Structures of the human mitochondrial ribosome bound to EF-G1 reveal distinct features of mitochondrial translation elongation

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The mammalian mitochondrial ribosome (mitoribosome) and its associated translational factors have evolved to accommodate greater participation of proteins in mitochondrial translation. Here we present the 2.68–3.96 Å cryo-EM structures of the human 55S mitoribosome in complex with the human mitochondrial elongation factor G1 (EF-G1mt) in three distinct conformational states, including an intermediate state and a post-translocational state. These structures reveal the role of several mitochondria-specific (mito-specific) mitoribosomal proteins (MRPs) and a mito-specific segment of EF-G1mt in mitochondrial tRNA (tRNAmt) translocation. In particular, the mito-specific C-terminal extension in EF-G1mt is directly involved in translocation of the acceptor arm of the A-site tRNAmt. In addition to the ratchet-like and independent head-swiveling motions exhibited by the small mitoribosomal subunit, we discover significant conformational changes in MRP mL45 at the nascent polypeptide-exit site within the large mitoribosomal subunit that could be critical for tethering of the elongating mitoribosome onto the inner-mitochondrial membrane.
Mitochondria are thought to have originated through an early endosymbiotic event between an α-proteobacterium and a primitive eukaryotic host cell. However, the structural, functional, and compositional organization of the mitochondrial ribosomes (mitoribosome) is dramatically different from its cytoplasmic and bacterial counterparts. The ribosomal RNA (rRNA) to ribosomal protein ratio in mammalian mitoribosome (~1:2) is reversed as compared to that in the eubacterial ribosomes (~2:1). The first cryo-EM study of the mammalian mitoribosome identified several unique structural features in both its subunits: the large 39S subunit (LSU) and the smaller 28S subunit (SSU). Subsequent high-resolution structures provided molecular description of previously identified features, such as heavily shielded rRNA cores by mitoribosomal proteins (MRPs), the presence of a significantly modified entrance of the mRNA channel and nascent polypeptide-exit tunnel (NPET), and a P-site finger. In addition, the high-resolution structures revealed that one of the mitochondrial tRNAs (tRNAsmt) partially substitutes for the role of bacterial 5S rRNA by becoming a structural component of the mammalian mitoribosomal LSU.

Similar to bacterial translation, the mechanism of the mammalian mitochondrial translation is roughly divided into four stages: initiation, elongation, termination, and ribosome recycling. Each of these stages are facilitated by translational factors that are homologous to their bacterial counterparts but carry mitochondria-specific (mito-specific) segments. Biochemical and structural studies have shown that the mito-specific segments in translational factors play important functions in mitochondrial translation. The distinct structural features in both the mitoribosome and its binding translational factors therefore suggest unique molecular interactions and mechanism during each mitochondrial translation step. The critical step of tRNA and mRNA translocation on the ribosome is promoted by elongation factor G (EF-G) in eubacteria and homologous EF-2 in eukaryotic cytoplasm. In mammalian mitochondria, there are two isoforms of EF-G mt: EF-G1 mt and EF-G2 mt, which catalyze tRNA translocation on the mitoribosome, whereas EF-G2 mt is involved exclusively in mitoribosome recycling. A mutation in human EF-G1 mt leads to fatal hepatocerebralopathy, indicating that this isoform is essential for mitochondrial protein biosynthesis in humans. In addition, defects in mitochondrial protein synthesis are associated with numerous human diseases that directly involve mutations in MRPs and tRNAsmt.

The bacterial EF-G is composed of five structural domains, namely G (or domain I) and domains II – V. The structural and functional aspects of EF-G-catalyzed translocation on bacterial ribosomes have been extensively studied in various functional states, using both cryo-EM and X-ray crystallography. Like other translocases, the mammalian EF-G1 mt (molecular weight ~80 kDa) is a single polypeptide that possesses mito-specific extensions at both its termini with an additional 47 amino acids (aa), including the signal sequences, as compared to its bacterial homologs. Human EF-G1 mt is 751 aa long, while the first 36 residues at the N-terminus constitute the mitochondrial targeting signal, which is cleaved off in the functional form. The functional human EF-G1 mt shows ~45% sequence identity with its bacterial counterpart, with a major difference being the presence of an 11 aa mito-specific extension at its C terminus. We have determined near-atomic-resolution cryo-EM structures of the human 55S mitoribosome in complex with the human EF-G1 mt to investigate the roles of the mito-specific MRPs and C-terminal extension in EF-G1 mt translocation in mammalian mitochondria. Our study reveals several distinct features, including mito-specific molecular interactions during EF-G1 mt-mediated tRNA translocation on the human mitoribosome. In addition, we identify conformational changes associated with translation elongation at the exit of the NPET within the mitoribosome that could be necessary for facilitating the release of the nascent polypeptide chain through the NPET and anchoring of the mitoribosome on to the inner mitochondrial membrane.

Results and discussion

Overall structure of the 55S-EF-G1 mt complex. To obtain the 55S-EF-G1 mt complex a non-hydrolysable analog of GTP, GMPPCP, was used to lock EF-G1 mt on the mitoribosomes (see Methods). A cryo-EM structure for the 55S-EF-G1 mt-GMPPCP complex with an overall resolution of 2.7 Å (Supplementary Figs. 1 and 2) was obtained (see Methods). 3D classification of all selected 55S mitoribosome images yielded two major classes that were refined to 2.96 Å and 2.97 Å resolution, respectively, and a minor class that was refined to 3.96 Å resolution (Supplementary Figs. 1 and 2; Supplementary Table 1). All three 55S mitoribosome maps show well-defined densities for EF-G1 mt, but reveal significant differences in the relative orientation of the 28S subunit with respect to the 39S subunit. The cryo-EM structure with the 28S subunit in its fully rotated state is referred to as Class I, the structure with the 28S subunit in its unrotated canonical state is referred to as Class III (Fig. 1a, b), and the structure with the 28S subunit in an intermediate state between the Class I and Class III conformations is referred to as Class II. Each of these structures shows variable densities for tRNAsmt in the mitoribosomal peptidyl (P) and exit (E) sites (Fig. 1a, b; and Supplementary Notes).

The overall conformation of EF-G1 mt on the 55S mitoribosome in all three maps is analogous to the structure of EF-G in the cytoplasmic ribosomal complexes, where the factor has been trapped either with the help of the antibiotic fusidic acid (FA) or by using a non-hydrolysable GTP analog or using a non-hydrolysable analog of GTP. In addition to determining the structure of complete EF-G1 mt with all of its 715 aa residues that fold into five globular domains (Fig. 1c), high-resolution features in our cryo-EM maps (Supplementary Fig. 3) allow us to model 75 rRNA residues and 1,082 aa residues that are absent in the currently available human mitoribosome structure. Furthermore, we identify species-specific structural differences among mammalian MRPs. The EF-G1 mt binding stabilizes the flexible C-terminal domain (CTD) of the uL10–L12 stalk and enables modeling of one copy of the uL12m CTD that interacts with the G′ subdomain of EF-G1 mt (Fig. 1d). EF-G1 mt induced movement also brings the N-terminal domain (NTD) of uL11m by 5 Å closer to the uL12m CTD (Supplementary Fig. 4c), thereby enabling the latter to simultaneously interact with the uL11m and the G′ subdomain to form an arc-like structure. A direct interaction between uL12m CTD and EF-G1 mt suggests uL12m’s role in factor recruitment during mitochondrial translation.

Interactions of EF-G1 mt with the GTPase-activating center of the mitoribosome. Translocation of tRNAs and mRNA is an intrinsic property of the ribosome but binding of EF-G-GTP and subsequent hydrolysis of GTP on EF-G enhances the rate of translocation by several orders of magnitude. Using our higher resolution map (Complex III), a complete de novo model of the nucleotide-binding pocket and the interactions of switch I with other domains of EF-G1 mt and the adjacent ribosomal components could be constructed. The bound GMPPCP is held in position through a large network of hydrogen bonds and van der Waals interactions with several highly conserved EF-G1 mt residues, notably D56 and K59 from the P-loop, T101 from the...
switch I region, and H124 from the switch II region (Fig. 2a). A crucial Mg$^{2+}$ ion positioned close to γ phosphate of GMPPCP is coordinated by T60 from the P-loop and T101 from the switch I region (Fig. 2b). H124 is known to be essential for catalyzing the hydrolysis of GTP, as mutation of this crucial residue in bacterial EF-Tu severely inhibits its ribosome-stimulated GTP hydrolysis. In our maps, the catalytic H124 is oriented towards the γ phosphate of the bound GMPPCP molecule (Fig. 2c), representing an active nucleotide-binding pocket similar to previously described ratchet-like inter-subunit reorganization of the bacterial ribosomes. In addition to this ratchet-like motion, significant head swiveling was also observed in the Class I complex, where the 28S subunit head region has rotated ~3° relative to the body in a roughly orthogonal direction to the ratchet-like motion (Fig. 3a). The Class II complex presents a previously unknown EF-G-bound conformational intermediate, where the head region of the 28S subunit has swiveled as in the ratcheted Class I complex (Fig. 3b), while the conformation of the 28S body region is similar to that in the unrotated Class III complex (Fig. 3c). The presence of a Class I complex-like conformational state with ratcheted and head rotated SSU in a previous EF-G mt -unbound map but the absence of such a conformation in our control maps (Supplementary Fig. 6) suggest that the Class I complex is formed either upon binding of EF-G mt to a subpopulation of mitoribosomes that carries only a single tRNA at the E-site (Fig. 3d), or to a population that carries loosely bound P-site tRNAs that are all translocated to the E-site. Overall, we find that EF-G mt binding brings a greater proportion of mitoribosomes into unratcheted state, when compared with the distribution in our control population (Supplementary Fig. 6). The Class II complex represents the smallest of the three populations and shows a strong density for the E-site tRNA but a somewhat fragmented tRNA density at the P site (Fig. 3e), whereas the Class III complex shows densities for both P- and E-site tRNAs (Figs. 1a, 3f).

Interactions of domain IV of EF-G mt in the A site of the 28S subunit in three conformational states. In all three 55S-EF-G mt complexes, the EF-G mt is held in position by interacting with several components of both the large and small mitoribosomal subunits but domain IV of EF-G mt and mitoribosomal components interact differently among these complexes. In the Class I 55S-EF-G mt complex, the 28S subunit has undergone a ~9.5° counter-clockwise rotation relative to the 39S subunit (Fig. 3a), similar to previously described ratchet-like inter-subunit reorganization of the bacterial ribosomes. In addition to this conformational state with ratcheted and head rotated SSU in a previous EF-G mt -unbound map but the absence of such a conformation in our control maps (Supplementary Fig. 6) suggest that the Class I complex is formed either upon binding of EF-G mt to a subpopulation of mitoribosomes that carries only a single tRNA at the E-site (Fig. 3d), or to a population that carries loosely bound P-site tRNAs that are all translocated to the E-site. Overall, we find that EF-G mt binding brings a greater proportion of mitoribosomes into unratcheted state, when compared with the distribution in our control population (Supplementary Fig. 6). The Class II complex represents the smallest of the three populations and shows a strong density for the E-site tRNA but a somewhat fragmented tRNA density at the P site (Fig. 3e), whereas the Class III complex shows densities for both P- and E-site tRNAs (Figs. 1a, 3f).
Domain IV of the bacterial EF-G is known to play a crucial role in tRNA and mRNA translocation. In all three classes, Domain IV of the EF-G1mt is inserted into the 28S subunit decoding center such that it would sterically overlap with the anticodon arm of an A-site tRNA (Fig. 3g-i), as found in case of analogous bacterial complex. Minimum mitoribosomal interactions with domain IV occur in Class I complex, an intermediate number of interactions occur in Class II complex, and the maximum interactions occur in Class III complex (Fig. 3g-i). In the Class III complex, Domain IV makes contacts with multiple 12S rRNA components of the 28S subunit such as helix 24 (h24), h30, h44, and the anticodon arm of the tRNA bound in the P/P position (Fig. 3i). [We have adopted the bacterial numbering to refer to the mitochondrial rRNA in SSU and LSU. The rRNA nucleotide numbering is according to Amunts and coworkers.] In the 28S P-site region, aa residues S543, G544 and G545 from the loop1 region of domain IV interact with the backbone phosphates of h44 bases C1561 and G1562, while base A1078 from h24 is placed within hydrogen-bond forming distance from G544 and G545 of domain IV (Fig. 3i). The Class II and Class III maps show density for a P-site tRNA (Fig. 3e, f) bound in the classical P/P state. However, the P-site density is relatively weak, because it represents an averaged density of the endogenously bound multi-sized tRNAmt, some of which are known to have much smaller T-loops compared to their bacterial counterparts. Nevertheless, conserved segments such as anticodon and CCA arm of tRNA could be docked into corresponding densities. Accordingly, nucleotides 33 and 34 from the anticodon of the P-site tRNA are positioned within 3 Å of residues M618 and V619 (Fig. 3i) from the loop3 region of domain IV. In the 28S head region of Class III complex, the backbone phosphates of the 12S rRNA bases U1209 and U1210 interact with aa residues S576 and N577 from the loop2 region of domain IV (Fig. 3i). The size variability in tRNAmt also affect the density corresponding to the anticodon region of the E-site tRNAmt (Supplementary Notes and Supplementary Fig. 7).

Simultaneous interactions of domain IV with both the head and shoulder regions of the 28S subunit and the anticodon region of the tRNA in Complex III would stabilize the tRNA in the P site and prevent the anticodon end of translocated P-site tRNA from slipping back to the A site, as also suggested by structural studies on bacterial translocation. The small subunit of a bacterial 70S ribosome is also found in an unrotated conformation with similar interactions in the crystallographic structure of the 70S EF-G-GDP-tRNA post-translocational complex, suggesting that our Class III 55S·EF-G1mt complex represents an authentic post-translocation state of the human mitoribosome. The core 12S LSU rRNA regions of the mitochondrial and bacterial ribosomes that are known to interact with A- and P-site tRNAs in eubacteria are generally conserved, and the relative orientations of bound A- and P-site tRNAs are also similar (Supplementary Fig. 8), despite the presence of a significantly altered and MRP-enriched environment around the tRNA binding sites in the LSU of the mitoribosome as elaborated under the next heading.

The tip of EF-G1mt domain IV in the Class I complex is positioned ~10 Å away from 12S rRNA helices h24 and h30, closer to the 28S shoulder or the A site than its position in the Class III complex (Fig. 3g, also see Supplementary Fig. 9). Domain IV does not interact with the 28S head region in Class I complex, and the only 28S subunit element that still interacts with Domain IV is the 12S rRNA h44 (Fig. 3g). This is not surprising since simultaneous interactions of Domain IV with both the head and platform components would impede the head rotation, and a
combination of subunit ratcheting and head swiveling help translocate the A- and P-site tRNAs into the P and E sites, respectively. Unlike the Class III complex, the Class I map does not have enough density to model a P-site tRNA but superimposing the Class III P-site tRNA density onto the Class I map suggests that the interactions of domain IV with anticodon of the P-site tRNA in the P/P state would not be established in the Class I complex (Fig. 3g). Interestingly, these contacts can be restored if the anticodon end of the P-site tRNA is moved by 6–7 Å towards the A site of the 28S subunit (Fig. 3g), which indicates that the Class I 55S-EF-G1\textsubscript{mt} complex represents a key early translocation intermediate where the domain IV has moved only partially into the A site following the movement of A-site tRNA into an intermediate chimeric ap/P state\textsuperscript{31}, preceding the Class II state (Fig. 3h) that is followed by the Class III state (Fig. 3i). Domain IV of EF-G1\textsubscript{mt} synchronizes the ratcheting motion of the ribosome along with the movement of tRNAs, as it appears to closely follow the anticodon arm of the A-site tRNA during its translocation into the P site\textsuperscript{31,32,35,52}.

**Role of P-site finger and other MRPs that directly interact with tRNAs\textsubscript{mt} and EF-G1\textsubscript{mt}.** One of the major structural differences between the bacterial and mitochondrial ribosomes is the loss of several rRNA segments in mitochondria that are partially compensated by the acquisition of new MRPs and extensions in homologous MRPs\textsuperscript{3,34–36}. This also changes the compositional landscape of the ribosomal intersubunit space that provides the corridor for the mRNA and tRNA movement on the mammalian mitoribosome during translation elongation. In mammalian mitochondria, protein bL5 is lost from the P site while bL25 and the A-site finger (23S rRNA helix 38) are lost from the A site\textsuperscript{2,4,5}. The loss of these structural elements that interact with bound tRNA molecules is compensated by a unique finger-like structural element called the P-site finger (PSF) that interacts with both the A- and P-site bound tRNAs\textsuperscript{4,5}. In the Class III complex, the PSF is found interacting with both the T-loop and the D-loop of P-site tRNA (Fig. 4a). The role of PSF appears to be to correctly position the A- and P-site tRNAs and prevent the elbow region of the P-site bound tRNA from reverting back to the A site during and
after its translocation from the A to the P site. In comparison to empty 55S mitoribosomes, the PSF has undergone a significant conformational change and moved closer towards the P-site bound tRNA in all our EF-G1mt-bound complexes (Fig. 4b) as well as in the mammalian mitochondrial initiation complex. Tight interactions with the PSF could be one of the reasons for the frequent co-purification of mitoribosomes with a P-site bound tRNA. In our maps, we found a previously unassigned tubular density in the region between the C-terminus of mito-specific mL64 and the PSF. This extra density is readily attributable to an α-helix-forming 32 aa residues of the C-terminus of mL64 (Fig. 4c), which extends in the 39S subunit between the P- and E-site tRNAs while interacting with the D and T loop regions of both the tRNAs (Fig. 4a). Along with the mito-specific protein mL48 and the mito-specific segments of MRPs uL11m and uL16m, PSF and the C-terminus of mL64 span all three tRNA binding sites on the 39S subunit (Fig. 4a), structurally compensating for the absence of some of the bacterial homologs of r-proteins and rRNA components that are known to be involved in tRNA positioning, stabilization and translocation in the bacterial ribosome.

In all three EF-G1mt-bound complexes the uL11m stalk-base region within the mitoribosomal LSU moves by 5 Å towards the domain V of EF-G1mt (Supplementary Fig. 4a, b), as compared to that in the empty 55S mitoribosomes, in the initiation, and in mitoribosome recycling complexes. However, in the mammalian mitoribosome the conformational change is associated with a direct contact between the domain IV of EF-G1mt and the mito-specific segment of uL11m (Fig. 4d). K192 from the mito-specific C-terminus α-helix of uL11m interacts with the E562 from the domain IV through a hydrogen-bond interaction (Fig. 4d). Interestingly, the uniquely placed E562 is absent in EF-G2mt. The presence of E562 and the mito-specific CTE in EF-G1mt and their absence in EF-G2mt, along with presence of four small insertion segments within corresponding domains II and III of EF-G2mt (Supplementary Fig. 10), appear to be the key
Role of the C-terminal extension in EF-G1mt. Both the Class I and Class III complexes show an additional density adjacent to the conserved C-terminal end of the EF-G1mt domain IV that could readily accommodate its mito-specific C-terminal extension (CTE) (Fig. 5a), which is not resolved in the Class II complex. The lysine-rich CTE folds into an α-helix and extends into the 39S subunit enabling the EF-G1mt to interact with rRNA and tRNAmt segments that would be inaccessible to the bacterial EF-Gs. The CTE is positioned close to the nucleotides U2606-G2608 segment of the 16S LSU rRNA helix 71 (H71) (Fig. 5a). In its current orientation, the CTE would overlap with the inner bend of A-site tRNAmt elbow primarily involving tRNAmt’s CCA arm. The interaction of lysine-rich CTE with H71 would also prevent the reverse translocation of the P-site tRNA to the A site. The fact that EF-G1mt remains active on the E. coli ribosomes, but E. coli EF-G remains inactive on mitoribosomes37, suggests that the observed interaction of EF-G1mt’s CTE with the mitoribosome and the A-site tRNAmt in our structure could also be associated with EF-G1mt’s GTPase activity on the ribosome. A significantly altered landscape of the mitoribosomal intersubunit space described in the previous section and the location of EF-G1mt’s CTE on the mitoribosome suggest that the MRPs and translational factors have coevolved with its unique tRNAmt to structurally and functionally compensate for the lost bacterial RNA segments.

Conformational changes at the nascent polypeptide-exit site. The newly synthesized protein chain exits the ribosome through a tunnel-like feature in the large subunit38,39, known as the nascent polypeptide-exit tunnel (NPET). The NPET originates from the peptidyltransferase center (PTC) and ends on the opposite side at the solvent interface, which is referred to as the polypeptide-exit site (PES). The structural composition of this tunnel is substantially different between the bacterial and the mammalian mitochondrial ribosomes24,43. Domains I and III of the 23S rRNA that line the bottom portions of NPET in bacteria are greatly reduced in the analogous mitochondrial 16S LSU rRNA53. The loss of these important structural components surrounding the tunnel is compensated through the acquisition of larger bacterial r-protein homologs with extended N and C termini5,54,56. A mito-specific protein mL45 is also present near the PES24. During the initiation phase, the entire NPET is blocked by the insertion of the N-terminus (NT) residues 38–64 of mL45 into the NPET9. The N-terminal region of mL45 also interacts with MRPs ul23m and ul24m near PES. Mutational studies have shown that deletion of the mL45 NT severely inhibits mitochondrial translation9.

Though the blocked NPET might not pose any problem for an initiating mitoribosome, a vacant tunnel would be necessary to accommodate the growing nascent polypeptide chain during the translation elongation phase. In all our complexes, we found an unassigned density that is connected to the CCA end of the P-site tRNA and reaches close to the NT of the ribosomal protein mL45 inside the NPET (Fig. 6a). This density that could accommodate up to 5 aa residues can be readily attributed to a nascent peptide chain (NPC). In our structure, a conserved adenine residue (A2725) from a loop region between the 16S LSU rRNA helices H73 and H74 intercalates between the NT of NPC and aa R40 from the NT of mL45 (Fig. 6a). By simultaneously interacting with the NPC and NT of mL45, A2725 might play a crucial role in triggering a conformational change in the large mitoribosomal subunit that eventually results in the retraction of NT of mL45 from the NPET to make room for the growing NPC. Indeed, we observed a significant conformational change involving aa residues R61 to D73 (Fig. 6b). Compared to their position in the initiation complex9, these residues have moved substantially, ~9 Å away from the tunnel and toward ul24m, which also shifts in conjunction with the mL45 movement. The EF-G1mt-induced conformational change in the large subunit captured in our structure likely represents a functional state, as mL45 prepares to retrieve its NT from the NPET to allow the insertion of incoming nascent polypeptide from the PTC side of the NPET.

In addition, the residues T101-Y128 located in the core region of mL45 undergo a large conformational change (Fig. 6c) between the initiation9 and our elongation complex. In the initiation complex, these residues form two separate α-helices with an angle of ~120° between them. Of these, residues T109–T115 from the N-terminal helix rotate by ~60° to become part of a single long α-helix in the elongation complex, leaving residues S101–R108 in an open conformation. This large conformational change, involving a secondary structure rearrangement, may be necessary for anchoring of the mammalian mitoribosome to the inner mitochondrial membrane (IMM). mL45 happens to be the homolog of Mba1, the IMM-associated receptor necessary for the co-translational insertion of nascent polypeptides into the IMM in yeast80. Interestingly, this positively charged segment (aa residues 101–114) of mL45 has been implicated in mediating the association of 55S mitoribosomes with the negatively charged
In summary, our study presents the most complete structure for the human 55S mitoribosome, and shows that the EF-G_{1mt}-bound mitoribosome can adopt at least three different conformational states irrespective of the GTP hydrolysis state. The major variation occurs in the relative orientation of its entire 28S subunit, or only its head domain, suggesting an unusual adaptability of the 28S subunit during translocation (Fig. 3). Direct structural evidence is presented that the mito-specific components in both the mitoribosome and EF-G_{1mt} are involved in tRNA_{m} translocation. Our study also shows how mito-specific ribosomal proteins, such as PSF and mL64 in the mitoribosome’s tRNA_{m} interaction sites (Fig. 4), and the addition of a mere 11 aa residues in the C-terminus of EF-G_{1mt} (Fig. 5), allow the mitochondrial translation system to adapt to a massive reduction in mitoribosomal RNA components as compared to their bacterial counterparts. For example, the absence of 23S RNA helix 38, also known as the A-site finger that dynamically interacts with both A- and P-site tRNAs during the tRNA translocation in eubacteria, is structurally and functionally compensated by the PSF protein in the mammalian mitoribosome. Similarly, the missing eubacterial E-site tRNA interacting RNA components are replaced by protein mL64. Finally, the large conformational changes between the initiation and elongation states involving mito-specific protein mL45 in the NPET’s exit site (Fig. 6), seem to be associated with the mitoribosomal anchoring to the IMM.

**Methods**

**Isolation of mitochondria from HEK cells.** Mitochondria were isolated from human embryonic kidney cells lacking N-acetyl-glucosaminyltransferase I (HEK293S GnTI) that were cultured in roller bottles using FreeStyle™293 media (Gibco, Life Technologies) supplemented with 5% fetal bovine serum (Gibco, Life Technologies). After centrifugation at 1000 × g for 7 min, the HEK293S GnTI cell pellet was transferred to a glass homogenizer and resuspended in buffer containing 50 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. After homogenization, the supernatant was separated from the cell debris by spinning at 950 × g for 15 min. The supernatant was then spun at 11,000 × g for 15 min, and the resulting pellet that contains crude mitochondria was resuspended in SEM buffer (250 mM sucrose, 20 mM HEPES-KOH pH 7.5, 1 mM EDTA, and 1 mM EGTA). Dounce homogenization was performed in a Beckman polylameller tube by layering 2.5 ml of 60%, 4 ml of 32%, 1 ml of 23%, and 1 ml of 13% sucrose solutions in 10 mM HEPES-KOH pH 7.5 and 1 mM EDTA. The treated sample was loaded on the discontinuous gradient and centrifuged for 1 h at 135,000 × g using Ti70 rotor in a Beckman ultracentrifuge. The brownish-orange layer containing pure mitochondria was carefully separated and re-suspended in SEM buffer.

**Isolation of mitoribosomes from mitochondria.** Mitoribosomes were isolated by adding four volumes 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) to the mitochondrial-pellet and then incubating for 15 min at 4 °C. The sample was centrifuged at 30,000 × 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, 2 mM DTT) for 60 min. The supernatant was loaded on a continuous sucrose density gradient (Mitobuffer, using the gradient making apparatus) and centrifuged for 17 h at 305,000 × g using Ti70 rotor in a Beckman ultracentrifuge, a minimal volume of Mitoribosome (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 20 mM MgOAc, and 2 mM DTT) enough to dissolve the pellet was added. 10–30% continuous sucrose density gradients were prepared in Mitoribosome buffer, using the gradient making apparatus (C.B.S. Scientific Co.). The resulting pellet was suspended in Polybuffer (5 mM HEPES-KOH pH 7.5, 100 mM KCl, 20 mM MgOAc, and 2 mM DTT) for 17 h using Ti70 rotor in a Beckman ultracentrifuge. The gradient was fractionated on ISCO gradient analyzer (Teledyne ISCO, Inc), and the fractions corresponding to the mitoribosomes were collected and pooled. Finally, the pooled mitoribosomes were concentrated by spinning them at 130,000 × g for 6 h using Ti70 rotor and the pellet was resuspended in Polybuffer (5 mM HEPES-KOH pH 7.5, 100 mM KCl, 20 mM MgOAc, 5 mM NH₄Cl, 0.5 mM CaCl₂, 1 mM EDTA, 1 mM spermidine, and 8 mM putrescine).

**Cloning and expression of human EF-G_{1mt}.** An expressed sequence tag coding for human EF-G_{1mt} was obtained from GeneCopenia (No. GC-W1058). Using PCR, the sequence corresponding to the mature form of EF-G_{1mt} (amino acids 36–751) was amplified by employing forward 5'-GGAAATCCCATGTCATGCAGTTGGAGTACAC-3' and reverse 5'-AACGGCTGAGTTCTGCTGTTCGTTCCCTTTTTT
AAC-3' primers19. The PCR product was cloned into pET 21c (+) (Novagen) and this vector provides a sequence encoding six His residues (His-tag) at the C-terminus. The recombinant construct was transformed into E. coli ER2276 and subsequently transformed into E. coli BL21 (DE3) (RIL) for over-expression.

**Purification of human EF-G1mt**

The cultures were grown to mid-log phase and induced with 50 μM isopropyl-1-thio-galactopyranoside (IPTG). After centrifugation at 5,000 rpm for 15 min at 4°C, the cells were harvested, shock-frozen, and stored at −80°C. The frozen cells were disrupted by grinding with double the cell weight of Alumina Type A-5 (Sigma) for a total of 20 min. The paste was resuspended in Buffer B (50 mM Tris–HCl, pH 7.6, 40 mM KCl, 7 mM MgCl2, 7 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol), and the debris was removed by centrifugation at 10,000 rpm for 4°C at 10 min. This is followed by DNase I (5 μg/mL) treatment and centrifugation at 15,000 rpm at 4°C for 20 min. The resulting supernatant was mixed with 0.6 M NaCl of a 50% slurry of Ni-NTA resin equilibrated in Buffer B and relatively pure EF-G1mt was obtained using affinity chromatography39. In order to achieve high-level purity, ion exchange chromatography technique was employed where the partially purified EF-G1mt from the Ni-NTA purification was processed on a cation exchange TSKgel SP-SW column (TosohAs, Japan).

**Preparation of the human mitoribosome+EF-G1mt+GMPPCP complex**

To obtain the 55S-EF-G1mt complex, a non-hydrolysable analog of GTP, GMPPCP, was used to lock EF-G1mt on the mitoribosomes. The complex was formed by incubating 150 nM 55S mitoribosomes with 5 μM EF-G1mt and 1 mM GMPPCP (Sigma-Aldrich, USA) at 37°C for 5 min in the HEPES polyimix buffer.

**Cryo-electron microscopy and image processing**

In all, 4 μl of the 55S-EF-G1mt+GMPPCP complex was applied to Gatan planer copper 12/13 grids that were prepared on a copper mesh grid (200 mesh; 2–3 mm thick) of home-made continuous carbon film and glow-discharged for 30 s on a plasma sterilizer. After incubating the grids for 15 s at 4°C and 100% humidity, they were blotted for 4 s and immediately flash-frozen into the liquid ethane with the help of a Vitrobot (FEI company). Data were collected on a Titan Krios electron microscope (FEI company) equipped with a Gatan K2 Summit direct-electron detecting camera at 300 kV. We used a defocus range of −1.0 to −3.0 μm at a calibrated magnification of ×105,000, yielding a pixel size of 1.096 Å. A dose rate of 7 electrons per pixel per s and an exposure time of 2.6 Å range (Supplementary Fig. 2). However, to lower the resolution for the previous human model where the side-chain density of L7/L12 stalk was not adequately described39

**Model building and optimization**

Coordinates corresponding to the small and large subunits of the published human ribosome (PDB ID: 3R9M39 were used as the initial template. The higher resolution of our maps (Supplementary Fig. 3) enabled us to build multiple protein and RNA segments that were not present in the previous human ribosome structures22,25. Highly resolved secondary structural elements (SSE) and amino acid side-chain features guided the manual building of the majority of protein models using UCSF Chimera 1.1457 and COOT84. For modeling the relatively low-resolved regions such as the L7/L12 stalk, regions in the homology model that do not fully accommodate into the side-chain density were modeled de novo using Chimera 1.1457 and COOT84. Lower resolution in our cryo-EM maps corresponding to the density of EF-G1mt C-terminal extension (CTE) restricted modeling of this region in the side-chain model but permitted building the carbon backbone guided by recognizable SSEs. For the final optimization of the models into the cryo-EM densities, we used the “Real-space refinement” function in PHENIX32. The models were validated using Molprobity server53 and the overall statistics of EM reconstruction and molecular modeling are listed in Supplementary Table 1.

**Data availability**

The data that support this study are available from the corresponding authors upon reasonable request. The cryo-EM maps and atomic coordinates have been deposited in the Electron Microscopy and PDB Data Bank (wwPDB) under accession codes EMD-21233 [https://www.ebi.ac.uk/pdb/entry/emdb/EMD-21233] and PDB 6VLZ [https://doi.org/10.2210/pdb6VLZ/pdb] for the EF-G1mt-bound 55S mitoribosome (Complex I), and EM 21243 [https://www.ebi.ac.uk/pdb/entry/emdb/EMD-21243] and PDB 6VMY [https://doi.org/10.2210/pdb6VMY/pdb] for the EF-G1mt-bound 55S mitoribosome (Complex III). Cryo-EM maps of the Complex II and bovine 55S mitoribosome have been deposited with access code EMD-22212 [https://www.ebi.ac.uk/pdb/entry/emdb/EMD-22212] and EMD-22209 [https://www.ebi.ac.uk/pdb/entry/emdb/EMD-22209], respectively. All raw micrographs and particle images used in 3D reconstructions will be made available through empiar, an electron microscopy public archive image, https://www.ebi.ac.uk/pdb/emdb/empiar/.

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**References**

1. Gray, M. W., Burger, G. & Lang, B. F. The origin and early evolution of mitochondria. *Genome Biol.*, 2, reviews1018.1–reviews1018.5 (2001).
2. Annunts, A., Brown, A., Toots, J., Scheres, S. H. W. & Ramakrishnan, V. Ribosome. The structure of the human mitochondrial ribosome. *Science* 348, 95–98 (2015).
3. Desai, N., Brown, A., Annunts, A. & Ramakrishnan, V. The structure of the yeast mitochondrial ribosome. *Science* 355, 528–531 (2017).
4. Greber, B. J. et al. Ribosome. The complete structure of the 55S mammalian mitochondrial ribosome. *Science* 348, 303–309 (2015).
5. Sharma, M. R. et al. Structure of the mammalian mitochondrial ribosome reveals an expanded functional role for its component proteins. *Cell* 115, 97–108 (2003).
6. Florent Waltz, H. S., Bochler, A., Giegé, P., Hashem, Y. Cryo-EM structure of the RNA-rich plant mitochondrial ribosome. *bioRxiv*, https://doi.org/10.1101/773429 (2019).
7. Ramrath, D. J. F. et al. Evolutionary shift toward protein-based architecture in trypanosomal mitochondrial ribosomes. *Science* 362, eaau7735 (2018).
8. Korippella, R. K., Sharma, M. R., Risteff, P., Keshavan, P. & Agrawal, R. K. Structural insights into unique features of the human mitochondrial ribosome recycling. *Proc. Natl Acad. Sci. USA* 116, 8283–8288 (2019).
9. Kummer, E. et al. Unique features of mammalian mitochondrial translation initiation revealed by cryo-EM. *Nature* 560, 263–267 (2018).
10. Christian, B. E. & Spremulli, L. L. Mechanism of protein biosynthesis in mammalian mitochondria. *Biochim. Biophys. Acta* 1819, 1035–1054 (2012).
11. Sharma, M. R., Kaulash, P. S., Gupta, M., Banavali, N. K. & Agrawal, R. K. In Translation in Mitochondria and Other Organelles (ed. Duchêne A.-M.) Ch. 1, 1–28 (Springer, 2013).
12. Bhargava, K. & Spremulli, L. L. Role of the N- and C-terminal extensions on the activity of mammalian mitochondrial translational initiation factor 3. *Nucleic Acids Res.* 33, 7011–7018 (2005).
13. Gaur, R. et al. A single mammalian mitochondrial translation initiation factor 3. *Proc. Natl Acad. Sci. USA* 108, 1809–1814 (2011).
14. Haque, M. E. & Spremulli, L. L. Roles of the N- and C-terminal domains of mammalian mitochondrial translation initiation factor 3 in protein biosynthesis. *J. Mol. Biol.* 384, 929–940 (2008).
15. Rorbach, J. et al. The human mitochondrial ribosome recycling factor is essential for cell viability. *Nucleic Acids Res.* **36**, 5787–5797 (2008).

16. Koppella, R. K. et al. Structure of human mitochondrial translation initiation factor 3 bound to the small ribosomal subunit. *Science* **12**, 76–86 (2019).

17. Yassin, A. S. et al. Insertion domain within mammalian mitochondrial translation initiation factor 2 serves the role of eubacterial initiation factor 1. *Proc. Natl Acad. Sci. USA* **108**, 3918–3923 (2011).

18. Hamsamund, M. et al. Identification and characterization of two novel human mitochondrial elongation factor genes, hEFG2 and hEFG1, phylogenetically conserved through evolution. *Hum. Genet.* **109**, 542–550 (2001).

19. Tsuboi, M. et al. EF-G2mt is an exclusive recycling factor in mammalian mitochondrial ribosome. *J. Mol. Biol.* **332**, 689–699 (2003).

20. Frank, J. I. & Agrawal, R. K. Ratchet-like movements between the two ribosomal subunits: their implications in elongation factor recognition and tRNA translocation. *Cold Spring Harb. Symp. Quant. Biol.** **66**, 67–75 (2001).

21. Coenen, M. J. et al. Cytochrome c oxidase biogenesis in a patient with a mutation in COX10 gene. *Ann. Neurol.* **56**, 560–564 (2004).

22. Abbott, J. A., Francklyn, C. S. & Robey-Bond, S. M. Transferase, RNA. and protein. *Proc. Natl Acad. Sci. USA* **106**, 6419–6424 (2009).

23. Bugiardini, E. et al. MRPS25 mutations impair mitochondrial translation and oxidative phosphorylation. *Hum. Mol. Genet.* **23**, 55–66 (2014).

24. Chen, Y., Feng, S., Kumar, V., Ero, R. & Gao, Y. G. Structure of EF-G-ribosome complexes trapped in intermediate states of translocation. *EMBO J.* **13**, 3669–3677 (1994).

25. Czwerko, J., Wang, J., Steitz, T. A. & Moore, P. B. The crystal structure of elongation factor G complexed with GDP, at 2.7 A resolution. *EMBO J.* **13**, 3668–3673 (1994).

26. Agrawal, R. K., Penczek, P., Grassucci, R. A. & Frank, J. Visualization of elongation factor G bound to the ribosome. *Nat. Struct. Biol.* **12**, 723–735 (2005).

27. Frank, J. & Agrawal, R. K. A ratchet-like inter-subunit reorganization of the ribosome during translocation. *Nature* **406**, 318–322 (2000).

28. Ramrath, D. J. et al. Visualization of two transfer RNAs trapped in transit during elongation factor G-mediated translocation. *Proc. Natl Acad. Sci. USA* **110**, 20964–20969 (2013).

29. Ohtsuki, T., Kawai, G. & Rodnina, M. V. The minimal tRNA: unique structure and mechanism of translocation. *Science* **300**, 643–647 (1999).

30. Gao, Y. G. et al. The large subunit of the mammalian mitochondrial ribosome. *J. Mol. Biol.* **358**, 193–212 (2006).

31. Chen, Y., Feng, S., Kumar, V., Ero, R. & Gao, Y. G. Structure of EF-G-ribosome complexes in a pretranslocation state. *Nat. Struct. Mol. Biol.* **12**, 709–716 (2005).

32. Agrawal, R. K., Heagle, A. B., Penczek, P., Grassucci, R. A. & Frank, J. EF-G-dependent GTP hydrolysis induces translocation accompanied by large conformational changes in the 70S ribosome. *Nat. Struct. Biol.* **6**, 643–647 (1999).

33. Tourigny, D. S., Fernandez, I. S., Kelley, A. C. & Ramakrishnan, V. Elongation factor G bound to the ribosome in an intermediate state of translocation. *Science* **340**, 1235490 (2013).

34. Zhou, J., Lancaster, L., Donohue, J. P. & Noller, H. F. Crystal structures of EF-G-ribosome complexes trapped in intermediate states of translocation. *Science* **340**, 1236086 (2013).

35. Zhou, J., Lancaster, L., Donohue, J. P. & Noller, H. F. How the ribosome hands over the A-site tRNA to the P site during EF-G-catalyzed translocation. *Science* **345**, 1188–1191 (2014).

36. Bhargava, K., Templeton, P. & Spremuli, L. L. Expression and characterization of isofrom 1 of human mitochondrial elongation factor G. *Protein Expr. Purif.* **37**, 368–376 (2004).

37. Diaconu, M. et al. Structural basis for the function of the ribosomal L12/12 stalk in A-site binding and GTPase activation. *Cell* **121**, 991–1004 (2005).

38. Molter, M. et al. The ribosomal stalk binds to translation factors EF2, EF-Tu, EF-G and RF3 via a conserved region of the L12 C-terminal domain. *J. Mol. Biol.* **365**, 468–479 (2007).

39. Rodnina, M. V., Peske, F., Peng, B. Z., Belardelli, R. & Wintemer, Y. Converting GTP hydrolysis into motion: versatile translational elongation factor G. *Biol. Chem.* **401**, 131–142 (2019).
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Author contributions
R.K.A. conceived this study. K.B. and L.L.S. contributed to reagents, including purified EF-G1mt and clones for EF-G1mt; P.K. purified human mitoribosomes and human EF-G1mt; R.K.K. prepared the human 55S-EF-G1mt complex. R.K.K., M.R.S., and N.K.B. collected cryo-EM data and performed image processing. M.R.S., P.P.D., and P.S.K. performed purification and reconstructions of bovine mitoribosomes; R.K.K., M.R.S., and N.K.B. performed molecular modeling; R.K.K., M.R.S., and R.K.A. analyzed the data and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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