High-resolution structures of a thermophilic eukaryotic 80S ribosome reveal atomistic details of translocation

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Ribosomes are complex and highly conserved ribonucleoprotein assemblies catalyzing protein biosynthesis in every organism. Here we present high-resolution cryo-EM structures of the 80S ribosome from a thermophilic fungus in two rotational states, which due to increased 80S stability provide a number of mechanistic details of eukaryotic translation. We identify a universally conserved 'nested base-triple knot' in the 26S rRNA at the polypeptide tunnel exit with a bulged-out nucleotide that likely serves as an adaptable element for nascent chain containment and handover. We visualize the structure and dynamics of the ribosome protective factor Stm1 upon ribosomal 40S head swiveling. We describe the structural impact of a unique and essential m1acp3Ψ 18S rRNA hyper-modification embracing the anticodon wobble-position for eukaryotic tRNA and mRNA translocation. We complete the eEF2-GTPase switch cycle describing the GDP-bound post-hydrolysis state. Taken together, our data and their integration into the structural landscape of 80S ribosomes furthers our understanding of protein biogenesis.

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Ribosomes are complex macromolecular assemblies consisting of ribosomal proteins (RPs) and ribosomal RNA (rRNA), which catalyze protein synthesis in all cells. In the past decades, huge advances in understanding eukaryotic protein biosynthesis have been made with key contributions from structure determination of vacant, mRNA, tRNA, and different associated factors containing ribosomes from various organisms. For the late phase of translation, in order to complete the elongation cycle after peptide-bond formation, the deacylated P-site tRNA needs to be translocated to the E-site, and the mRNA accordingly one codon forward.

Translocation is induced by rotation of the small ribosomal 40S subunit in respect to the large 60S subunit, and rotation is complemented by swiveling of the 40S head perpendicular to the rotation axis of the 40S body. During translocation, in both prokaryotes and eukaryotes, the tRNAs move from the non-rotated A/A P/P PRE state, via various A/P P/E rotated hybrid states, to the translocated P/P E/E POST state.

In a classical mechanistic view of translation, mostly derived from the bacterial system, the whole machinery translocates the tRNAs and attached mRNA without frame shifting, and stops in the forward position (induced by a *doorstop or pawl*) that keeps the tRNA3-mRNA module in the translocated POST state and prevents a backward movement of the tRNAs. The mRNA translocation step is physically driven by correlated tRNA movements guided by the framework of the ribosome that by itself acts as a ‘Brownian-ratchet’.

The general architecture of the ribosome is highly homologous to *C. thermophilum* 80S ribosomes. In order to obtain suitable samples of *C. thermophilum* ribosomes for high-resolution single-particle cryo-EM analyses, we adapted well established protocols for the purification of mesophilic and mammalian 80S monosomes (Supplementary Fig. 1). Cryo-EM reconstructions have been refined to an average resolution of 2.9 Å for the PRE-POST and 3.0 Å for the pe/E (TI)-POST state (Supplementary Figs. 2 and 3, Supplementary Table 1). Both states are devoid of mRNA and only the (TI)-POST state contains an undefined tRNA bound in the pe/E hybrid conformation. The resolution enabled us to build a high-precision model for a cytoplasmic 80S ribosome and to re-evaluate mechanisms of protein translation. The general architecture of the *C. thermophilum* 80S ribosome (Fig. 1) is similar to *T. lanuginosus* and *S. cerevisiae*. It contains 80 core RPs that are present in all eukaryotic cytoplasmic 80S ribosomes. *C. thermophilum* specific RPs were not found, based on complete density interpretation. On the contrary, some of the identified and built RPs were not annotated in the online resource for the *C. thermophilum* genome and a few proteins did not match the sequence deposited in the database (Supplementary Table 2). Due to its intrinsic flexibility, the L1-stalk was not built in either of the two structures.

The presence of *C. thermophilum* 80S RPs was validated by mass spectrometric analysis using intensity-based absolute quantification (iBAQ). Proteins and complexes from this fungus have proved beneficial for structural analyses, e.g. of proteins involved in ribosome biogenesis and assembly. Thermophiles had an immense impact in ribosome research due to their enhanced stability allowing for structure determination at relatively high resolution. However, while this powerful tool was mainly used for prokaryotic ribosomes, its exploitation for eukaryotes lags far behind. Using isolated 80S ribosomes from *C. thermophilum*, we present single-particle cryo-electron microscopy (cryo-EM) structures of an idle PRE state ribosome and of a hybrid pe/E tRNA translocation-intermediate (TI)-POST state (0° rotation, 7° swiveling) bound to eEF2-GDP-Mg2+. An additional factor has been applied in both states, although no starvation protocol has been applied during cell growth, is the protein Stm1, both a protective and translation suppressing factor. Stm1 is described here in detail, and we find it also bound to the non-rotated (and non-swiveled) POST state, which was not observed so far. Despite treatment with the antibiotic puromycin that is commonly used for forcing nascent chain (NC) release, NCs are present in both ribosomal states. We define a universal and adaptable RNA tertiary motif narrowing the very end of the polypeptide tunnel exit. Furthermore, we observe numerous chemical modifications of the rRNA and ribosomal proteins including N-terminal acetylation and hundreds of ions modelled as magnesium. We describe details of the Dph modification of eEF2 bound to GDP-Mg2+ in contact with Stm1 in the ribosomal A-site. The invariant m’acpr’Y 185 rRNA modification in the 40S head serves as part of a ‘wobble-seal’ and seems involved in in-frame shifting during translocation. Finally, we detail the eEF2-GTPase switch cycle, which until now was not fully understood due to the lack of high-resolution structures of all eEF2 states in context of the ribosome.

**Results**

**Overall structure of *C. thermophilum* 80S ribosomes.** In order to obtain suitable samples of *C. thermophilum* ribosomes for high-resolution single-particle cryo-EM analyses, we adapted well established protocols for the purification of mesophilic and mammalian 80S monosomes (Supplementary Fig. 1). Cryo-EM reconstructions have been refined to an average resolution of 2.9 Å for the PRE-POST and 3.0 Å for the pe/E (TI)-POST state (Supplementary Figs. 2 and 3, Supplementary Table 1). Both states are devoid of mRNA and only the (TI)-POST state contains an undefined tRNA bound in the pe/E hybrid conformation. The resolution enabled us to build a high-precision model for a cytoplasmic 80S ribosome and to re-evaluate mechanisms of protein translation. The general architecture of the *C. thermophilum* 80S ribosome (Fig. 1) is similar to *T. lanuginosus* and *S. cerevisiae*. It contains 80 core RPs that are present in all eukaryotic cytoplasmic 80S ribosomes. *C. thermophilum* specific RPs were not found, based on complete density interpretation. On the contrary, some of the identified and built RPs were not annotated in the online resource for the *C. thermophilum* genome and a few proteins did not match the sequence deposited in the database (Supplementary Table 2). Due to its intrinsic flexibility, the L1-stalk was not built in either of the two structures.

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Even though the *C. thermophilum* ribosome is highly homologous to *S. cerevisiae* 80S (Sc80S), it also shares similarities to the human ribosome, especially concerning RP tails. For example, while *S. cerevisiae* eS26 has a short C-terminus, the *C. thermophilum*
protein has an extended C-terminal tail (even longer than in *H. sapiens* 80S (Hs80S)) bridging over the mRNA exit channel (Supplementary Fig. 6a). Another example is eS6, which in *S. cerevisiae* has a short α-helical C-terminus. In *C. thermophilum* RP eS6 extends towards expansion segment 6 (ES6), and in human ribosomes it contacts ES62 (Supplementary Fig. 6b). In *Ct*80S, RPs eL6 and eL28 interact with ES7 thus stabilizing and fixing its position on the ribosomal surface. This interaction has also been observed in the *Hs*80S, but is not present in the *Sc*80S ribosome (which lacks eL28) (Supplementary Fig. 6c).

In order to analyze the stability of *C. thermophilum* ribosomes, we compared them with ribosomes from mesophilic *S. cerevisiae* (Sc) using nanoDSF. The melting temperature of *Ct*80S is 22 °C higher than for *Sc*80S (T_m of 62.5 °C and 40.5 °C, respectively) (Supplementary Fig. 7). While we exploit this increased stability of *Ct*80S primarily as a tool to obtain high-resolution structures, it implies significant adaptations, which are, however, difficult to dissect in a quantitative manner. Overall, thermophilic RP adaptations concern amino acid composition (bulky IVYWREL amino acids +5%, arginines +11%, and prolines +13%) as found in bacteria and archaea, and as also validated for *C. thermophilum* 27. Increased bulkiness also confers to protein length, and *Ct*80S RPs often have terminal extensions compared with yeast. Whether thermophily of *Ct*80S also relates to the generally higher abundance of some RPs 38 or their higher affinity to rRNA as observed for thermophilic bacteria is not known so...
A key factor that usually contributes to the increased stability is the G + C content of the rRNA. C. thermophilum 18S and 26S rRNA have a 4.5% higher G + C content than S. cerevisiae, and this trend also shows for the SS and 5.8S rRNA, however, less pronounced (Supplementary Table 4). However, the G + C content is similarly increased in the mesophilic fungus Chaetomium globosum and thus might also be a phylogenetic attribute of certain molds. We also observe an increase of repeats of three or more nucleotides with the same nucleobase (G and C homoiterons), which are known to support RNA secondary structure and stability of association with RPs41 (Supplementary Fig. 11). For 18S rRNA, especially Cs7 is much shorter than human Cs7, and even ES7 is much shorter than human ES7 and even slightly shorter and more compact than the yeast one. For 26S rRNA, segments H15, H30, ES19, and ES27 are shorter than their counterparts of the Sc26S. G + C repeats are very common in ESs, where they partake a crucial role in reducing the flexibility of the peripheral rRNA.

The general analysis of the C. thermophilum rRNA revealed a total of 1794 nucleotides for 18S rRNA and 3338 nucleotides for 26S rRNA, numbers similar to S. cerevisiae and sharing 89% and 85% sequence identity, respectively. The rRNA structures in Ct80S are analogous to Sc80S, with the most obvious differences in the ESs (Supplementary Figs. 9–11). For 18S rRNA, especially C. thermophilum ES7 is much shorter than human ES7, and even slightly shorter and more compact than the yeast one. For 26S rRNA, segments H15, H30, ES19, and ES27 are shorter than their counterparts of the Sc26S. G + C repeats are very common in ESs, with e.g. ES27 having four additional homoiterons alone. Also, the Ct18S ES6A and ES6B, and ES12 adopt different conformations (Supplementary Fig. 9). Taken together, the general criteria described for thermostability are met by the Ct80S RPs and rRNAs, and the high stability of Ct80S allowed us to determine high-resolution structures in two rotational states.

Nascent chains and exit tunnel constrictions. The cryo-EM reconstructions show that the C. thermophilum ribosomes contain density throughout the exit tunnel in both the idle and pe/E (TI)-POST states, which can be explained satisfactorily only as mixture of nascent chains (NCs) (Fig. 3a). This came as a surprise, as puromycin (a protein synthesis inhibitor) treatment during ribosome preparation typically results in premature NC termination during translation and the growing peptides are released as peptidyl-puromycin42. However, puromycin treatment of Ct80S did not result in significant NC release and our iBAQ analyses show no additional stress factors apart from Stm1. Following a purification protocol adapted for S. cerevisiae ribosomes, we initially performed puromycin treatment of Ct80S at 30 °C. As C. thermophilum grows at 30–55 °C, we tested whether NC stalling can be overcome at higher temperature and performed puromycin incubation of Ct80S at 50 °C. Subsequent cryo-EM structure determination again resulted in the idle and pe/E (TI)-POST states that both still contained NCs in the exit tunnel revealing that puromycin treatment at higher temperature also does not release NCs from Ct80S. In contrast, human ribosomes analogously prepared with the same puromycin batch did not retain any NCs as judged by cryo-EM.

In order to see whether there is a structural explanation for the presence of NCs in Ct80S, we carefully inspected the polypeptide exit tunnel. The observed density for the NCs results from a mixture of different peptides present in the exit tunnel and was modelled as a poly-alanine chain of 25 amino acids. In both rotated states, we were able to trace the NC from the beginning of the empty peptidyl transferase center (PTC) up to the very tunnel exit. Closer inspection shows the two established constrictions (Supplementary Fig. 12a). The first one is created by uL4 and...
Fig. 4 Ribosome-associated factor Stm1 and the m<sup>1</sup>acp<sup>54</sup>Ψ hypermodification. a Surface representation of the 80S in both rotational states with bound Stm1 (red), eEF2 (orange), and pe/E tRNA (blue). b Zoom on the pathway of Stm1 following the P- and A-sites, and the mRNA entry tunnel. A-, P-, and E-sites are marked with circles. c In the idle POST state Stm1 occupies the mRNA position in the P-site. The strictly conserved m<sup>1</sup>acp<sup>54</sup>Ψ hypermodification in eukaryotic 18S rRNA (CU1188) lines the P-site (cyan line). Cryo-EM map is shown for central features (2 e). d In the rotated (TI)-POST state of 80S, Stm1 conformation is changed and m<sup>1</sup>acp<sup>54</sup>Ψ1188 forms an interaction with the pe/E-site tRNA-ASL in the E-site. e In presence of the codon-anticodon<sup>2</sup>, the modified nucleotide (not built originally) forms a lid described here as *wobble-seal* together with a conserved cytosine stacking on the wobble.

uL22 loops, localizes at the NC residues 10–12, and is conserved in all domains of life<sup>43,44</sup>. The second one is created by the uL4 protein, localizes at NC residue 16, and is specific for eukaryotic ribosomes<sup>44</sup>. However, apart from these two conserved constrictions, we observe a third constriction at the very end of the 80S exit tunnel that has previously not been described. Here, guanosine G1485 of 26S rRNA H50 is bulged-out towards the tunnel thus narrowing the passageway to only about 12 Å (Fig. 3b and Supplementary Fig. 12b). G1485 forms the largest direct contact with the NC. For comparison, in Sc80S (without NC) the corresponding cytosine (C1502) is not bulged-out and hidden in the 26S rRNA<sup>45</sup>, which results in a much wider diameter of the exit tunnel of about 20 Å (Fig. 3c and Supplementary Fig. 12c). In a cryo-EM structure of the yeast ribosome/NatA (pull-out with a mixture of NCs)<sup>45</sup>, C1502 seems to remain hidden in the rRNA, however, also with no electron density of the NC visible next to this cytosine. This suggests that in C80S the NCs might be stabilized in the exit tunnel by the additional interaction with the bulged-out G1485.

Guanosine G1485 is not only present in *C. thermophilum*, but also in the 28S rRNA of mammals and other eukaryotes. It is the central element of an unusual minimal nested RNA pseudoknot, which we describe as a ‘nested base-triple knot’ with two intertwined triples and an exposed base stack forming a platform for the bulged-out nucleotide (Fig. 3d). This RNA pseudoknot has so far not been annotated although it is present in ribosomes of all eukaryotes (e.g. *H. sapiens*, *S. cerevisiae*; Fig. 3e), prokaryotes (e.g. *E. coli* or *Thermus thermophilus*)<sup>46</sup> and archaea (e.g. *Haloarcula marismortui*)<sup>47</sup> structurally characterized so far, and based on sequence comparisons it seems universally conserved (Supplementary Fig. 13). The C80S structures of this study are to our knowledge the first reported examples with a bulged-out nucleotide originating from one of the base-triples (base-triple 2). In order to analyze this further, we carefully revisited mammalian 80S cryo-EM structures obtained with and without NCs. In a recent 3.0 Å 80S structure with ribosomes stalled on the NC of the ribosome-arresting XBP1u protein<sup>48</sup>, the EM-density indicates at least partial flipping of the base into the tunnel although this feature (and the adjacent NC) was not built. Similarly, in a stalled mammalian RNC in complex with the signal recognition particle (SRP)<sup>4</sup> (Fig. 3f), the EM-density also indicates that the nucleotide is partially bulged-out although it was not built. Finally, in the high-resolution cryo-EM structure of the idle human 80S ribosome<sup>49</sup>, the careful re-evaluation of the cryo-EM-density indicates nucleotide dynamics also in the absence of a NC. Taken together, these observations show that this base-flipping into the exit tunnel can occur independent of a NC and is a more general feature that so far has escaped attention.

Two conformations of the ribosome-associated factor Stm1. Both of our C80S structures contain the ribosome inhibitory protein Stm1 (Suppressor of target of Myb protein 1). Stm1 and its analogues have so far only been described in context of rotated ribosomal states<sup>1,2,50</sup>. Interestingly, we observe Stm1 not only in the rotated pe/E (TI)-POST state with bound eEF2-GDP but also in the back-rotated idle POST state, although in a markedly different conformation (Fig. 4a, b). We could not assign the entire polypeptide of Stm1, but for the (TI)-POST state we were able to build residues 26–49, 60–80, and 89–154. Here, Stm1 adopts an extended toothpick-like structure starting from the central pro-tuberance (CP) of the 60S up to 40S body/head interface, following the mRNA track backwards and passing through the P- and A-sites (here occupying the codon-anticodon space), and the mRNA entry tunnel. In the CP, Stm1 wraps around the 5S rRNA, H84 of 26S rRNA, ul5, and el42 (Supplementary Fig. 14a). Stm1 then passes the intersubunit bridge B2a and thus stabilizes the rotated state as observed earlier<sup>50</sup>. Of note, the intersubunit bridges in C80S are similar as reported for other eukaryotic ribosomes<sup>1,3</sup> (Supplementary Table 5). Importantly, Stm1 not only occupies important functional sites of the ribosome, but also inserts an extended α-helix (residues 127–154) in the 40S body/
Fig. 5 Ct80S modifications and ligands. a N-terminal acetylation of Ser2 of uL13 (SAC2) is shown in its cryo-EM-density and with close interactors (highlighted by color and with dashed connector lines). b Conserved 2'-O methylation in the PTC. The high resolution allows for detailed modelling of sugar methylations and conformations (OMG2578), and of magnesium coordination. The peptidyl-tRNA is modeled from a superposed high-resolution 5c80S structure. c Typical example for a magnesium ion (magenta) with its hydration shell tethering two rRNA helices of 5.8S and 26S rRNA, respectively. Interactions are indicated by dashed lines. All cryo-EM maps are contoured at a 2.5 σ level.

head interface next to the mRNA entry tunnel, thus interfering with back-swiveling of the 40S head (Supplementary Fig. 14b). The extended α-helix forms two salt bridges to conserved arginines of uS3 that are known to be important for pre-initiation complex stabilization. The mRNA entry tunnel is entirely blocked by Stm1 (111-126), located between uS3 and uS5. Multiple contacts with the 40S include uS3, eS5, eS10, eS12, uS13, uS19, a tight interaction with 18S mRNA bases it can be regarded here as an mRNA placeholder. In the idle POST state, Stm1 Arg94 takes the wobble position of the mRNA within the P-site and is RRGGFRGRGKREE-101). In the idle POST state, Stm1 Arg94 shifts Arg94 above the 40S head in the (TI)-POST state, Stm1 Arg94 in the 40S head (residues 127-147) is found to be important for extending the 40S body and, the swiveling-block is released (Supplementary Fig. 14d, e). Electron density is not visible at the 60S subunit or in the mRNA entry tunnel and the A-site, and Stm1 binding to the P-site is also altered with Stm1 folding into an open hairpin structure. The A- and P-sites in both Ct80S structures are devoid of mRNA and tRNA (pe/E state-free major parts of P-site) and Stm1 fills the mRNA-void with an extremely flexible and surprisingly positively charged sequence fingerprint (89-RRGFRGRGKREE-101). In the idle POST state, Stm1 Arg94 takes the wobble position of the mRNA within the P-site and is involved in π-cation stacking with an invariant cytosine (CiC1633) from H44 of 18S rRNA (Fig. 4c). The swiveling of the 40S head in the (TI)-POST state shifts Arg94 above the wobble position allowing the Stm1 region around the 94-RGR motif to contact the anticodon of the pe/E tRNA (Fig. 4d). Comparison with a yeast 80S structure containing the mRNA (Fig. 4e) shows that Stm1 mainly occupies the mRNA binding region. With its positively charged residues taking the space of the mRNA bases it can be regarded here as an mRNA placeholder. Having observed this particular binding mode of Stm1, we performed a sequence comparison with the corresponding proteins in yeast and humans (Supplementary Fig. 15). Sequence conservation is rather local, like e.g., a WG-motif (CTV123) binding in the mRNA entry tunnel, or comprises extended RG-repeats occupying the A- and P-sites as found in this study. These repeats are also found in the C-termini, which are not resolved in any cryo-EM structure.

Protein and rRNA modifications. In our structure of the idle Ct80S ribosome at 2.9 Å extra density for putative chemical modifications is present as described in detail for other eukaryotes. However, modifications have not yet been analyzed and validated (e.g., by mass spectrometry) for C. thermophilum ribosomes. Therefore, we focus here on two strictly-maintained rRNA modifications and two examples for protein modifications, an N-terminal RP acetylation and the diphthamide (Dph) modification of eEF2 (see below). N-terminal acetylation is a widespread protein modification among eukaryotes and alters lifespan, folding characteristics and binding properties of the acetylated proteins. N-terminal acetylation of RPs was also shown to influence translational fidelity in yeast. In our MS analysis, 31 out of the 70 most abundant RPs were found to be modified and most often when the methionine is cleaved-off. Interestingly, these RPs are most of the time detected in similar amounts with or without modification (Supplementary Table 3). As an example of how N-terminal acetylation stabilizes protein-protein interactions in the ribosome, uL13 Ser2-Ac of the 60S subunit is shown (Fig. 5a).

Most of the rRNA modifications are known to cluster around the central functional sites as the PTC, A- and P-sites, the DC, or intersubunit bridges. In eukaryotes, the most abundant RNA modifications are 2'-O methylation of the ribose and the isomerization of uridine to pseudouridine (Ψ). For example, in the PTC the penultimate cytosine of the peptidyl-tRNA forms a Watson-Crick base pair with a conserved guanine that always carries a ribose methylation (OMG2578 in Ct26S rRNA). Despite the absence of the P-site tRNA, this methylation is clearly identified in our idle Ct80S structure, highlighting the level of detail resolved in high-resolution cryo-EM reconstructions (Fig. 5b). The modification is found at the tip of the short H80 stem-loop with the 2'-O-Me moiety in plane with the nucleotide base, which has been described to be important for extending base-stacking. The quality of the density allows us to confirm the sugar pucker as the 3'-endo conformation as observed for non-flipped rRNA bases. The same region also highlights the importance of magnesium ions for ribosome integrity, and overall, more than 550 ions could be localized with many of them tethering rRNA helices (Fig. 5c). The H80 closing uridine (U2576) forms a U-turn and is ligated to magnesium as a mediator to close phosphates and bound to an uL10 arginine side-chain (R116) at the heart of the PTC, respectively.

A function for the unique m1acp3Ψ hypermodification. An important and conserved rRNA modification in eukaryotic ribosomes is the 1-methyl-3-α-amino-α-carboxyl-propyl pseudouridine (m1acp3Ψ) hypermodification in 18S rRNA of the 40S head. In our idle Ct80S structure, m1acp3Ψ (U1188 in Ct18S,
U1191 in Sc18S, and U1248 in Hs18S rRNA) lines the P-site at the tRNA wobble position (Fig. 4c) as also found in the idle human 80S ribosome49. The mRNA codon wobble position is filled by Stm1 as described above. The comparison with a yeast 80S cryo-EM structure, including mRNA and P-site tRNA stalled on an obligate A1 wobble base pair in the POST state (modification not built, but defined in the same orientation)5, allows to derive a specific role for this modification. It forms a ‘wobble-seal’ with intimate contacts to the nucleotide in position 1 of the anticodon (Fig. 4e). In detail, while the α-amino group hydrogen-bonds to the inosine phosphate as seen in the A1 wobble, the α-carboxyl binds to the exocyclic N2 of an invariant cytosine of 18S rRNA (C1633, Sc1637, Hs1701; at the base of H44) that stacks on top of the wobble base pair. Moreover, the plane of the modified base, extended by the 1-methyl group, stacks upon the inosine-ribose, as typical for carbohydrate-binding to hydrophobic side chains. These interactions indicate that the hyper-modification is essential to close the seal over the wobble. Prokaryotes do not have this modification, however, the exchange of the uracil for the larger and (m2)-modified guanine (essential for bacterial fitness56) perfectly matches the hypermodification as defined for the E. coli system57 (Supplementary Fig. 16). Here, as also in yeast5, a conserved arginine at the C-terminus of uS9 aids tRNA binding by stabilizing the anticodon from the side. This arginine is exchanged in C. thermophilum to a lysine that is turned away (Fig. 4c).

Interestingly, despite the (TI)-POST structure presenting an off-pathway intermediate, it corresponds to a late intermediate of translocation according to its rotational state58. During rotation between our two observed states, m3aca3Ψ1188 follows the swirling motion as part of the 40S head; however, it still binds to the ASL of the translocating pe/E tRNA the same way as it would as part of the wobble-seal with a PRE P/P tRNA as described above (movement of 12.5 Å; Fig. 4d and Supplementary Fig. 17). Due to the tight interaction of this large rRNA modification grabbing around the whole nucleotide, and the importance of the ASL for tRNA binding10, it seems likely that the tRNA rotates back and forth during ribosomal Brownian-ratcheting together with m3aca3Ψ1188 and staying in direct contact throughout.

The eEF2-GTPase switch cycle and ribosomal rotation. The elongation factor eEF2 undergoes large conformational changes upon ribosome binding and rotation18. Our late pe/E tRNA (TI)-POST ribosome structure allows the assignment of eEF2-GDP-Mg2+ that corresponds to the GTP post-hydrolysis conformation just before eEF2 leaves the full-translocated ribosome. In this state, the Dph modification of a histidine (C1701) at the apex of eEF2 domain IV is located deeply inserted into the ribosomal A-site and is surrounded by the Stm1 protein (Fig. 6a and Supplementary Fig. 14c). Overall eEF2-ribosome interactions are detailed in Supplementary Fig. 18. Although the Stm1-bound structure is an off-pathway intermediate and contacts are weak, the Dph modification is found in the same position as observed in structures of the mammalian ribosome in different (TI)-POST states including a complete tRNA•mRNA module10,18 (Fig. 6a). Here, Dph was found in tight contact with the minor groove of the P-site codon-anticodon, whereas in the PRE state24, it has been found moved outwards from the A-site in close contact to the so-called ‘monitoring adenes’ of the DC. In absence of a tRNA•mRNA module, our late pe/E tRNA (TI)-POST state bound to eEF2-GDP reveals the monitoring adenes of the DC hidden in H44 of the 40S head (Fig. 6a).

Efficient translocation of the tRNA•mRNA module of the processive ribosome relies on eEF2-GTP hydrolysis. Distant from Dph, the active site of the eEF2-GTPase is located in domain 1, which is hooked on the SRL24,59 (Fig. 6b). Our pe/E tRNA (TI)-POST structure with eEF2-GDP-Mg2+ now provides insights into a late (TI)-state after GTP hydrolysis (Fig. 6c). GTP hydrolysis relies on two flexible switch regions (1 and 2) of the GTPase. The switch 2 region, harboring the catalytic histidine (CH1108) is ordered and two magnesium ions are found to bridge between negatively charged side chains and the phosphoribose backbone of the SRL. Comparing unbound elongation factors (EF-G or eEF2) with ribosome-bound ones23,60 shows how the SRL generally pushes on the GTPase switch 2 region (Fig. 6c, d) and thereby activates the catalytic histidine, which flips into the active site to position the catalytic water molecule (not modelled in other cryo-EM structures). GTP hydrolysis, and probably subsequent phosphate release, as shown very recently by detailed time-resolved cryo-EM studies for the bacterial system61,62, impacts on eEF2-ribosome interactions by modulating and loosening contact sites, as observed in our eEF2-GDP structure between the P-loop and the SRL.

GTP hydrolysis also requires ordering of the switch 1 region to allow completion of the active site usually by an arginine finger, which is often supplied for GTPase activation either in cis or in trans by a GAP (GTPase-activating protein)63. In our (TI)-POST structure the whole switch 1 effector loop (residues 42–67) of eEF2 is disordered (Fig. 6b, e) indicating major rearrangements along the GTPase switch cycle. A high-resolution X-ray structure of eEF2-GTP is lacking, however, also in a bacterial EF-G-GMPPNP structure60 switch 1 is disordered and an arginine finger was not defined. Mutational studies on switch 1 arginines of E. coli EF-G only showed moderate inhibition and thus an arginine finger hypothesis was discarded64. Interestingly, in an 80S-eEF2-GMPPCP structure in context of the full-rotated PRE ribosomal state24 as well as in previous low-resolution structures18,19, switch 1 adopts a defined conformation and establishes a contact to the 40S body (ScA416 of H5 in 18S rRNA) (Fig. 6b). Although not described, in this full-rotated structure an inbuilt arginine finger (ScArg55 of eEF2, CtArg55) is indeed placed on top of the scissile bond (Fig. 6b, f). Interestingly, a corresponding arginine seems to be absent in bacteria and archaea (Supplementary Fig. 19). Of note, eukaryotic (and archael) switch 1 loops have a four-residue deletion inducing a conformational change, which makes a direct structural comparison difficult.

Finally, the ordering of the eukaryotic switch 1 region is transmitted to eEF2 domains II and III by a relay system. As deduced from yeast eEF224 (Fig. 6f), a positively charged residue (ScArg440) on the tip of an extended hairpin of domain II is fixed in a pocket below the arginine finger. Similarly, domain III is held via at least one salt bridge (ScArg60/Asp548). However, in our (TI)-POST eEF2-GDP structure, the conformational changes induced upon GTP hydrolysis and phosphate release result not only in a disordered switch 1, they also abrogate the contacts with domains II and III that transmit the hydrolysis event to domain IV (Fig. 6e). Overall, eEF2-GDP is now ready to leave the ribosome.

Discussion
Understanding the molecular mechanisms of the ribosome has for decades been a central theme in molecular cell biology. Here, we provide structural data for the thermophilic fungus Chaetomium thermophilum and deduce functional implications for the eukaryotic 80S ribosome integrating the vast database of ribosome research. The structural analyses are complemented by quantitative mass spectrometry (iBAQ). The iBAQ data nicely mirror the structural findings by giving an inventory for the ribosome and its associated factors, and reveal important...
information on the N-terminal acetylation status of the RPs. The high-resolution structures provide a blueprint for a thermophilic 80S ribosome and give some insights into thermal adaptations both on protein and RNA levels. Overall, RPs contain more bulky side chains and elongated tails; and in a unique case an eL41 duplicate fills an rRNA packing void. The ribosome shows an increased GC content with respective homoiterons as known for bacterial thermophiles and the ESs are shorter than in mesophilic 80S ribosome structures to a lower extent (so far undetected), the equilibrium of base-pairing is shifted in C80S. Revisiting a cryo-EM reconstruction of mammalian RNCs in complex with the co-translational targeting SRP complex, we find evidence for a partially flipped guanosine (G2416 in Oc28S rRNA) only 10 Å away from the SRP54M domain and about 25 Å from the signal peptide present in the structure (Fig. 3f and Supplementary Fig. 21). Therefore, bulging-out of this base might directly serve in NC handover to downstream factors. Already available cryo-EM reconstructions of mammalian RNCs in complex with ribosome-arresting NCs provide further evidence for such function.

Our structures represent snapshots of two rotational states along the eukaryotic ribosomal translocation cycle, although mRNA is missing: a late (TI)-POST state with pe/E tRNA and eEF2-GDP and the idle POST state. Both structures contain the ribosomal recycling factor Stm1, which acts as protective and stabilizing factor for hibernating ribosomes upon cellular stress conditions. Stm1 stabilizes the rotated state by spiking that likely prevents NC release despite puromycin treatment. This adaptable constriction seems to be a more general feature of eukaryotic ribosomes. While it is present in mesophilic 80S ribosome structures to a lower extent (so far undetected), the equilibrium of base-flipping is shifted in C80S. Revisiting a structure of mammalian RNCs in complex with the co-translational targeting SRP complex, we find evidence for a partially flipped guanosine (G2416 in Oc28S rRNA) only 10 Å away from the SRP54M domain and about 25 Å from the signal peptide present in the structure (Fig. 3f and Supplementary Fig. 21). Therefore, bulging-out of this base might directly serve in NC handover to downstream factors. Already available cryo-EM reconstructions of mammalian RNCs in complex with ribosome-arresting NCs provide further evidence for such function.
Fig. 7 Scheme of eEF2-catalyzed ribosome translocation. From left to right: Schematic for eukaryotic ribosomal states along a translocation cycle starting with the PRE state after peptide-bond formation. The m^3^acP^5^ hypermodiﬁcation (magenta bracket) within the 40S head, on top of position one of the anticodon in the wobble, helps as part of the wobble-seal in coupling ribosomal rotation to tRNA_mRNA module movements between the classical A/A P/P and the A/P P/E hybrid states. Dph (highlighted in blue) within domain IV of eEF2-GTP acts as a stopdoor (pawl) in the A-site and the fully-rotated state is stabilized. The full-rotated ribosome acts as GAP inducing GTP hydrolysis in eEF2, marking the transition to the translocated POST state. Back-rotation of the 40S head is uncoupled from the tRNA_mRNA module and Dph acts as `post-pawl` in contact with the P-site. eEF2-GDP binding is weakened due to the internal relay and SRL-contact loosing and upon back-swiveling of the 40S head, eEF2-GDP is released. Translocation is complete in the P/E E/P state.

Through both subunits and occupying important functional sites following exactly the mRNA trace, as observed in our (TI)-POST structure. In this state, Stm1 is known to inhibit ribosomal subunit splitting by the Dom34 recycling system. However, we find Stm1 also present in the non-rotated idle POST state, where it adopts a significantly different conformation leaving the A-site and mRNA entry tunnels empty. Here, these sites could be readily occupied by the Dom34 ribosomal subunit splitting protein (Supplementary Fig. 22) in the same manner as observed in yeast. Thus, we reason that only in the rotated state Stm1 protects from subunit splitting. Further, the Stm1 structure at least partially resembles the binding mode of the alternative ribosomal rescue factor ArfB. ArfB rescues bacterial and mitochondrial ribosomes on stalled nonstop mRNAs by releasing NCs from the P-site tRNA. The C-terminal part of both proteins pass via the A-site and are threaded through the entire mRNA entry channel making mRNA binding impossible (Supplementary Fig. 23). However, while Stm1 follows the mRNA path, the folded N-terminal hydrolase domain of ArfB mimics a release factor by inserting a GGQ motif into the PTC.

The high resolution of both C80S structures permits to build protein and RNA modifications with atomic precision and, in context with previous ribosomal data, provides new insights into the translocation process (Fig. 7). We observe the unique m^3^acP^5^ hypermodiﬁcation within 18S rRNA in the 40S head in atomic detail and describe it as an essential component of a codon-anticodon wobble-seal in the P-site. Its central importance is underlined by the loss of this modiﬁcation (or hypo-modiﬁcation) being implicated in about 50% of all rectal colon cancers. Due to its size and presence of additional functional groups, it ensures a tight association with the anticodon position 1 that seems to be important for wobble stability and for efﬁcient and in-frame translocation of the tRNA_mRNA module from the P- to the E-site. Furthermore, comparing our and other (TI)-POST structures including the complete tRNA_mRNA module from the P- to the E-site. Moreover, the assignment of the Dph conformation to a deﬁned post-state (a Dph post-pawl position) upon GTP hydrolysis. The post-state conformation of domain IV corresponds to the time-resolved cryo-EM data obtained very recently for EF-G, however, Dph is not present in bacteria.

Finally, we ﬁne the GTPase switch cycle of eEF2. Although, the pe/E tRNA-bound state in complex with eEF2 is common for structures determined upon puromycin treatment of mammalian and yeast ribosomes, the nucleotide load could not be assigned previously due to limited resolution and/or the mechanistic deﬁnition of the eEF2 switch cycle was not in the focus of these studies. Our C80S (TI)-POST state shows eEF2 in the GDP-Mg^2+ bound state in relatively loose contact with the SRL (mediated by magnesium ions) and the switch 2 region within the GTPase domain I. As expected after GTP hydrolysis and phosphate release, the switch 1 region is disordered. Our structure is similar to 80S-eEF2-GDP-ires structures from yeast stabilized by the antifungal sordarin. This study deﬁned 80S rotational dynamics of in total ﬁve eEF2-GDP-bound intermediates; however, the eEF2-GTPase switch cycle was not described. Further, revisiting and integrating previous structures with non-hydrolyzable eEF2-GTP reveals an intrinsic arginine finger within switch 1 that is susceptible to the ribosomal rotational state. Only in the transient full-rotated PRE state bound to eEF2-GTP, the 18S RNA within the 40S body pushes on switch 1 and places the arginine on top of the scissile bond. Thus, comparison of our ribosomal eEF2-GDP structure to the eEF2-GTP structures shows that the full-rotated ribosome acts as classical ‘GAP’ (GTPase-activating protein) for eEF2; the rotated 18S RNA places the switch 1 arginine finger and the SRL the switch 2 catalytic residue. Thus, it is not eEF2-GTP that stabilizes the full 80S rotation, but the rotation in situ ‘stabilizes’ eEF2-GTP in the full-activated conformation. How bacterial EF-G is activated by the rotating ribosome is left unexplained here, but recent time-resolved cryo-EM data indicate a different mechanism of sw1 compaction without an arginine finger and including a SRL twisting that modulates sw2 in the transition state of GTP hydrolysis.

Of note, eEF2 is a member of the ancient TRAFAC (translation factor) superfamily of GTPases that co-evolved with the ribosome at the same time as the SRP GTPases (SRP54, SRa) regulating co-translational targeting. Most TRAFAC GTPases seem to be activated by RNA (this study and refs. 68–70) and thus open a window to an ancient eukaryotic RNA world. While the GTPase switch cycle is stimulated by ribosome binding, translocation per se is uncoupled from GTP hydrolysis and it is only eEF2 release that depends on it. Upon back-rotation of the 40S subunit, phosphate release is communicated in a relay system via switch 1 to the adjacent domains II and III. Finally eEF2 relaxes, in agreement with the general ‘loaded-spring’ model, as discussed also for EF-G. The back-swiveling of the 40S head as last step of translocation is then able to release eEF2-GDP and to set the stage for a new round of polypeptide elongation.

Methods

Publication of C. thermophilum 80S ribosomes. The protocol for the isolation of C. thermophilum non-translating 80S (C80S) ribosomes was adapted from previously described methods. C. thermophilum cells were grown in a rotary shaker at 55 °C for 3 d, harvested with a vacuum ﬁlter and immediately frozen in
liquid nitrogen. Frozen mycelium cells were ground to fine powder by Cryo Mill (Retch) (5 mm, frequency 36%) and stored at −80 °C. The powdered mycelium was resuspended in 2 mM HEPES-KOH (pH 7.5), 10 mM magnesium acetate, 2 mM DTT, and 0.5 mM PMSF and vortexed until no clumps remained. Insoluble material was removed by centrifugation (48,254 × g, 1 h, JA25-50 rotor, Beckman, 35 min). Ribosomes were pelleted through a high-salt sucrose cushion (20 mM HEPES-KOH (pH 7.5), 500 mM potassium acetate, 1.5 M sucrose, 5 mM magnesium acetate and 2 mM EDTA) at 105,000 × g for 1 h (Thermo Scientific) for 18 h before they were resuspended in 20 mM HEPES-KOH (pH 7.5), 50 mM potassium acetate, 5 mM magnesium acetate, 2 mM DTT and 0.5 mM PMSF. The C.80S ribosomes were then incubated with 1 mM neutralized puromycin as solution and 1 mM GTP for 1 h at 30 °C (or 50 °C—in a high temperature control). Ribosomes were further purified in a 15–40% sucrose gradient (20 mM HEPES-KOH (pH 7.5), 150 mM potassium acetate, 5 mM magnesium acetate, 15–40% sucrose, 2 mM DTT and 0.50 mM PMSF) spun at 60,076 × g in a Surespin 630 rotor (Sovall) for 15 h. Peak fractions containing C80S monosomes were pooled and precipitated by adding 7% (w/v) of PEG20K. After a 10 min centrifugation, the pellets were resuspended in 20 mM HEPES-KOH (pH 7.5), 50 mM potassium acetate, 5 mM magnesium acetate, 2 mM DTT and 0.5 mM PMSF, and used for cryo-EM grid preparation or stored at −80 °C.

In-gel tryptic digestion. LC-MS/MS analysis, and database search. Samples were purified and sequenced by IDB-PAGE, followed by trypsin digestion. Trypsin was extracted from the gel pieces, concentrated in a SpeedVac vacuum centrifuge and dissolved with 15 μL 0.1% TFA. Nanoflow LC-MS2 analysis was performed with an Ultimate 3000 liquid chromatography system coupled to an Orbitrap Elite mass spectrometer (ThermoFischer, Bremen, Germany). Five microliters of sample injection to a self-packed analytical column (75 μm × 500 mm, Poroshell 120C18-AQ; Agilent Technologies) and eluted with a flow rate of 300 nL/min in an acetonitrile-gradient (3–40%). One survey scan (res: 60,000) was followed by 15 information dependent product ion scans in the ion trap. For quantification, the local resolution variations were calculated with ResMap39. The model of C80S was initially built on a predicted 80S ribosome structure (PDB ID: 4A8P) with some proteins being built by homology modelling using the SWISS-MODEL Workspace40. Sequences of C. thermophilium proteins were assigned by BLAST41 and the C. thermophilium genome resource42. The models were manually built and corrected in Coot43. As amino acid side-chain densities and nucleotides were clearly resolved, real-space refinement was efficiently used for model building in Phenix44. Magnesium ligands were built as hexa-aqua complexes according their size in the EM-density and when present as validated osmium-hexamine complexes in a S80S X-ray structure45. Octahedral geometry of the ligand complexes was set free only in the last round of refinement. Atomic models were validated using Phenix and MolProbity46.

Measurement of rotations and figure preparation. 40S subunit rotation and head swiveling were measured in UCSF Chimera47 using the command ‘measure rotation’. For the 40S body, the structures were aligned on 26S rRNA, and the rotation between a pair of 18S rRNA (1:1150; 1620–1796) was measured. For the 40S head, the structures were aligned on the 40S body, and the rotation between a pair of 18S rRNA (1150; 1620–1796) was measured. Figures were prepared in GraphPad Prism, Pymol, UCSF Chimera and UCSF ChimeraX48.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support this study are available from the corresponding author upon reasonable request. Cryo-EM maps have been deposited in the Electron Microscopy Data Bank under accession codes EMD: EMD-12976 for idle POST and EMD: EMD-12977 for (TI)-POST conformational states. The atomic models have been deposited in the Protein Data Bank under accession codes EMDB: EMD-12976 for C.79

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88. T.R. performed mass spectrometry analysis. M.K. prepared cryo-EM samples, collected and processed EM data. M.K. and K.W. built the structural models. M.K., K.W., and I.S. interpreted the data. M.K., K.W., and I.S. wrote the manuscript with contributions from all authors. M.K., K.W., and I.S. conceived the study and designed the research.

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