 Å toward the 5S rRNA (Fig. 3f). H68 is the binding site for AF Sda1, which is important for rotation of the 5S RNP in Nog2.
**Fig. 3 Aberrant conformations of rRNA helices in rpl4Δ63-87 mutant particles.**

a  Two major density maps (R1 and R2 particles, shown in light gray) obtained from Nog2-associated rpl4Δ63-87 particles are overlaid with their respective atomic models. Enlarged view of the conformational changes in rRNA helices from the two major classes of the rpl4Δ63-87 mutant. Compared with wild-type Nog2-particles (state 1), H75-76 (magenta) and H68-69 (red) have undergone dramatic conformational changes in class R1 but not in class R2. c  rRNA helices from the atomic model of wild-type Nog2 state 1 particles are compared with those in the atomic model of the rpl4Δ63-87 mutant class R1. Positions of relevant rRNA helices in relation to uL4 and Nog1 in the NPET in RPL4 wild-type (left) and rpl4Δ63-87 mutant (right) Nog2 particles. Helix 74–75 extend into the NPET. H74 is located in close proximity to the uL4 TD (cyan) and the CTD of Nog1 (dark purple). H74-75 are located below H68-69 (gray for wild-type and gold for mutant). The 5S rRNA (black) is shown to provide a frame of reference. d  Inset showing the interaction of the TD of uL4, the Nog1 CTD, and the rRNA linker between H73 and H74. e  H74-75 from rpl4Δ63-87 mutant particles (hot pink) are shifted relative to those in wild-type Nog2 state 1 particles (light blue). H74 is displaced up to -4 Å while H75 is shifted up to -33 Å. f  H75 in rpl4Δ63-87 mutant particles is shifted to an aberrant position (hot pink) that clashes with the native position of H68 (gray). This displaces H68 in rpl4Δ63-87 mutant particles (gold), causing it to shift up to -62 Å.
particles and acts as an intersubunit bridge in mature wild-type ribosomes. Once structured in Nog2 state 1, this helix remains stably docked throughout middle and later stages of 60S subunit assembly\(^1\). Therefore, deviations from this stable position of H68, as seen in class R1, likely have negative consequences as a result of improper NPET construction during the assembly of 60S subunits.

Consistent with the displaced H74-75, the 3-way junction of rRNA helices 75, 76, and 79 is affected in class R1. Specifically, the L1 stalk (H76 and uL1) adopts an unusual, deflected conformation not observed in any previously characterized wild-type particles (Supplementary Fig. 4) and H79 is unstructured. As a result, the internal loop of uL15, which interacts with H75 in Nog2 state 1, cannot be visualized. Likewise, the N-terminus of el8, which interacts with the 3-way junction of H75, H76, and H79 and was previously shown to be necessary for middle stages of 60S subunit assembly\(^2\), is also flexible (Supplementary Fig. 5a-d). These observations further characterize the perturbation of this 3-way junction as a result of improper NPET construction.

A misassembled NPET can block Sda1 binding to the pre-60S. To help clarify the consequences of the aberrant rRNA conformations observed in rpl4Δ63–87 mutant pre-60S subunits, we utilized SDS-PAGE, western blotting, and iTRAQ mass spectrometry to analyze the protein composition of these particles. Importantly, levels of uL4 do not change in the rpl4Δ63–87 mutant preribosomes compared to wild-type, indicating that the mutant uL4 protein is stable and able to efficiently assemble into pre-60S subunits. However, we did observe a number of changes in amounts of other proteins in the mutant preribosomes. Most striking was that levels of Sda1 decreased and levels of Rpf2 and Rrs1 increased (Fig. 4a, b). In wild-type cells, the AFs Rpf2 and Rrs1, which are bound to the 5S RNP, are thought to exit from pre-60S subunits in this mutant, and Rpf2 and Rrs1 fail to exit from preribosomes. Furthermore, amounts of downstream AFs Real1, and the Rix1 complex (Rix1, Ipi1, and Ipi3), which depend on Sda1 for recruitment onto pre-60S subunits and are required to stabilize 5S RNP in its rotated state, also are decreased relative to wild-type (Fig. 4b). Consistent with a failure of Real1 to enter assembling pre-60S subunits, levels of Rsa4, which is removed by Real1, are increased.

The C-terminal domain of Nog1 facilitates 60S assembly. Since the Nog1 CTD is in close contact with the TD of uL4 in wild-type cells and fails to enter the NPET in rpl4Δ63–87 mutant particles, we wanted to investigate whether deleting the Nog1 CTD results in similar or different 60S subunit assembly defects compared to those observed in the rpl4Δ63–87 mutant. Thus, we generated a series of genomic mutations to sequentially truncate the CTD of Nog1 (Fig. 5a and Supplementary Fig. 6a). We also constructed the triple mutant nog1Δ595–647 rei1Δ31-393 reh1Δ 380-432 (nog1Δrei1AC reh1AC) lacking the NPET-occupying sequences of Nog1, Rei1, and Reh1. As an orthogonal approach to deleting Nog1 sequences inserted into the tunnel, we constructed a strain expressing Nog1 fused to GFP at its C-terminus (NOG1-GFP). Because an immature NPET is already formed before the Nog1 CTD enters the tunnel (Fig. 1a), this bulky GFP tag may prevent insertion of the Nog1 CTD into the tunnel.

All of these mutants and the NOG1-GFP strain exhibit an extreme cold-sensitive growth defect (Fig. 5a). In contrast, an analogous C-terminal truncation of Rei1 has been shown to have minimal effects on cell growth at all temperatures, while the complete absence of Reh1 has no obvious growth defect\(^22,29\). To examine whether this cold-sensitivity reflects a defect in 60S subunit assembly, we used sucrose gradient fractionation to assay amounts of free ribosomal subunits, 80S monosomes, and polyribosomes in extracts prepared from each strain. In each case, levels of free 60S ribosomal subunits are decreased relative to 40S subunits, polyribosomes are decreased, and halfmier polyribosomes are present, indicating a defect in production of 60S subunits (Supplementary Fig. 6b).

To determine which interval of 60S subunit assembly is perturbed in each mutant, we examined pre-rRNA processing. When cells were shifted to 16°C for 5 h, 27SB pre-rRNA accumulates in all mutant strains relative to the wild-type NOG1 yeast strain (Supplementary Fig. 6c). Together, these results indicate that the presence of the CTD of Nog1 in the NPET is important during late nucleolar and nucleoplasmic stages of 60S subunit assembly.

The C-terminal domain of Nog1 scaffolds uL4. We performed cryo-EM of Nog2-containing particles affinity purified from the nog1ΔC rei1AC reh1AC mutant, shifted to 16°C for 5 h. We focused on this triple mutant because it exhibited the most severe growth defect and we expected that the CTD’s of Re1 and Reh1 might partially rescue defects of a nog1Δ595–647 mutant. We obtained structures of four stable intermediates at resolutions ranging from 3.0 to 4.3 Å, which we refer to as classes N1–N4 (Supplementary Figs. 7 and 8). The most striking observation from these structures is that the interior of the NPET in all four nog1ΔC rei1AC reh1AC particle classes lacks density for the TD of uL4 (Fig. 5b). This result indicates that the presence of the Nog1 CTD in the NPET is required to stabilize the TD of uL4. In contrast to the rpl4Δ63–87 mutant particles, eL39 was present in classes N1–N4 (Fig. 5b), Classes N1 and N2 closely resemble class R2 from the rpl4Δ63–87 mutant, except that the ITS2 structure is absent in class N2 (Fig. 5c). This latter observation is consistent with previous findings that removal of ITS2 can occur independently from other remodeling events such as 5S RNP rotation\(^30,31\). Class N3 particles resemble class R1 from the rpl4Δ63–87 mutant, including the aberrant rRNA rearrangements, deflected L1 stalk, and absence of Sda1 (Fig. 6a, b).

However, while class R1 makes up about 27% of total particles obtained from the rpl4Δ63–87 mutant, class N3 only comprises about 7% of total particles recovered from the nog1ΔC rei1AC reh1AC mutant. Most notable is class N4. Unlike any classes observed in the rpl4Δ63–87 mutant, class N4 represents improperly assembled Nog2 state 2 particles; it displays weak density for the Rix1 complex and lacks density for Real1. Consistent with inefficient recruitment of the Rix1 complex and Real1, the Real1-dependent removal of Rsa4 has not yet occurred in class N4 (Fig. 7c). These results indicate that the nog1ΔC rei1AC reh1AC mutant is distinct from the rpl4Δ63–87 mutant. In this mutant, a smaller fraction of intermediates appears to be blocked at the stage of Nog2 state 1, and the second block appears to be at the state 2 stage, rather than the state 3 stage, of Nog2 particles. These differences between the rpl4Δ63–87 mutant and the nog1ΔC rei1AC reh1AC mutant may in part result from the absence of the TD of uL4 in the former, versus its being present but in a flexible conformation in the latter.

To further characterize the differences between rpl4Δ63–87 and nog1ΔC rei1AC reh1AC mutant particles, we assessed the protein composition of Nog2-associated pre-60S subunits using SDS-PAGE, western blotting, and iTRAQ mass spectrometry. Purifications were done using wild-type or mutant cells grown at 30°C and then shifted to 16°C for five hours. SDS-PAGE followed by silver staining or western blotting indicated that...
deletion of the Nog1 CTD does not affect the stability or recruitment of Nog1 into pre-60S subunits (Fig. 7a). In contrast to the rpl4Δ63–87 mutant, levels of Sda1, Rpf2, and Rrs1 do not change in nog1Δ rei1Δ reh1Δ mutant particles relative to wild-type cells (Fig. 7b). Despite the observation that both the exit of Rpf2 and Rrs1 and the entry of Sda1 are unaffected in the nog1Δ rei1Δ reh1Δ mutant, levels of the Rix1 complex and Rea1, which are required for stabilization of the 5S RNP in its rotated state, are consistently decreased relative to wild-type (Fig. 7b). These results indicate that, for reasons that are still not entirely clear, nog1Δ rei1Δ reh1Δ mutant pre-60S subunits are able to recruit Sda1 but cannot recruit the other machinery necessary for 5S RNP rotation as efficiently as wild-type particles. This unusual protein composition most closely reflects what was observed by cryo-EM of class N4 of the nog1Δ rei1Δ reh1Δ mutant particles (Fig. 7c).

Fig. 4 Deviations in rRNA structure in rpl4Δ63–87 mutant particles result in H68 clashing with the binding site of Sda1. a SDS-PAGE gels followed by silver staining or western blotting show proteins in Nog2-associated particles purified from mutant rpl4Δ63–87 cells. Labeled bands were identified using mass spectrometry. b Samples prepared as in panel a were scaled up and subjected to semi-quantitative iTRAQ mass spectrometry to assay changes in relative amounts of pre-60S subunit proteins. Proteins were labeled with iTRAQ reagents and compared to wild-type counterparts. Each bar represents a biological replicate. All protein levels are normalized to the bait protein, Nog2. Ratios are represented on a log2 scale. Error bars represent standard deviation (n = 3). c The AF Sda1 (dark green) from Rix1 particles superimposed over wild-type Nog2 state 1 particles (left) and rpl4Δ63–87 mutant particles (right). H68 (gray for wild-type and gold for mutant) in rpl4Δ63–87 mutant particles clashes with almost the full length of observable Sda1.
Discussion

Here, we combined molecular genetics and biochemical approaches with high-resolution cryo-EM to uncover how construction of the NPET fits into the hierarchy of 60S ribosomal subunit assembly in yeast. Our work reveals that a misassembled NPET can delay or block at least three different stages of pre-60S subunit assembly and demonstrates interdependence between the TD of uL4 and the CTD of Nog1.

Previous work purifying Nog2-associated pre-60S particles from wild-type cells yielded three major stable assembly states, which were named Class N1, N2, and N3. In Class N1, the helical bundle and the G-domain of Nog1 are present, but the CTD is not. In Class N2, the CTD is fully assembled, but the helical bundle is not. In Class N3, both the helical bundle and the CTD are fully assembled.

Fig. 5 The Nog1 CTD stabilizes the tunnel domain of uL4. a Serial dilutions (1:10 to 1:10,000) of NOG1 wild-type and nog1 mutant cultures were spotted onto solid medium containing glucose, and incubated at 30 °C or 16 °C, for 8 days. Each construct is depicted in cartoon form (right). b Density maps of the wild-type Nog2 state 1 and N1–N4 mutant particles are aligned with the atomic model of wild-type Nog2 state 1 particles, displaying the interior of the NPET. Densities (gray) for the uL4 TD (cyan) are missing in the NPET in all four mutant classes (top). Densities for eL39 (green) can be visualized in classes N1–N4 but no densities for the Nog1 CTD (magenta) are visible (bottom). c View of nog1Δ rei1Δ reh1Δ classes N1–N3 from the subunit interface. Densities are fitted to the atomic model of wild-type Nog2 state 1 (PDB: 3jct). Exposed cartoon models of Arx1 (blue), the Nog1 CTD (magenta), Bud20 (red), Cgr1 (yellow), and Nug1 (cyan) represent missing densities for each respective protein.
intermediates (Nog2 states 1–3)\(^\text{17}\), with 5S RNP rotation occurring in Nog2 state 2. Our cryo-EM analysis of Nog2-associated pre-60S particles from the rpl4Δ63–87 mutant revealed seven different particle states. Based on our data as well as others’ data, we propose a model to explain the chronological progression and fate of each individual particle class through the 60S subunit assembly pathway (Fig. 8).

Using the atomic model of class R1 from the rpl4Δ63–87 mutant, we were able to visualize several rRNA conformational changes that are apparently initiated by the instability of the linker between H73 and H74, which results in increased flexibility of H74 (Fig. 3c, 3d). In wild-type particles, H74 is one of the last rRNA helices in the NPET to undergo maturation (Fig. 1b). The absence of the uL4 TD results in a flexible H74, which appears to be sufficient to cause H75 to adopt an aberrant conformation. Consequently, H68 cannot remain stably docked on pre-60S subunits, and the 3-way junction of H75, H76, and H79 becomes destabilized, causing the L1 stalk to adopt a deflected position. Notably, densities for a shifted H75 could also be observed in classes R4 and R6 (Supplementary Fig. 9a). Consistent with this observation, densities for H68 are not visible in either class (Supplementary Fig. 9b), and the L1 stalk is deflected in class R4, the same as R1, indicating that L1 stalk maturation may be affected in early stages of 60S assembly. In summary, we suggest that a flexible H74, which occurs as a consequence of an improperly formed NPET, can shift rRNA helices 75, 76, and 68 toward aberrant conformations that can affect several stages of 60S subunit assembly.

Classes R3 and R4 resemble the early nucleolar intermediates Nsa1 state B and E, respectively, which and do not usually co-purify with Nog2 particles\(^\text{16,17}\). However, class R4 contains density for the bait protein Nog2 and is therefore unlikely to be a contaminant. Nog2 may be entering pre-60S particles earlier due to their stalling\(^\text{32}\). This defect in early particles resulting from the absence of the TD of uL4 is consistent with the observation that the TD of uL4 is first visible (and therefore present in a stable form) in the NPET at this stage of wild-type 60S subunit maturation (Fig. 1a). Class R4 lacks densities for several AFs on particles (Erb1, Ytm1, and Has1) have not yet been released. Otherwise, failure of so many essential AFs to initially bind early assembly intermediates would result in their rapid turnover\(^\text{33}\). It remains unclear what the biological consequences of a deflected L1 stalk in class R4 may be. Therefore, classes R3 and R4 may represent on-pathway particles that are delayed in their progression to middle stages of assembly.

The rpl4Δ63–87 mutant particles that reach class R2 may subsequently become trapped in class R1, be unable to bind Sda1, and become destined for turnover (class R7). Alternatively, they might progress beyond the 5S RNP rotation checkpoint to class R6. Because densities for H68 are not visible in class R6, we suspect that this flexible intersubunit bridge results in particles destined for turnover (class R5). However, in the case of R6, this flexibility of H68 may not arise until after 5S RNP rotation occurs (Fig. 8). Together, our data suggest that proper maturation of the NPET affects the stability of specific rRNA helices and keeps pre-60S particles on-pathway. These mutant classes blocked at various stages may reflect the phenomenon of rerouting ribosome assembly to alternate pathways in response to stress\(^\text{34,35}\).

Deletion of the TD of uL4 consistently resulted in more extreme phenotypes than those observed in nog1 CTD mutants. For example, the rpl4Δ63–87 mutant is lethal at all temperatures, whereas the nog1 CTD mutants exhibited severely reduced growth only at 16 °C (Figs. 2a, 5a). The different extent to which maturation of pre-60S subunits is blocked in rpl4 compared to nog1 mutants may reflect the difference between the absence of

**Fig. 6** A minority of nog1ΔC rel1ΔC reh1ΔC mutant pre-60S particles display aberrant rRNA conformations. a Densities of classes N1–N3 of nog1ΔC rel1ΔC reh1ΔC mutant particles solved at a resolution of 3.0–6.0 Å. Classes N1 and N2 are aligned with the atomic model of class R2, while class N3 is aligned with the atomic model of class R1. Note that ITS2 is missing from classes N2 and N3. b View of H68-69 (red) and H75-76 (magenta) from classes N1–N3.
the uL4 TD (rpl4Δ63–87) vs. the TD being present but in a flexible state (nog1ΔC rei1ΔC reh1ΔC). This milder effect in the nog1 mutant is also consistent with the observation that class N3, which contains the same aberrant rRNA conformations as class R1, makes up only 7% of the total particles obtained from the nog1ΔC rei1ΔC reh1ΔC while class R1 makes up 27% of rpl4Δ63–87 mutant particles (Supplementary Figs. 2b and 7b). When flexible, the TD of uL4 might still stabilize H74 enough to maintain growth at 30 °C in rich media. However, the potential for this rRNA to become trapped in unproductive rRNA conformations might increase at lower temperatures and result in cold sensitivity.\(^{36}\) Alternatively, or in addition, the lack of eL39 in the NPET of rpl4Δ63–87 mutant particles could help explain the difference between the rpl4 and nog1 mutants. While eL39 is not essential for growth, rpl39Δ mutants do exhibit growth defects\(^ {37}\). Although it is unclear how eL39 is loaded into pre-60S subunits and why exactly deletion of the uL4 TD prevents this, eL39 may fine-tune NPET assembly.

Our results also suggest that Nog1 plays the most important role of the three tunnel-occupying AFs Nog1, Rei1, and Reh1 during ribosome assembly. Analogous C-terminal truncation of Rei1 has minimal effects on cell growth at all temperatures, while reh1Δ cells
have no obvious growth defect. Nevertheless, our triple truncation mutant, nog1ΔC rei1ΔC reh1ΔC, displayed a slightly slower growth rate at 30°C than the nog1Δ595–647 single mutant. This leaves the possibility that the NPET-occupying amino acids in the CTDs of either Rei1 or Reh1 perform a still unknown function.

Class N4 particles from the nog1ΔC rei1ΔC reh1ΔC mutant resemble Nog2 state 2 particles but lack density for Rea1 and display weak density for the Rix1 complex (Fig. 7c). The only other deviation from wild-type Nog2 state 2 in class N4 is that the L1 stalk adopts an open conformation as opposed to a

Fig. 8 NPET maturation in the 60S subunit assembly hierarchy. In the wild-type 60S subunit assembly pathway (middle, tan), Nog2 state 1 progresses to Nog2 state 2 particles, which have bound AFs Sda1 (dark green), the Rix1 complex (orange), and Rea1 (coral). The L1 stalk has closed, and the 5S rRNA has rotated ~180°. Rsa4 (light purple) has also shifted to face Rea1. These wild-type particles progress to Nog2 state three particles, which have released multiple AFs and are ready to exchange Nog2 for Nmd3 and be exported from the nucleoplasm to the cytoplasm. In the rpl4Δ63–87 mutant (top, purple) classes R3 and R4 are the earliest chronological particles to be observed. Some fraction presumably progresses to class R2. Class R2 particles are wild-type-like and likely progress to class R1 particles, which display a shifted rRNA helix H68 (gold) that prevents binding of Sda1 and progression to Nog2 state 2. These class R2 particles are likely precursors to class R7 on a pathway to turnover. Alternatively, some fraction of class R2 particles may progress past Nog2 state 2 to class R6, which displays aberrant conformations of multiple rRNA helices. Consequently, class R6 particles are likely precursors to class R5 on a pathway to turnover. In the nog1ΔC rei1ΔC reh1ΔC mutant, wild-type-like classes N1 and N2 (bottom, teal) progress to either N3, which resembles class R1, or class N4. Class N4 primarily displays density for the L1 stalk in an open position and Sda1 is bound, but weak densities (transparent colors) can be seen for the Rix1 complex, Rea1, and the L1 stalk in a closed position. Some fraction of class N4 particles likely progresses to Nog2 state 3. Two curved black lines represent flexibility in protein or rRNA structure. Solid vs. dotted arrows represent major and minor pathways, respectively.
closed one. While this particle makes up a minor fraction of total particles (5.6%), it is possible that failure to close the L1 stalk may explain why the Rix1 complex and Rea1 are not stably associated with pre-60S subunits. In this scenario, in wild-type cells, both Sda1 binding and closure of the L1 stalk may promote recruitment of the Rix1 complex and Rea1. The flexible L4 TD in the nog1ΔC rei1ΔC reh1ΔC mutant may subtly perturb the conformation of H74, which could be propagated to H75 and then to H76 in the L1 stalk. These perturbations might affect the closing of the L1 stalk in ways that we do not yet fully understand.

A consistent defect observed across all cryo-EM classes from both mutants was the lack of the nonessential AF and export adaptor, Arx1 (Figs. 2d and 5c). Portions of the Nog1 CTD that lie just outside the NPET interact with Arx1. We reasoned that the flexibility or absence of the Nog1 CTD in our mutants may prevent stable assembly of Arx1. Normally, Ret1 is necessary for the removal of Arx1 from pre-60S subunits. When this fails to occur, Arx1 remains bound to preribosomes as they are exported to the cytoplasm. However, when Arx1 cannot assemble into preribosomes, it is found in the nucleolus38. Consistent with the assembly defect of Arx1, we observed that upon truncation or tagging of the Nog1 CTD in an rei1Δ strain, Arx1-GFP was located in the nucleolus (Supplementary Fig. 10a, b). These results indicate that the Nog1 CTD recruits Arx1 to pre-60S subunits, contributing to the export competency of pre-60S subunits.

It is vital to the cell’s survival that the constriction sites in the NPET are assembled properly, since these sites impact protein synthesis. In bacteria, deletion of the internal loop of uL4 results in a cold-sensitive growth defect and a failure to respond to the cmlAChb pausing peptide during protein synthesis10. Mutations in the NPET have also been shown to confer resistance to the macrolide antibiotic, erythromycin14,39. Several previous studies offered early clues that mechanisms may operate to couple NPET construction with other remodeling events during 60S subunit assembly.5,40,41. For example, translating ribosomes that are stalled in response to small molecules, peptides, or antibiotics that bind to the NPET display a distorted PTC incapable of catalyzing peptide bond formation4. Furthermore, defects in bacterial ribosomes lacking a SS RNP can be partially rescued by the binding of a macrolide antibiotic, which could be propagated to H75 and then to H76 in the L1 stalk.

Methods

Construction of mutant plasmids. Plasmids containing each AF gene were obtained from the Yeast Genomic Tiling Collection (Open Biosystems), and used as templates in a multi-step PCR protocol for inserting unique restriction sites as templates in a multi-step PCR protocol for inserting unique restriction sites

Generation of yeast strains. All genomic epitope tags, GAL-promoter fusions, knockouts, or C-terminal truncations were constructed according to standard methods44. The nog1ΔC rei1ΔC reh1ΔC triple truncation strain was made by truncating one assembly factor gene at a time. A strain conditional for expression of only rpl4Δ63–87 was constructed as follows. First the endogenous promoter of RPL4A was replaced with the conditional GAL promoter, plus the HA-epitope in-frame with the RPL4A ORF. Then, RPL4B was knocked out by replacement of the ORF with KanMX. This GAL-HA-RPL4A rpl4b strain was transformed with a LEU2 plasmid containing rpl4bΔ63–87. Arx1-GFP strains were a generous gift from Arlen Johnson (University of Texas at Austin). All strains and plasmids are available upon request. (Supplementary Tables 2 and 3).

Growth assays. Yeast strains were grown to an OD600 of ~0.5 in permissive conditions and then serially diluted out to 10−4fold before pipetting 10−12 μL of culture onto appropriate galactose or glucose-containing solid media. Plates were checked daily after being incubated at 30 or 16°C.

Sucrose density gradient analysis. Pre- and polysomes were fractionated from 40 OD254 units of yeast whole-cell extracts on 7–47% (w/v) sucrose gradients47. Five milligrams of cycloheximide (Sigma) was added to 130 ml of culture 20 min before harvesting the cells. Extracts were made using lysis buffer (10 mM Tris-HCl pH 7.5, 0.1 M NaCl, 30 mM MgCl2, 50 μg/ml cycloheximide, 200 μg/ml heparin, and 0.2% diethyl pyrocarbonate). Cells were vortexed eight times for 30 s with glass beads (0.5 mm diameter, Biospec Products), kept on ice in between vortexing, and clarified by two consecutive centrifugations. Forty OD254 units of whole-cell extracts were layered on 7–47% (w/v) sucrose gradients, and polysomes, ribosomes, and polysomes were fractionated according to the manufacturer’s protocol (Teledyne IEC). The sucrose gradients were spun in an ultracentrifuge for 4 h. A Foxy R1 density gradient fractionator was used to fractionate and analyze gradients with continuous monitoring at OD254.

Fluorescence microscopy. The intracellular location of preribosomes or AFs was determined as follows: 35 μm glass bottom microwell dishes (MaTek) were coated with concanavalin A (Sigma) and allowed to dry for 40 min. Cells expressing GFP-tagged uL23 or Arx1-GFP were grown to an OD600 of 0.3–0.5 before plating 10 μL onto the glass bottom of the MaTek dish and incubating at room temperature for 20 min. Plates were then washed once with 1 mL of appropriate media before being overlaid with 2 mL of media and then imaged using a Zeiss LSM 880 confocal microscope. Images were processed using Fiji49.

Analysis of pre-rRNA processing. Steady-state levels of pre-rRNAs were assayed using northern blot and primer extension analysis50. Ten milliliters of cells were harvested, frozen, and RNA was extracted using phenol. Five microgram of RNA was used for primer extension reactions or loaded onto a formaldehyde/MOPS agarose gel for northern blotting. 32P-labeled oligonucleotide probes for specific pre-rRNAs were used in primer extension reactions and for hybridization in northern blots. For northern hybridization of small molecular weight RNAs (7, 6, 5.8, and 55 S), RNA samples were mixed with an equal volume of sample buffer (0.1x TBE buffer, 10 M urea, 0.1% xyline cyanol, 0.1% bromophenol blue) and subjected to electrophoresis on a 5% acrylamide/7 M urea gel for 4 h at 120 mA. Following electrophoresis, gels were electroblotted to a Nytran N membrane (GE Healthcare Life Sciences) using a Trans-Blot Plus Cell (BioRad), hybridized with an end-labeled oligonucleotide, washed, and exposed to X-ray film.

Affinity purification and analysis of preribosomes by silver staining, western blotting, and iTRAQ mass spectrometry. The protein composition profiles of affinity-purified preribosomes was analyzed by SDS-PAGE (4–20% Tris-glycine and 4–12% Bis-Tris, Thermo Fisher Scientific) followed by silver staining. Western blotting tested incorporation of specific proteins into preribosomes.30 Pre- and polysomes were purified by lysing frozen cells using acid-washed glass beads and TNM150 buffer consisting of 50 mM Tris HCl pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 0.1% NP-40 (Sigma–Aldrich), and 5 mM βME. Following lysis, lysates were incubated with IgG-conjugated Dynabeads while rocking for 1 h at 4°C. Dynabeads were washed with TNM150 buffer (for iTRAQ, this step excluded NP-40). Samples were eluted using TEV protease while rocking for 1 h at room temperature, then precipitated with 10% TCA and washed with 100% cold acetone. Purified samples were sent to the Penn State Hershey Core Research Facilities for trypsin digestion and 4-plex labeling with iTRAQ reagents 114, 115, 116, 117 (Applied Biosystems).

Purification of preribosomes for cryo-EM. Preparation of Nog2-associated particles described here required 20 L of cells grown to an OD600 of 0.8–1.0 in appropriate media. Frozen cell pellets were lysed using TAP lysis buffer consisting of 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl2, and 0.075% NP-40 (Sigma–Aldrich). Lysates were incubated with IgG-conjugated Dynabeads while rocking for 1 h at 4°C. Dynabeads were washed with TAP lysis buffer three times and then washed twice using TEV cleavage/resuspension buffer (TAP lysis buffer with NP-40 excluded). Samples were eluted using TEV protease while rocking for
1 h at room temperature. The eluate was then added to a 100 K centrifugal filter (Millipore), prewashed with TVE cleavage-resuspension buffer, and spun at 14,000 × g for 5 min. The flow through was discarded and the sample was recovered by inverting the filters and spinning again at 1000 × g for 2 min before being stored at −80 °C until preparation for cryo-EM.

Cryo-EM data acquisition. Vitrified specimens were prepared by adding 4 μL samples of nog1ΔC rei1ΔC reh1ΔC or rplΔΔ3–87 particles at a concentration of ~150 nM to a glow-discharged holey carbon grid (Quantifoil R1.2/1.3) covered with a freshly made thin carbon film. Grids were blotted for 0.5 s and plunge-frozen into liquid ethane using an FEI Vitrobot Mark IV (4 °C and 100% humidity). The cryo-grids were initially screened at a nominal magnification of 92,000× in an FEI Talos Arctica microscope, operated with an acceleration voltage of 200 kV. Good-quality grids were transferred to an FEI Titan Krios electron microscope that was operating at 300 kV. Images were recorded using a K2 Summit direct electron detector (Gatan) in counting mode at a nominal magnification of ×105,000 (for the rplΔΔ3–87 sample) and ×130,000 (for the nog1ΔC rei1ΔC reh1ΔC sample), respectively (pixel sizes on the object scale are 1.373 and 1.052 Å, respectively), with the defocus ranging from −1.0 to −2.0 μm. Comma-free alignment was manually optimized and parallel illumination was verified before data collection. 5 S RNP (in the premature rotated state) and classes R3–R7 also have well resolved structural features; but they lack densities for major domains of rRNA, suggesting that they are likely turnover products (Supplementary Fig. 3a–e).

For the nog1ΔC rei1ΔC reh1ΔC mutant sample, a total of 9115 micrographs were collected and 883,649 particles were picked for a cascade 2D and 3D classification with a binning factor of two. About 57% of particles were removed during several rounds of 2D and 3D classification, and 382,748 particles were split into ten classes during the final round of 3D classification (Supplementary Fig. 2). After the final round of 3D classification, a total of 103,319 particles were applied for high-resolution refinement (without binning), resulting in a 3.1 Å map (gold-standard FSC 0.14 contrast transfer function parameters of each micrograph were estimated by Gctf, and adjusted with COOT55. The atomic model of classes R1 and R2 of the final round of 3D classification into ten classes. Around 62.6% of these particles are mature or mutant particles were further refined against the density map of classes R1 and R2 of the rplΔΔ3–87 mutant particles were deposited in the Electron Microscopy Data Bank (EMDB) under accession numbers EMD-30170, EMD-30174, EMD-30172, EMD-30173, EMD-30175, and 54, respectively, and atomic models of the R1 and R2 classes of the rplΔΔ3–87 mutant particles have been deposited in the Protein Data Bank (PDB) under accession numbers 7BTG and 7BTB, respectively. Source data are provided with this paper.

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Cryo-EM data processing. Original image stacks were summed and corrected for drift and beam-induced motion at the micrograph level using the MotionCor2 program49. The SPIDER50 software was used for micrograph screening. The drift and beam-induced motion at the micrograph level using the MotionCor2 program49. The SPIDER50 software was used for micrograph screening. The SPIDER50 software was used for micrograph screening. The SPIDER50 software was used for micrograph screening. The SPIDER50 software was used for micrograph screening. The SPIDER50 software was used for micrograph screening. The SPIDER50 software was used for micrograph screening. The SPIDER50 software was used for micrograph screening. The SPIDER50 software was used for micrograph screening. The SPIDER50 software was used for micrograph screening. The SPIDER50 software was used for micrograph screening. The SPIDER50 software was used for micrograph screening.}

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Author contributions
D.W. and J.W. designed the study. D.W., M.G., and A.L constructed strains and plasmids, and D.W. and M.G. assayed effects of mutations on ribosome assembly. Y.L. collected the cryo-EM data and Y.L. and N.G processed the data. Y.L. built the atomic models, and together with D.W., N.G., and J.W., analyzed the structures. D.W., Y.L., N.G., and J.W. interpreted results and wrote the paper.

Competing interests
The authors declare no competing interests.

Additional information
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