Distinct mechanisms of the human mitoribosome recycling and antibiotic resistance

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Ribosomes are recycled for a new round of translation initiation by dissociation of ribosomal subunits, messenger RNA and transfer RNA from their translational post-termination complex. Here we present cryo-EM structures of the human 55S mitochondrial ribosome (mitoribosome) and the mitoribosomal large 39S subunit in complex with mitoribosome recycling factor (RRFmt) and a recycling-specific homolog of elongation factor G (EF-G2mt). These structures clarify an unusual role of a mitochondria-specific segment of RRFmt, identify the structural distinctions that confer functional specificity to EF-G2mt, and show that the deacylated tRNA remains with the dissociated 39S subunit, suggesting a distinct sequence of events in mitoribosome recycling. Furthermore, biochemical and structural analyses reveal that the molecular mechanism of antibiotic fusidic acid resistance for EF-G2mt is markedly different from that of mitochondrial elongation factor EF-G1mt, suggesting that the two human EF-Gmt5s have evolved diversely to negate the effect of a bacterial antibiotic.
The process of protein synthesis in all living cells is orchestrated by highly complex macromolecular assemblies called ribosomes, in coordination with mRNA, tRNAs, and multiple translational factors. Mitochondrial ribosomes (mitoribosomes) and their associated translation machinery are distinct from those in the cytoplasm and display features reminiscent of prokaryotic translation\(^1\), in line with the assumption that mitochondria have evolved from endocytosis of an α-proteobacterium by an ancestral eukaryotic cell\(^2\). However, cryo-electron microscopy (cryo-EM) structures have revealed that the mammalian mitoribosomes have diverged considerably from bacterial ribosomes and acquired several unique features\(^3-8\). A striking difference is the reversal in the protein to RNA ratio, as the bacterial ribosomes are high in ribosomal RNA (rRNA) whereas the mammalian mitoribosomes are high in protein. The increase in protein mass is the result of acquisition of multiple mito-specific ribosomal proteins (MRPs) and addition of extensions to many MRPs, that are homologous to bacterial ribosomal proteins. Though the steps of mitochondrial translation closely resemble those of prokaryotic translation in the general sequence of events and the homologous accessory protein factors involved, they also show significant structural and functional differences\(^9,10\).

The complex process of protein synthesis is accomplished in four essential steps of initiation, elongation, termination, and the ribosome recycling. The transition between translation termination and ribosome recycling is well characterized in eubacteria. During translation termination, the nascent polypeptide chain attached to the peptidyl tRNA is released from the ribosome with the help of a class I release factor (RF) that interacts with the stop codon exposed at the ribosomal decoding site, or aminoacyl-tRNA binding site (A site)\(^11,12\). Subsequently, the class I RF is dissociated from the ribosome with the help of a class II RF in a GTP hydrolysis-dependent manner\(^13,14\). At the end of the termination, the translated mRNA and the deacylated tRNA remain associated with the ribosome\(^15,16\), a state referred to as the post-termination complex (PoTC). In order to initiate a new round of protein synthesis, the ribosome must be split into its two subunits and its bound ligands must be removed. In eubacteria, the disassembly of the PoTC requires the concerted action of two protein factors, the ribosome recycling factor (RRF) and the elongation factor G (EF-G)\(^15,17,18\). RRF binds to the PoTC as the 70S ribosome adopts a ratcheted conformation\(^19,20\), in which the small (30S) subunit of the ribosome rotates in an anticlockwise direction with respect to the large (50S) subunit\(^21\). This is followed by the binding of EF-G in conjugation with guanosine 5'-triphosphate (GTP) to the RRF-bound PoTC and the dissociation of the 70S ribosome into its large and small subunits, a process that requires the hydrolysis of GTP on EF-G\(^22-24\). Though the involvement of a third factor, initiation factor 3 (IF3) in the recycling process is generally agreed upon, its precise function has been debated\(^16,18,25\).

Unlike most eubacteria, where a single form of EF-G is known to participate in both the elongation and ribosome recycling steps\(^22,23\), mammalian mitochondria utilize two isoforms of EF-G, EF-G\(_{1\text{mt}}\) and EF-G\(_{2\text{mt}}\). While EF-G\(_{1\text{mt}}\) specifically functions as a translocase during the polypeptide elongation step\(^26,27\), EF-G\(_{2\text{mt}}\) has been reported to act exclusively as a second recycling factor together with RRF\(_{\text{mt}}\). Human RRF\(_{\text{mt}}\) is about 25–30% identical to its eubacterial homologs but carries an additional 79 amino acids (aa) long extension at its N-terminus\(^29\). The recent high-resolution cryo-EM structures of RRF\(_{\text{mt}}\) bound to the 55S mitoribosomes\(^7\), and an in vivo formed mitoribosomal complex\(^30\) revealed that the structurally conserved segment of the RRF\(_{\text{mt}}\) is similar to its bacterial analog on\(^14,19,20,31-36\) and off\(^37,38\) the 70S ribosome in terms of its overall size and domain composition. However, the unique mito-specific N-terminal extension (NTE) in RRF\(_{\text{mt}}\) extends towards the GTPase-associated center and interacts with the functionally important 16S rRNA elements of the mitoribosomal 39S subunit, including the rRNA helices 89 (H89), H90, and H92\(^27\).

Valuable mechanistic inferences about the bacterial ribosome recycling process were made from the structures of the 70S\(_{\text{RRF}}\) (e.g., Agrawal et al\(^31\)) and dissociated 50S\(_{\text{RRF}}\cdot EF-G\(_{\text{1\text{mt}}}\) complex. Capturing the simultaneous binding of both factors on the 70S ribosome is challenging, however, owing to the rapid rate of 70S ribosomes dissociation into subunits by the combined action of RRF and EF-G\(_{\text{2\text{mt}}}\). To slow down this reaction, a heterologous system with \(T.\ thermophilus\) RRF and \(E.\ coli\) EF-G was used to capture both factors on the 70S ribosome by cryo-EM\(^34\). Subsequently, a time-resolved cryo-EM study was also able to capture various 70S\(_{\text{RRF}}\cdot EF-G\(_{\text{functional}}\) intermediates, albeit at low resolution\(^36\). More recently, a bacterial ribosome recycling complex containing both RRF and EF-G was obtained by X-ray crystallography by stabilizing EF-G on the 70S ribosome through a fusion between EF-G and ribosomal protein bL\(^35\). All these structures conclude that binding of EF-G to the 70S\(_{\text{RRF}}\) complex induces rotation of RRF domain II towards the helix 44 (h44) region of the 30S subunit, destabilizing the crucial intersubunit bridges B2a and B3, and thereby facilitating the dissociation of the 70S ribosome into its two subunits.

With a molecular weight of 87 kD, human EF-G\(_{2\text{mt}}\) is slightly larger than EF-G\(_{1\text{mt}}\) (83 kD), as well as the two isoforms of bacterial EF-Gs, EF-G (78 kD), and EF-G2 (73 kD), identified in certain bacterial species but without known function for the second bacterial isoform\(^39-41\), except in case of a spirochete\(^42\). EF-G\(_{2\text{mt}}\) has about 36% aa sequence identity to EF-G\(_{1\text{mt}}\) and about 30% aa identity to both its bacterial homologs. Some mammalian mitochondrial translation steps are now better understood through determination of the cryo-EM structures of the initiation\(^13-15\) and the elongation\(^44\) complexes at high-resolution. Our previous study of the human mitoribosome recycling complex of the RRF\(_{\text{mt}}\)-bound 55S provided useful insights into the mito-specific aspects of the recycling process, but a complete 55S mitoribosomal recycling complex comprising both RRF\(_{\text{mt}}\) and EF-G\(_{\text{mt}}\) remained elusive.

In this work, we investigate the role of mito-specific aa segments of RRF\(_{\text{mt}}\) and EF-G\(_{\text{2\text{mt}}}\) in recycling the 55S mitoribosome PoTC by determining cryo-EM structures of the key intermediate mitoribosome•RRF\(_{\text{mt}}\)•EF-G\(_{\text{2\text{mt}}}\) complexes, and determine the effect of fusidic acid, an antibiotic that is known to inhibit the GTPase activity of bacterial EF-G\(_{\text{1\text{mt}}}\), on the GTPase activity of EF-G\(_{\text{2\text{mt}}}\). Our study reveals distinct features of the mechanism of human mitoribosome recycling and of the mechanism of fusidic-acid resistance shown by EF-G\(_{1\text{mt}}\) and EF-G\(_{\text{2\text{mt}}}\).

Results and discussion

Structure of the human mitoribosome recycling complex.

To investigate the molecular mechanism of ribosome recycling in mammalian mitochondria, we first prepared a model post-termination complex (PoTC) by incubating the human 55S mitoribosome with puromycin\(^47\). The model PoTC was briefly incubated with human RRF\(_{\text{mt}}\) and human EF-G\(_{\text{2\text{mt}}}\)–GMPPCP to obtain the mitoribosome recycling complex. (see “Methods” section). Single-particle cryo-EM analysis on this complex yielded three major classes that each represent a major functional state formed during human mitoribosome recycling, referred henceforth to as Class I, Class II, and Class III (Fig. S1). Class I corresponds to the intact 55S mitoribosome that carries a strong density for RRF\(_{\text{mt}}\) and was refined to 3.5 Å (Figs. 1 and S1a). Class II, a relatively small class with only 28,929 particle images, corresponds to the 55S mitoribosome that carries both RRF\(_{\text{mt}}\) and
EF-G2\textsubscript{mt}, and was refined to 3.9 Å (Fig. S2b). In this class, the densities corresponding to the large (39S) mitoribosomal subunit, RRF\textsubscript{mt}, and EF-G2\textsubscript{mt} are well resolved, but the small (28S) mitoribosomal subunit appears to be loosely bound and present in multiple poses (Figs. 1 and S2). The most populated Class III corresponds to the dissociated 39S subunits that carry both RRF\textsubscript{mt} and EF-G2\textsubscript{mt}, and was refined to 3.15 Å (Figs. 1 and S2c). Class II likely represents an ensemble of low-population intermediate states of mitoribosome recycling that occur between the states represented by Classes I and III. In addition to these three recycling complexes, we have also obtained a class of particles consisting of 55S mitoribosomes without either of the two factors, where the 28S subunit was rotated by about 8° around its long axis such that its shoulder side moves closer to the 39S subunit while its platform side moves away from it (Fig. S3a). A similar orientation for the small subunit relative to the large subunit, termed as “subunit rolling” has been reported earlier for the 80S ribosomes\textsuperscript{49} and the 55S mitoribosomes\textsuperscript{5,7}.

RRF\textsubscript{mt} binding stabilizes the rotated state of the 28S subunit in the 55S mitoribosome. Superimposition of our Class I complex with the RRF\textsubscript{mt}-unbound human\textsuperscript{3}, bovine\textsuperscript{8} and porcine\textsuperscript{4} 55S mitoribosomes showed that the small 28S subunit was rotated counter-clockwise by about 8.5° with respect to the large 39S subunit (Fig. S3b), similar to the “ratchet-like inter-subunit rotation” observed in the bacterial 70S ribosome\textsuperscript{21} and the 55S mitoribosomes complexed with translational factors\textsuperscript{6,7,44,46}. In addition to the inter-subunit rotation, the head domain of the 28S subunit is rotated by about 4° towards the tRNA exit (E) site in a direction roughly orthogonal to the inter-subunit motion (Fig. S3b), similar to “head swiveling” in the bacterial 70S ribosomes\textsuperscript{50,51}. As expected, the structure of the Class I complex matches the previously published 3.9 Å resolution map of the analogous 55S-RRF\textsubscript{mt} complex\textsuperscript{7}.

The Class I map showed the characteristic “L” shaped RRF\textsubscript{mt} density and a density corresponding to a pe/E-state tRNA within the inter-mitoribosomal subunit space. The overall positioning and domain arrangement of RRF\textsubscript{mt} in the Class I map is similar to the bacterial RRF on\textsuperscript{14,19,20,31-36} and off\textsuperscript{37} the 70S ribosomes, and also to the structures of RRF bound to the 70S chloroplast ribosome\textsuperscript{52,53} and RRF\textsubscript{mt} bound to the human mitochondrial 55S in our previous study\textsuperscript{7}. As observed in bacteria, domain I is positioned close to the peptidyl transferase center (PTC) and extends towards the α-sarcin-ricin loop (SRL). A striking difference between the human RRF\textsubscript{mt} and its bacterial counterpart is the presence of a 79 aa long N-terminal extension (NTE) in RRF\textsubscript{mt}. We could model the last 14 aa residues of the NTE of RRF\textsubscript{mt} into an additional density contiguous with the α-helix1 from domain I. As discussed in our previous study\textsuperscript{7}, the NTE is strategically positioned in the intersubunit space between domain I and several functionally important 16S rRNA structural elements such as H89, H90, H92 (A-loop), and MRP L16 (Fig. S4) and interacts with several nucleotides (nts) and aa residues in its vicinity\textsuperscript{7}. Interestingly, unlike the α-helical nature inferred for the part of this segment of NTE\textsuperscript{7}, we find that its higher resolution density to be partially unstructured. A similar observation of a relatively unstructured NTE has been recently
reported in an in vivo state complex. However, the mitochondrial components interacting with RRFm have essentially remain unaltered.

We found a small density in a tight pocket surrounded by the outer bend of the junction between domains I and II of RRFm, MRP uS12m, and the large subunit’s 12S rRNA helix h44, and the large subunit’s 16S rRNA helices H69 and H71 (Fig. 2a). Except for our previous lower resolution map of the 55S•RRFm complex, this additional density is not observed in any of the available 55S mitoribosomal structures, whether complexed with other translational factors or not. Since our complex was reconstituted from purified components, this additional density should correspond to an RRFm NTE segment, that has been stabilized through interactions with multiple mitoribosomal components in its vicinity. Though bacterial and mitochondrial ribosomes exhibit significant differences in their overall shape, composition, and conformation, their internal rRNA core regions are largely conserved. Comparison of the RRF binding sites between the bacterial and mitochondrial ribosomes reveals that the H69 of 16S rRNA is slightly shorter in the mammalian mitoribosomes (Fig. 2b). This minor shortening of H69 is critical because it directly impacts the interaction of H69 with domain II of RRFm. The small density, most likely corresponding to N-terminus segment of the mitoribosome, appears to compensate for the shortened H69 by mediating the interactions between RRFm and H69 (Fig. 2b). We generated a model that would place 10 aa residues (Ala2-Val11) at the N-terminus of NTE into this density (Fig. 2b). This model was guided by the observed sidechain density for two consecutive large side chains of Phe8 and Arg9 within the first 11 aa (Fig. 2c). Since there are other possibilities for two consecutive aa residues with large sidechains in the remaining 54 aa residues in the unmodelled segment of NTE, further confirmation of this assignment may need a well-resolved density for the entire NTE.

It should be noted that the RRFm-bound 55S mitoribosomes (present work and ref.7) were never observed in the unrotated state, suggesting that the RRFm binding locks the ribosome in a fully rotated state. This is in contrast to the bacterial 70S•RRF complexes that were found both in their rotated and unrotated conformational states. The rotation of the 55S mitoribosome seems to prime subunit dissociation by either destabilizing or completely breaking seven out of fifteen inter-subunit bridges in the unrotated 55S mitoribosome. The simultaneous interactions of N-terminus segment of the RRFm NTE with RRFm’s structurally conserved domain I, MRP uS12m, h44, H69, and H71 likely help prevent the back-rotation of the small 28S subunit. The rotated state of the 55S mitoribosome could serve as an ideal substrate for the subsequent binding of EF-G2mt to complete subunit dissociation. In this context, it is important to note that the entire 79 aa long NTE is an integral part of the mature protein and is known to be essential for RRFm function during the mitoribosome recycling.

RRFm domain II motion helps split the 55S mitoribosome into its two subunits. Using fast-kinetics, it has been shown that the splitting/recycling of the 70S ribosome by the concerted action of RRF and EF-G happens in the sub-second time scale. Both RRF and EF-G have been captured on the 70S ribosome with time-resolved cryo-EM. It is more challenging without time-resolved techniques, but RRF and EF-G were also captured on the 70S ribosomes by using the factors from different species or by crosslinking the EF-G with one of the ribosomal proteins. In the present work, collection of very large cryo-EM datasets altogether 21,752 micrographs (Fig. S1), enabled the isolation of a small subset of 55S particles (Class II) that contained both RRFm and EF-G2mt (Fig. 1). However, the 28S subunit density was found to be weak and present in multiple destabilized conformations relative to the 39S subunit in this complex.

Both Class II and Class III maps showed readily recognizable densities corresponding to RRFm, EF-G2mt, and E-site tRNA. The Class III complex had superior resolution, which enabled more accurate analysis of molecular-level interactions between the two factors and the mitoribosome, and their functional implications, while the Class II map was useful for interpreting large-scale conformational changes. In line with the bacterial 70S/50S•RRF•EF-G complexes, the conformation of RRFm is substantially different between the Class I and Class III recycling complexes. The conformation of RRFm domain I remains unchanged among all three classes. In the Class II and III maps, domain II was rotated by about 45° towards the small subunit compared to its position in the Class I complex (Fig. 3a). This large conformational change is enabled by a highly flexible hinge regions between domain I and domain II in RRFm. Due to this rotation, the tip of domain II moved by about 40 Å towards the h44 of 12S rRNA. When the maps of Class II and III were superimposed, this motion resulted in a major steric clash between the RRFm domain II and the 28S subunit elements h44 and MRP uS12m (Fig. 3a). In the 55S mitoribosome, h44 is involved in the formation of two intersubunit bridges B2a and B3 by pairing with H69 and H71, respectively. By displacing h44,
Landmarks on the thumbnails are same as in Fig. 2.

EF-G2mt binding induces conformational changes in RRFmt large subunit. is captured in multiple positions during its separation from the intermediates of mitoribosome recycling, where the small subunit supports it being an ensemble of authentic, short-lived functional a poorly resolved 28S subunit remained unchanged. This 55S mitoribosomal particles with a well-resolved 39S subunit and reference-based 3D classi

cation was employed, but this class of domain III reported in the bacterial 50S 14 and 70S complexes34,36. A
domain II strongly interacts with Glu495 from RRFmt domain I through a hydrogen bond with Ser112 from the hinge region of RRFmt and the loop regions from domain III of EF-G2mt. Several aa residues from the RRFmt hinge region (Pro183–Thr186) interact with EF-G2mt domain III residues Glu495–Leu499 (Fig. S6b) via hydrogen bonds and hydrophobic interactions. Arg187 from the a-helix following the hinge region of RRFmt domain II has close hydrogen-bonding interactions with Tyr556 of EF-G2mt domain III (Fig. S6b). A second set of contacts formed between the two factors involves residues Ile109–Arg110 from the second hinge region of RRFmt and residues Ser527–Gln529 from the domain III of EF-G2mt (Fig. S6c).

Domain IV of EF-G2mt presses against domain II of RRFmt through multiple interactions. The surface residues of a-helix 1 (Asn629, Ser633, and Leu636) and a-helix 2 (Thr664, Met665, Ser667, and Ala668) from domain IV of EF-G2mt interact with residues in b-strand 3 (Ser134–Met138), and its adjoining loop region (Gln132 and Ile133) from domain II of RRFmt through a combination of electrostatic and hydrophobic interactions (Fig. S6d). Gln637 from the a-helix 1 of EF-G2mt also shares a hydrogen bond with Ser112 from the hinge region of RRFmt (Fig. S6d). Contacts are observed between the C-terminal a-helix of EF-G2mt domain IV and the a-helix 3 from the triple-helix bundle of RRFmt domain I. In bacteria, the analogous C-terminal a-helix of EF-G is often considered as part of domain V though the first atomic models of EF-G35,36 grouped it with domain IV. While residues Ser776–Leu778 from EF-G2mt domain IV pair with residues Arg251 and Val255 from RRFmt domain I through hydrogen bonds (Fig. S6e), Arg775 from EF-G2mt domain IV strongly interacts with Glu259 from RRFmt domain I through a salt-bridge (Fig. S6e). Direct interactions of EF-G2mt domain III with RRFmt domain II at its hinge regions, known to confer interdomain flexibility to the bacterial factor35, likely help trigger the dissociation of 55S mitoribosomes into subunits by enabling the repositioning of RRFmt domain II. At the same time, the

**Fig. 3 Direct involvement of RRFmt domain II and EF-G1mt domain IV in human mitoribosome recycling.**

- **a** Comparison of the overall conformation of RRFmt between the Class I (gray) and Class III (green) complexes revealed that domain II rotates by about 45° towards the 28S subunit. Such a rotation would sterically clash with the 125 rRNA helix 44 (h44, brown) and MRP uS12m (cyan) and destabilize a crucial inter-subunit bridge, B2a (yellow). Inset shows the magnified view of the inter-subunit bridge B2a with RRFmt domain II residues V121-K127 (shown with density in magenta) disrupting B2a by inserting between h44 residues (C1491 and A1492) and H69 residues (A2581 and A2582).
- **b** Superimposition of the Class III complex with the Class I complex shows a direct overlap between the loop1 region (dark cyan) of EF-G2mt domain IV (red) and the intersubunit bridge B2a (yellow) formed between h44 (brown) and H69 (blue). Inset shows the magnified view of EF-G2mt domain IV residues L582-R585 (shown with density in dark cyan) that will disrupt the bridge B2a by inserting between h44 residues (G1559 and U1560) and H69 residue (A2576) and pushing the 28S subunit (h44) away. Thumbnails to the left depict an overall orientation of the 55S mitoribosome, with semitransparent 28S (yellow) and 39S (blue) subunits, and overlaid position RRFmt.

RRFmt disrupts these crucial intersubunit bridges, thereby splitting the 55S mitoribosome into its two subunits.

A well-defined 28S structure was not seen within the Class II 55S mitoribosomal complex, but several inter-subunit bridges do appear to be destabilized or broken, and the small subunit seems to be dissociating from the large subunit. To remove any possible large subunit contamination from the Class II map, extensive reference-based 3D classification was employed, but this class of 55S mitoribosomal particles with a well-resolved 39S subunit and a poorly resolved 28S subunit remained unchanged. This supports it being an ensemble of authentic, short-lived functional intermediates of mitoribosome recycling, where the small subunit is captured in multiple positions during its separation from the large subunit.

**EF-G2mt binding induces conformational changes in RRFmt.**

The 55S•RRFmt complex (Class I) undergoes large conformational changes upon binding to EF-G2mt (Classes II and III). EF-G2mt would not be able to access its binding site on the mitoribosome without the significant movement seen in domain II of RRFmt in Class II and Class III complexes. This movement eliminates the direct spatial conflicts of EF-G2mt domains III, IV, and V with the initial position of RRFmt domain II in the Class I complex (Figs. 3a, b and Fig. S5). This change could be induced either during or upon binding of EF-G2mt. The domain II of RRFmt is repositioned into a cavity created by domains III, IV, and V of EF-G2mt (Fig. S6a) and an extensive network of interactions are formed between the two mitochondrial recycling factors. This is also in agreement with the location of RRF domain II reported in the bacterial 50S14 and 70S complexes34,36. A majority of the interactions are formed between the hinge regions that connect the two domains of RRFmt and the loop regions from domain III of EF-G2mt. Several aa residues from the RRFmt hinge region (Pro183–Thr186) interact with EF-G2mt domain III residues Glu495–Leu499 (Fig. S6b) via hydrogen bonds and hydrophobic interactions. Arg187 from the a-helix following the hinge region of RRFmt domain II has close hydrogen-bonding interactions with Tyr556 of EF-G2mt domain III (Fig. S6b). A second set of contacts formed between the two factors involves residues Ile109–Arg110 from the second hinge region of RRFmt and residues Ser527–Gln529 from the domain III of EF-G2mt (Fig. S6c).

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multiple interactions between domain IV of EF-G2mt and domain II of RRFmt appear to stabilize the RRFmt domain II in the altered position, which would push the 28S subunit away from the 39S subunit and prevent domain II from reverting back to its previous orientation, in order to maintain the 39S•RRFmt•EF-G2mt complex in a dissociated state.

In addition to aiding to the function of RRFmt, EF-G2mt plays a direct role in destabilizing the 55S mitoribosome. Superimposition of the maps of Class I and Class III complexes reveals a direct steric clash between the loop1 region of EF-G2mt domain IV and the 28S subunit component (rRNA h44), that participates in the formation of the inter-subunit bridge B2a (Fig. 3b). More importantly, the orientation of domain IV loop1 seems to be unique to EF-G2mt since the analogous region in EF-G1mt is positioned away from the intersubunit bridge B2a towards the decoding center6,46.

Role of mito-specific MRPs in stabilizing the deacylated tRNA.

The presence of E-site tRNA in Class III complex of the dissociated 39S subunit suggests a markedly different mechanism of deacylated tRNA removal during recycling of mitochondrial PoTC as compared to that in the bacteria, where the deacylated tRNA goes with the small ribosomal subunit during the 70S ribosome splitting36. This observation is particularly intriguing since a majority (11 of 12) of the rRNA segments in bacterial 23S rRNA that are known to interact with the E-site tRNA are absent in the mito-16S rRNA57. We find that the E-site tRNA in the 39S complex is stabilized by multiple interactions with Arg-rich and Lys-rich electro-positive segments of MRPs, including mL6449, uL33m, and uL1m58 (Fig. 4). With the tightly held body of the deacylated tRNA through conserved interaction of its CCA end with the rRNA H88 and multiple new interactions with MRPs, transition of its anticodon end from a pe/E state7 in Class I complex to E/E-state in Class II and III complexes could in part facilitate the dissociation of the two mitoribosomal subunits. Moreover, the mechanism of subsequent release of deacylated tRNA from the 39S subunit remains unknown.

Dynamic interactions among uL11m, CTD of uL12m, and EF-G2mt. EF-G2mt binding resulted in a prominent conformational change in the uL11m stalk-base region of the 39S subunit. The uL11m stalk-base region moved towards the CTD of uL12m, a component of the L10–L12 stalk, and assumed a unique conformation not reported previously44,46,74. The 16S rRNA H43 of the uL11m stalk-base moved by 3 Å towards the CTD of uL12m, oriented parallel to the domain V of EF-G2mt, while the NTD of MRP uL11m moved about 5 Å away from the EF-G2mt domain V (Fig. 5a). This is in sharp contrast to the 55S•EF-G1mt translocation complexes44,46,74, where the uL11m stalk-base region was observed to move 5 Å closer towards the domain V of EF-G1mt (Fig. 5b). The movement of uL11m away from the EF-G2mt domain V and towards the CTD of uL12m is essential for the binding of EF-G2mt in the present conformation, to avoid the steric clash between the NTD of uL11m and domain V of EF-G1mt (Fig. 5a). It is also possible that the conformation of EF-G2mt observed in our 39S•EF-G2mt complex (Class III) was attained after the dissociation of 39S subunit from the 55S complex (Class II).

In addition to the unique conformation of the uL11m stalk-base region, the CTD of uL12m was also observed in a distinct conformation. In the 55S•EF-G1mt complexes44,46, the CTD of uL12m is positioned close to EF-G1mt so that α-helices 1 and 2 of uL12m CTD would have close interactions with the G′ subdomain of EF-G1mt (Fig. 5c), thereby providing stability to the otherwise flexible uL12m CTD. In contrast, the CTD of uL12m rotates by about 60° and shifts away by about 7 Å from the G′ subdomain of EF-G2mt in the 39S•EF-G2mt complex (Fig. 5d). As a result of this large rotational movement, interactions between the uL12m CTD α-helix 2 and the G′ subdomain of EF-G2mt are lost, while the contacts between the α-helix 1 and the G′ subdomain of EF-G2mt are maintained (Fig. 5d). Since protein uL12 is known to play a central role in the recruitment of translational factors to the bacterial ribosome59–62, the semi-stable conformation of uL12m observed in the 39S•EF-G2mt complex represents a late-stage conformation of uL12m prior to its detachment from the EF-G2mt as EF-G2mt’s participation in the 55S ribosome recycling process nears completion.
Structural basis for use of EF-G2m in mitoribosomal recycling instead of EF-G1m. In most bacterial species, a single EF-G is involved in both translocation and ribosome recycling. Mammalian mitoribosomes have evolved to utilize the two isoforms EF-G1mt and EF-G2mt to cope with the significantly altered environment in mitochondria as compared to the bacterial cytoplasm and perform two separate functions. EF-G1mt is used during translation elongation while EF-G2mt is used along with RRFmt for the recycling of the 5S5 mitoribosome. The overall position and domain arrangement of EF-G2mt is similar to that of EF-G1mt in the recently published human and porcine translocational complexes (Fig. S6). There are, however, some specific structural distinctions between the two factors that assign them specialized functional roles. The most striking of these is the presence of a C-terminal extension (CTE) in EF-G1mt domain IV (Fig. 6a). Besides its CTE, the size of the conserved C-terminal α-helix of EF-G1mt domain IV is substantially longer (16 aa) than that of EF-G1mt in the recently published human and porcine translocational complexes (Fig. S6). There are, however, some specific structural distinctions between the two factors that assign them specialized functional roles. The most striking of these is the presence of a C-terminal extension (CTE) in EF-G1mt domain IV (Fig. 6a). Besides its CTE, the size of the conserved C-terminal α-helix of EF-G1mt domain IV is substantially longer (16 aa) than that of EF-G1mt in the recently published human and porcine translocational complexes (Fig. S6).

The sturdier and longer C-terminal α-helix of EF-G1mt domain IV and its 11 aa CTE would not permit the coexistence of RRFmt on the mitoribosome due to a major steric clash between domain I of RRFmt and the C-terminal region of EF-G1mt (Fig. 6b). Even a reorientation of the C-terminal α-helix and its CTE away from the domain I of RRFmt would not resolve the problem as they would then clash with H89 of the 16S rRNA and the NTE of RRFmt, that has been positioned in the inner subunit space between the domain I of RRFmt and 16S rRNA helix, H89 (Fig. 6b). Structural analysis of the bacterial EF-Gs from various species has revealed that the length of their C-terminal α-helices are about 12 aa long, suggesting that only EF-G2mt can function alongside RRFmt during subunit splitting. Interestingly, the C-terminal α-helices of EF-G2 from T. thermophilus and EF-G2mt are similar in size (Fig. S7) though the function of EF-G2 in T. thermophilus is not understood. Moreover, the interaction between the C-terminal regions of EF-G2mt domain IV and RRFmt domain I is essential for stabilizing the bound RRFmt. This tight anchoring of RRFmt domain I to the mitoribosome would prevent RRFmt dissociation from the mitoribosome, when its domain II undergoes substantial rotation to displace the h44 region of the 28S subunit. This agrees with the observation that deletion of the last few aa residues from the C-terminal region of bacterial RRF adversely effects its function during 70S ribosome recycling.

Now the question is why EF-G1mt mediates the translocation step during mitoribosomal elongation and not EF-G2mt? The most probable answer is that the C-terminal α-helix of EF-G1mt domain IV and its 11 aa CTE would not permit the coexistence of RRFmt on the mitoribosome due to a major steric clash between domain I of RRFmt and the C-terminal region of EF-G1mt (Fig. 6b). Even a reorientation of the C-terminal α-helix and its CTE away from the domain I of RRFmt would not resolve the problem as they would then clash with H89 of the 16S rRNA and the NTE of RRFmt. Structural analysis of the bacterial EF-Gs from various species has revealed that the length of their C-terminal α-helices are about 12 aa long, suggesting that only EF-G2mt can function alongside RRFmt during subunit splitting. Interestingly, the C-terminal α-helices of EF-G2 from T. thermophilus and EF-G2mt are similar in size (Fig. S7) though the function of EF-G2 in T. thermophilus is not understood. Moreover, the interaction between the C-terminal regions of EF-G2mt domain IV and RRFmt domain I is essential for stabilizing the bound RRFmt. This tight anchoring of RRFmt domain I to the mitoribosome would prevent RRFmt dissociation from the mitoribosome, when its domain II undergoes substantial rotation to displace the h44 region of the 28S subunit. This agrees with the observation that deletion of the last few aa residues from the C-terminal region of bacterial RRF adversely effects its function during 70S ribosome recycling.

Specific sequence differences in a critical region (loop1) within the domain IV of EF-G2mt can also make it ineffective in driving translocation. During EF-G-catalyzed translocation in bacteria, the presence of two universally conserved glycine residues at the tip of domain IV (loop 1) region facilitate the insertion of domain IV into the decoding center (DC), stabilizing the codon-anticodon interactions of the DC.
mRNA-tRNA duplex with the universally conserved 16S rRNA bases A1492 and A1493 in the 30S A site thereby aiding the A-site tRNA along with its associated codon to translocate into the P site67. The loop 1 region is conserved in EF-G1mt but is significantly altered in EF-G2mt (Fig. 6e). EF-G1mt retains both the glycine residues (Gly544 and Gly545) in its domain IV loop 1 region (Fig. 6c) whereas the second glycine is replaced by an aspartic acid in EF-G2mt (Fig. 6d), which alters the conformation and flexibility, and thereby making its insertion into the DC during translocation unfavorable. 

The boxed aa sequence corresponds to the loop 1 situated at the tip of domain IV, which is conserved between EF-G1mt and the T. thermophilus EF-G. First of the two universally conserved loop 1 glycine residues (green) is substituted by an alanine in B. burgdorferi EF-G, while the second one is replaced by an aspartic acid in EF-G2mt.

**Mammalian 55S mitoribosome recycling does not require GTP hydrolysis by EF-G2mt.** The overall G domain structure in the 39S•RRFmt•EF-G2mt complex (Class III map) is similar to the G domain in the 55S•EF-G1mt complex6,46. The well-ordered density in our maps of highly conserved translational GTPase consensus motifs such as the P-loop, switch I and switch II regions, allowed complete modeling of these essential regions. A well-defined density corresponding to a bound GMPPCP molecule is also readily identifiable in the nucleotide binding pocket. GMPPCP is stabilized through interactions with universally conserved aa residues, such as Asp80 and Lys83 of P-loop, Thr122 of switch I and His145 of switch II (Fig. S8a). As in the 55S•EF-G1mt complex6, a crucial Mg2+ ion is positioned near the γ phosphate of GMPPCP and is coordinated by Thr84 and Thr122 from the P-loop and switch I regions, respectively (Fig. S8a). The catalytic His145 (His124 in EF-G1mt), known to play a central role in the hydrolysis of the bound nucleotide70,71, is found oriented towards the γ phosphate of the bound GMPPCP (Fig. S8a), suggesting an active conformation of the factor prior to GTP hydrolysis.

The highly conserved α-sarcin–ricin stem-loop (SRL) region is known to be essential for the GTTPase activity of all the translational G proteins47,70,71. Base A3129 from the SRL was...
found to be contacting the Switch II His145 through hydrogen bonding interactions and thereby stabilizing His145 in its activated conformation poised to perform the hydrolysis reaction (Fig. S8b). While EF-G-dependent GTP hydrolysis is essential for an efficient splitting of the 70S ribosome into its subunits\textsuperscript{16,18,22}, dissociation of the mammalian 55S mitoribosomes does not require EF-G\textsubscript{2mt}-dependent GTP hydrolysis, which is only needed for the release of EF-G\textsubscript{2mt} from the dissociated large subunit\textsuperscript{27}. Our structural results strongly corroborate the Tsuboi and coworkers\textsuperscript{27} observation as a significant proportion (82\%) of the EF-G\textsubscript{2mt} was found complexed to the 39S subunits as compared to the small proportion (18\%), that remained associated with the 55S mitoribosomes (see Fig. S1). Even though GTP hydrolysis by EF-G\textsubscript{2mt} is not necessary for 55S mitoribosome splitting, the presence of GDP or its non-hydrolysable analogs GDPNP/GMPPCP in the nucleotide binding pocket is essential, as the presence of GDP or the absence of any nucleotide does not split the 55S mitoribosome\textsuperscript{27}.

Divergent mechanisms of fusidic acid (FA) resistance by EF-G\textsubscript{1mt} and EF-G\textsubscript{2mt}. FA is a fusidane class antibiotic that is used to treat bacterial skin infections along with chronic bone and joint infections. It is effective against several species of gram-positive bacteria and is clinically used to treat methicillin-resistant \textit{S. aureus} (MRSA). FA prevents the release of EF-G\textsubscript{GDP} from the 70S ribosome after GTP hydrolysis by preventing the switch II from attaining its GDP-bound conformation\textsuperscript{47}. Prior structural studies have demonstrated that FA binds in an interdomain pocket between the G domain, domain II, and domain III of EF-G\textsuperscript{47,72}. Recent cryo-EM structures of EF-G\textsubscript{1mt} bound to the 55S mitoribosomes have presented the switch I in a well-defined conformation\textsuperscript{46} (Fig. 7a), in contrast to bacterial 70S\textsubscript{EF-G} complexes where the density for switch I has been consistently poorly resolved (Fig. 7b)\textsuperscript{47,67,70,72}. It was proposed that the increased resistance observed for EF-G\textsubscript{1mt} towards FA resulted from a small insertion in the switch I region of EF-G\textsubscript{1mt}\textsuperscript{46}. The two positively charged lysine residues (Lys80 and Lys82) in this insertion form salt bridges with the negatively charged phosphate backbone of the SRL from the 39S subunit (Fig. 7b), and hence were hypothesized to confer additional stability to the switch I of EF-G\textsubscript{1mt}\textsuperscript{46}. Biochemical evidence showed EF-G\textsubscript{1mt} to be more resistant towards FA than its bacterial counterpart\textsuperscript{28,73}.

FA is known to inhibit both the translocation and the ribosome recycling steps in bacteria, though there is conflicting evidence on which step of translation is primarily targeted by FA\textsuperscript{22,25}.
Comparison of the FA binding pocket between the bacterial EF-G, EF-G\textsubscript{1mt}, and EF-G\textsubscript{2mt} revealed that key aa residues (Fig. S9a) reported to be necessary for the stable binding of FA\textsuperscript{47,72} are highly conserved (Fig. S9c), thereby suggesting a similar binding mechanism for FA for all the three EF-Gs. The three aa insertion in EF-G\textsubscript{1mt}, that confers resistance to FA is not present in EF-G\textsubscript{2mt} and the corresponding region in EF-G\textsubscript{2mt} does not contain any positively charged aa residues (Fig. 7e), that could strongly interact with the SRL. However, the cryo-EM map of EF-G\textsubscript{2mt} shows a well-resolved density for the switch I region (Fig. S9b) and enabled its modeling (Fig. 7c), indicating an alternative mechanism for the stabilization of switch I in EF-G\textsubscript{2mt}. Sequence alignment showed that the switch I region composition in EF-G\textsubscript{2mt} is significantly different as compared to EF-G\textsubscript{1mt} and the bacterial EF-G (Fig. 7e). Three new salt bridge interactions were identified within the switch I of EF-G\textsubscript{2mt}. Arg98 forms the first two salt bridges by pairing with Asp105 and Asp107, respectively, while Arg117 and Asp104 are involved in the formation of the third salt bridge (Fig. 7c). Furthermore, stronger interactions are observed between the switch I and domain II in EF-G\textsubscript{2mt} compared to EF-G\textsubscript{1mt}. Thr110 from switch I is placed in the close proximity of Asp419 of domain II with the possibility of hydrogen bond formation (Fig. 7c), while the corresponding interaction in EF-G\textsubscript{1mt} is between Asp404 and Val88, a much weaker interaction with no possibility of hydrogen bond formation. There is also potential for a tight T-stacking interaction between Phe417 and Tyr96 in EF-G\textsubscript{2mt} (Fig. 7c), while the corresponding residues in EF-G\textsubscript{1mt} being His402 and Arg72 offer no such interaction. Overall, through a combination of internal salt bridges and additional contacts with domain II, switch I gets highly stabilized in EF-G\textsubscript{2mt}. Since a stabilized switch I region occludes the binding site of FA (Fig. 7a, b), EF-G\textsubscript{2mt} is expected to exhibit strong resistance towards FA in the lines of EF-G\textsubscript{1mt}. To test this hypothesis, we measured the GTPase activity of EF-G\textsubscript{2mt} alongside EF-G\textsubscript{1mt} and \textit{E. coli} EF-G in the presence of FA under multiple-turnover conditions (see Methods). Our data shows that while 1 mM FA has no effect on the GTPase activity in \textit{E. coli}, significant inhibition is observed at higher concentrations of FA. In contrast, FA has almost no effect on the GTPase activity of either EF-G\textsubscript{1mt} or EF-G\textsubscript{2mt} even up to 10 mM FA. Our results are consistent with the earlier finding that EF-G\textsubscript{1mt} is highly resistant to FA compared to the bacterial EF-G\textsubscript{28,73}, and also consistent with recent finding that showed EF-G\textsubscript{2mt} is not susceptible to inhibition by FA\textsuperscript{74}. Our results and analysis suggest FA resistance in EF-G\textsubscript{2mt} occurs by a structural mechanism fundamentally different from that in EF-G\textsubscript{1mt}.

In conclusion, structures of three distinct functional states formed during the process of human mitoribosome recycling are presented (Fig. 1). A previous biochemical finding that GTP hydrolysis is not required for the RRF\textsubscript{mt}-EF-G\textsubscript{2mt}-mediated splitting of the post-termination mitoribosomal complex is corroborated. We also show that a mito-specific segment of the RRF\textsubscript{mt}'s NTE compensates for the slightly reduced size of the H69 within the 16S tRNA of the mitoribosomal large subunit (Fig. 2). This is the first evidence showing a translational factor compensating for an rRNA segment lost, perhaps during the evolution of the mammalian mitochondrial translation machinery. Our structures reveal how RRF\textsubscript{mt}'s domain II and EF-G\textsubscript{2mt} domain IV directly help in disrupting the central inter-subunit bridge, B2a (Fig. 3), how the deacylated tRNA is held with the dissociated 39S subunit components (Fig. 4), and suggest how the dynamics of the interactions among the uL11mt, CTD of uL12mt, and the G' domain of EF-G\textsubscript{2mt} alters between elongation and recycling steps (Fig. 5). Structural analysis of domain IV of EF-G\textsubscript{1mt} and EF-G\textsubscript{2mt} explains their specific roles in two distinct steps of elongation and recycling, respectively (Fig. 6). Analysis of their GTPase domains complemented by GTPase assays reveal two distinct mechanisms of antibiotic fusidic acid resistance adopted by two homologous GTPases (Fig. 7). These observations allow us to highlight distinct features of the main steps of human mitoribosomal recycling (Fig. 8). Future studies using time-resolved cryo-EM should help further resolve the short-lived intermediates that form during the transition from Class I to Class III states.

**Methods**

**Purification of 55S mitoribosomes.** The source of mitochondrial ribosomes was human embryonic kidney cells lacking N-acetyl-glucosaminyltransferase I (HEK293S GnTI) that were cultured in roller bottles using FreeStyle\textsuperscript{TM}293 media (Gibco, Life Technologies) supplemented with 5% fetal bovine serum (Gibco, Life...
After centrifugation at 1000 x g for 7 min, the HEK293S GmTt cell pellet was transferred to a glass homogenizer and resuspended in buffer containing 30 mM HEPES-KOH pH 7.5, 10 mM KCl, 1.5 mM MgOAc, 70 mM sucrose, 210 mM mannitol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF. The cells were homogenized by applying 120 strokes and the supernatant was separated from the cell debris by spinning at 950 x g for 15 min. The supernatant was then spun at 11,000 x g for 15 min, and the resulting pellet that contains crude mitochondrial was washed with buffer containing 20 mM HEPES-KOH pH 7.5, 1.7% Triton X-100, 2 mM DTT and 1 mM PMSF. The brownish-orange layer containing pure mitochondria was carefully separated and added to the mitochondria pellet and then incubated for 15 min at 4 °C. The sample was added to the mitochondria pellet and then incubated for 15 min at 4 °C. The sample was centrifuged at 130,000 x g for 30 min in a Beckman ultracentrifuge. After centrifugation at 17 h at 90,000 x g, the supernatant was subjected to a Hi-spin Ni²⁺- columns and the SUMO-tagged protein was eluted from the column using elution buffer (25 mM NaCl, 1× PBS, 4 mM β-mercaptoethanol, and 10 mM imidazole) using standard affinity purification protocols. The purified protein was dialyzed in buffer (20 mM Tris-HCl pH 8.0, 250 mM NaCl, 4 mM β-mercaptoethanol, and 50 mM EDTA and 4°C). The resuspended pellet was subjected to 10-30% continuous sucrose density gradient centrifugation at 60,000 x g for 17 h using Sw32 rotor in Beckman ultracentrifuge. After incubating the EF-G2mt with 3C pre-cleavage protease overnight, relatively pure EF-G2mt was eluted by passing the elution buffer (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 20 mM MgOAc, 1% Triton X-100, and 2 mM DTT) through a column (GE healthcare, USA) and pure protein was eluted by running a gradient (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 20 mM MgOAc, 1% Triton X-100 and 2 mM DTT) enough to dissolve the pellet was added. 10 vol. of lysis buffer (25 mM HEPES-KOH pH 7.5, 100 mM KCl, 25 mM MgOAc, 1.7% Triton X-100, 2 mM DTT and 1 mM PMSF) was added to the mitochondrial-pellet and then incubated for 15 min at 4 °C. The sample was centrifuged at 30,000 x g for 20 min and the supernatant was loaded on top of 1 M sucrose cushion in buffer (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 20 mM MgOAc, 1% Triton X-100, and 2 mM DTT). After centrifugation for 17 h at 90,000 x g using a T70 rotor in Beckman ultracentrifuge, a minimal volume of Mitobuffer (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 20 mM MgOAc, and 2 mM DTT) enough to dissolve the pellet was added. 10 vol. of lysis buffer (25 mM HEPES-KOH pH 7.5, 100 mM KCl, 25 mM MgOAc, 1% Triton X-100, and 2 mM DTT) added to the crude mitochondria and incubated at 4 °C for 1 h in a rocking platform to allow gentle mixing. A discontinuous gradient was prepared in a Beckman polycellumator by layering 2.5% of 400 ml of 20 mM HEPES-KOH pH 7.5, 100 mM KCl, 20 mM MgOAc, 1% Triton X-100, 2 mM DTT, 1.5% sucrose solutions in buffer containing 10 mM HEPES-KOH pH 7.5 and 1 mM EDTA. DNase-treated sample was loaded on the discontinuous gradient and centrifuged for 1 h at 135,000 x g using a T70 rotor in Beckman ultracentrifuge. The brownish-orange layer containing pure mitochondria was carefully separated and re-suspended in SEM buffer.

Preparation of the 55S mitoribosome: Frozen cells (10 g wet weight) were homogenized by applying 120 strokes and the supernatant was separated from the cell debris by spinning at 80 °C. The frozen cells were homogenized by applying 120 strokes and the supernatant was separated from the cell debris by spinning at 80 °C. The frozen cells were resuspended in lysis buffer (1× PBS, 0.5 mM CaCl2, 1 mM DTT, 1 mM spermidine, and 8 mM putrescine). The frozen cells were homogenized by applying 120 strokes and the supernatant was separated from the cell debris by spinning at 80 °C. The frozen cells were resuspended in lysis buffer (1× PBS, 0.5 mM CaCl2, 1 mM DTT, 1 mM spermidine, and 8 mM putrescine).

Overexpression and purification of RRFmt. The GST-tagged RRFmt was cloned into pGEX6.1 vector and over-expressed in Rosetta (pLysS strain) and pure protein over-expression was induced by adding 100 mM isopropyl-1-thio-D-galactopyranoside (IPTG). The cell culture was left overnight at 16 °C for optimal induction. Two 55S mitoribosome classes, Class I (93,212 particles) and Class II (132,008 particles) was re-determined from the 39S subunit Class III (162,354 particles), 39S subunits (419,699 particles), 28S subunits (235,896 particles) and poorly aligned particles (322,802 particles). To obtain more homogenous sub-populations, particles corresponding to the 55S mitoribosomes, 39S and 28S subunits were each subjected to additional rounds of 3D classification that finally yielded three stable classes representing three distinct functional states formed during the human mitoribosome recycling process. Two 55S mitoribosome classes, Class I (93,212 particles) and Class II (28,929 particles), were finally refined to 3.49 and 3.91 Å, respectively, while the 39S subunit Class III (132,008 particles) was refined to 3.15 Å.

Model building and optimization. Coordinates corresponding to the small and large subunits from our published human mitoribosome structures6 (PDB ID: 6Q1B) were used to dock independent rigid bodies into the cryo-EM density maps of the Class I, Class II, and Class III complexes using Chimera 1.14 (Pettersen EF 2004). To obtain optimal fitting into our cryo-EM densities, the models were subsequently refined in PHENIX 1.14 (Adams PD 2010) using the "real-space refinement" function. Coordinates belonging to the human RRFmt (Korippa et al.43) were placed independently into the corresponding cryo-EM densities of all the three maps as rigid bodies using Chimera 1.1446 and the models were further refined in PHENIX 1.14 to achieve optimal accommodation into the cryo-EM densities. The primary sequence of EF-G2mt was submitted to the I-TASSER server44 to generate the initial EF-G2mt homology model. The final model was used to interpret the corresponding cryo-EM reconstructions in the homology model that do not fully accommodate into the corresponding EF-G2mt density were modeled de novo using Chimera 1.1446 and COOT 0.9.51. For the final optimization of the model into the cryo-EM density, the "Real-space refinement" function in PHENIX 1.14 was utilized. Validation reports for all the models were evaluated using the program EM-REBOOk47 and the computational models and molecular modeling are listed in Table S2. All the figures in the manuscript were generated using ChimeraX 1.1148.
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