Interconnected assembly factors regulate the biogenesis of mitoribosomal large subunit

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Abstract

Mitoribosomes synthesize essential proteins encoded in mitochondria. They consist of RNA and protein components, coordinated assembly of which is critical for function. We used mitoribosomes with reduced RNA and increased protein mass from a mammalian parasite Trypanosoma brucei to provide insights into the biogenesis of mitoribosomal large subunit. Structural characterisation of a stable assembly intermediate revealed 16 assembly factors, 10 of which were not previously implicated in biogenesis. Most of the reported factors are also encoded in mammalian genomes. In the structure, the assembly factors form a protein network that spans over 180 Å, shielding the ribosomal RNA surface. The entire central protuberance and L7/L12 stalk are not assembled, and require removal of the factors and remodeling to become functional sites. The conserved mt-RbgA and mt-EngA are bound together in proximity to the peptidyl transferase center. A mitochondrial acyl-carrier protein plays a role in stably docking the L1 stalk that needs to be repositioned during maturation. Together, the extensive network of the assembly factors stabilizes the immature domains and connects between the functionally important regions.
Introduction

Mitoribosomes differ from bacterial and cytosolic ribosomes in their ribosomal RNA (rRNA), protein content, overall size, and structure. Their formation is an intricate and hierarchical process involving multiple proteins and a few RNA molecules working in coordination and under tight regulation (Pearce et al 2017). The cooperative effort involves regulation of two genomes, because rRNA is encoded by the organellar genome, and almost all the mitoribosomal proteins and assembly factors are encoded by the nuclear genome and therefore imported from the cytosol (Couvillion et al 2016). Finally, the fundamental process of the mitoribosomal assembly is complicated due to the localization of its large subunit (mtLSU) to the inner mitochondrial membrane. Therefore, stages of assembly were suggested to involve various mitochondrial milieu and specific kinetics (Bogenhagen et al 2014; Antonicka and Shoubridge 2015; De Silva et al 2015). The presence of different compositions is hypothesized to promote formation of defined pre-mitoribosomal complexes with as-yet-unknown organelle-specific auxiliary factors.

Mitochondria of Trypanosoma brucei provide a good model for studying the assembly process, because their mitoribosomes consist of over a hundred components, and the ratio of protein to rRNA is unusually high (Zikova et al 2008; Ramrath et al 2018). Since the rRNA forms a compact core of the mitoribosome, and proteins are mostly peripherally associated, an architecture based on the reduced rRNA and supernumerary mitoribosomal proteins would need additional stabilization for its assembly. Therefore, it increases the chances to characterize defined pre-mitoribosomal complexes, which are not stable enough for biochemical isolation in mitochondria of other species. Indeed, structural characterization of an assembly intermediate of the T. brucei mitoribosomal small subunit (mtSSU) provided insight into its assembly pathway with many additional proteins (Saurer et al 2019). However, the assembly of the mtLSU is poorly understood.

The mtLSU accommodates the peptidyl transferase center (PTC) that forms peptide bonds between amino acids, tRNA binding sites, the L7/L12 stalk that is responsible for the recruitment of translation factors, the L1 stalk, the central protuberance (CP) that facilitates communication between various functional sites, and exit tunnel for egress of synthesized protein. In bacteria, our understanding of the LSU assembly is relatively limited (Davis and Williamson 2017). It comes primarily from characterization of the final maturation stages (Li et al 2013; Jomaa et al 2014; Ni et al 2016), studies on incomplete LSU particles as a result of protein depletion (Davis et al 2016), and in vitro reconstitution studies with purified ribosomal RNA and protein components (Nikolay et al 2018). These studies identified different LSU precursors with assembly factors bound to rRNA components (Davis and Williamson 2017). In mitochondria, the mtLSU is lacking many of the rRNA components involved in the canonical pathways, and higher complexity of the interactions between the mitoribosomal proteins at the functional sites has evolved (Ott et al 2016; Greber and Ban 2016). A functional mtLSU require that the PTC and the rRNA regions which bind tRNAs are folded, a flexible L1 stalk, an extended L7/L12 protrusion, and a CP built from multiple mitochondria-specific proteins. However, only the final stage of the
mtLSU assembly has been visualized (Brown et al 2017; Itoh et al 2020), and no preceding steps in the formation of the functional sites have been characterised. To provide insight in the process of the mtLSU assembly, we determined the cryo-EM structure of a native T. brucei mtLSU assembly intermediate (pre-mtLSU) complexed with 16 factors. Most of the assembly factors have not been previously implicated in mitoribosomal biogenesis. The structural data suggests that the biogenesis relies on an extensive protein network formed by assembly factors that connect premature PTC, L1 and L7/L12 stalks with the CP. A homology search suggests that some of the newly identified assembly factors are also conserved in mitochondria from other species, including mammals, and therefore may represent a general principle for the mitoribosome assembly. Comparison with two bacterial assembly intermediates (Zhang et al 2014; Seffouh et al 2019) further provides insights into the conserved GTPases EngA and RbgA bound at the subunit interface.

Results and Discussion

Structural determination and composition of the native pre-mtLSU complex

We used a T. brucei procyclic strain grown in low-glucose medium that maintains translationally active mitochondria. Mitoribosomal complexes were rapidly purified directly from T. brucei mitochondria and analyzed by single-particle cryo-EM. During image processing, in addition to intact monosomes, we detected a pool of free subunits. We focused the analysis on this population and through 3D classification isolated a homogeneous subset of pre-mtLSUs that corresponded to ~3.5 % of the particles combined from five data sets. 896,263 particles were picked using Warp (Tegunov and Cramer 2019), and further processed using RELION (Kimanius et al 2016; Zivanov et al 2018). We performed reference-based 3D classification with references generated from a preliminary classification of a screening data set. This resulted in 207,788 particles corresponding to the mtLSU shape but distinct from that of a mature mtLSU of which we found 152,816 particles. Refinement of pose assignments and subsequent classification using fine-angular searches with a solvent mask identified 32,339 pre-mtLSU particles (AppendixFig S1). To improve the angles further, the particles were subjected to masked auto-refinement. Following the CTF refinement, we obtained a reconstruction of a pre-mtLSU that likely reflects a stable premature complex. This was evidenced by the presence of densities corresponding to conserved ribosomal assembly factors. The cryo-EM reconstruction was refined to 3.50 Å resolution. This allowed us to build a ~2.2 MDa model and assign 16 assembly factors, as well as additional mitoribosomal proteins, directly from the density (Fig 1, Fig 2). Five distinct features define the overall pre-mtLSU: 1) the rRNA domain V is well resolved and covered by newly identified mitochondria-specific assembly factors; 2) the subunit interface consists almost entirely of newly identified assembly factors and two conserved GTPases; 3) the proteinaceous CP is absent; 4) the L7/L12 stalk proteins are missing, and its rRNA platform is not folded, instead assembly factors occupy similar positions; 5) the L1 stalk is shifted inward ~30 Å and linked to the CP base by assembly
factors. Due to these features, compositional and conformational changes are required for the maturation of the pre-mtLSU. In terms of the mitoribosomal proteins, 18 previously identified proteins are missing from the structure of the pre-mtLSU. Seven of these have bacterial homologs (uL10m, uL12m, uL16m, bL27m, bL31m, bL33m and bL36m) and the rest are mitochondria specific (Fig 1, Appendix Fig S2, Appendix Fig S3). Additionally, we assigned sequences to two previously unidentified mtLSU proteins, which we named mL109 and mL110, and observed the presence of mitoribosomal protein uL14m which was not modeled in the mtLSU (Fig 2).

Following the previously identified mitoribosomal small subunit assembly factors (Saurer et al 2019), we adopt a similar nomenclature for the mitochondria specific large subunit factors. Therefore, we reference them as T. brucei Large subunit Assembly Factor(s) (TbLAF1-11), while proteins with bacterial homologs are referred to as their bacterial names with the prefix “mt-“. The identified assembly factors of the mitoribosome include two homologs of bacterial GTPase assembly factors mt-EngA and mt-RbgA, a homolog of the ribosome silencing factor mt-RsfS, a DEAD-box helicase (TbLAF10), two pseudouridinases (TbLAF4 and TbLAF6), a methyltransferase (TbLAF5), two copies of the mitochondrial acyl-carrier protein (mt-ACP), two LYR-motif-containing proteins (TbLAF1, TbLAF11), and other proteins with previously unassigned functions (TbLAF2, 3, 7, 8 and 9). In the model, we included only the parts for which clear secondary structure is apparent, and other regions with only partial density visible were modeled as UNK1-11 (Supplementary file 2).

**GTPase mt-RbgA is structurally linked to the mitoribosomal core via specific assembly linkers**

We started the structural analysis by searching for similar assembly intermediate architectures in bacterial counterparts. Particles with an absent CP were reported previously in RbgA-depleted Bacillus subtilis cells. RbgA was then added in vitro and shown to bind to the complex, which identified its role as an assembly factor (Seffouh et al 2019). RbgA is from the Ras GTPase family containing a typically low intrinsic GTPase activity, which is increased in the presence of a mature LSU subunit (Achila et al 2012). It has N-terminal GTPase domain and C-terminal helical domain, which forms a five-helix bundle (Pausch et al 2018). In the pre-mtLSU structure, we found a conserved mt-RbgA homolog. Studies in yeasts reported that deletion of mt-RbgA (yeast Mtg1) results in respiration deficiency (Barrientos et al 2003). In B. subtilis, where this assembly factor is essential, the LSU:RbgA complex showed that the N-terminal domain overlaps with rRNA H69 and H71, and the C-terminal helical domain interacts with H62 and H64 (Seffouh et al 2019). In this position, RbgA displaces the P-site and interacts with the surrounding rRNA, including H92 and H93. Therefore, the binding of RbgA requires contacts with rRNA.

In our map of the T. brucei pre-mtLSU, the corresponding regions forming the rRNA binding site for mt-RbgA are not observed, and its nucleotilde binding site empty (Fig 3b). However, the
comparison of our structure with the *B. subtilis* LSU:RbgA complex (PDB ID 6PPK) shows
conserved position of the catalytic site and nearly identical conformation of the factor on the pre-
mtLSU complex (Fig EV1). This includes the weak interaction between the mt-RbgA C-terminal
domain and the mitoribosomal protein uL14m is conserved (Fig 3).

We found that the conserved position of mt-RbgA in *T. brucei* is maintained through two
specialized assembly linkers (Fig 3). The first linker is established between the C-terminal
domain and the TbLAF5 N-terminal helix. The latter adopts a crescent shape around the C-
terminal domain of mt-RbgA, forming a series of contacts with four out of its five helices (Fig
3). The second linker is provided by TbLAF4 approaching from the mitoribosomal core. It
interacts with the mt-RbgA C-terminal domain and contributes a β-strand to a shared β-sheet
(Fig EV2). Therefore, RbgA is anchored to the flexible core via two dedicated factors that
compensate for the lack of rRNA contacts.

TbLAF4 belongs to a family of site-specific RluD pseudouridine synthases which are involved in
the bacterial LSU assembly and responsible for creation of pseudouridines at positions 1911,
1915 and 1917 (*E. coli* numbering) in the H69 end-loop (Gutgsell et al 2001; Gutgsell et al
2005). In pre-mtLSU structure, TbLAF4 encircles the immature rRNA nucleotides U1017-
U1021 and G1073-U1087 (Fig 4), and its active site is occupied by uridine 1017 likely present in
H89-93 of the mature rRNA (Fig EV2B). The N-terminal domain is positioned at the distance of
~80 Å facing towards the L7/L12 stalk. This connection plays a key role in coordinating the
factors between the different functional sites (Fig 5A).

TbLAF5 belongs to the family of SpoU RNA methyltransferases, but appears to have a closed
active site that does not allow the binding of its typical S-adenosyl methionine cofactor (Fig
EV2A). It is located peripherally, and bound to the mitoribosome via a C-terminal 24-residue
helix interacting with rRNA H41/42, and via contacts with TbLAF8 (Fig EV2).

Together, TbLAF4 and TbLAF5/TbLAF8 perform a structural scaffolding role for binding mt-
RbgA. A homology search of the assembly factors reveals that TbLAF4 and TbLAF5 are also
present in vertebrates, plants, fungi and ciliates (Fig 5C). Since their genomes encode mt-RgbA
as well, our data suggests that a cooperative action of the assembly factors might be conserved.

**GTPase mt-EngA is stabilized via protein extension**

In the subunit interface, we identified another conserved GTPase homolog, mt-EngA. It contains
two GTPase domains arranged in tandem as well as a C-terminal K homology (KH) domain
which is pointed towards the PTC. We could model two GTPs in the GTPase domains (Fig 3B).
Its positioning is identical to bacteria, suggesting functional conservation. The assembly factor
occupies the space between the PTC and the E-site (Fig EV1), and a role in chaperoning rRNA
has been proposed (Zhang et al 2012). The comparison with *E. coli* LSU:EngA complex reveals
conformational differences that highlight the nature of the mitochondrial protein-rich system, and
its role in the stabilization of the conserved assembly factors.
Firstly, the N-terminal GTPase domain is extended by 60 residues, with residues 101-108 stabilizing a helix-turn-helix motif (275-305), which remained unresolved in the bacterial complex (Fig EV1B). The N-terminal extension is generally present in mitochondria from other species (Fig EV3B, Appendix Figure S4). This motif is important for the stabilization of EngA, because one helix is stacked against a helix of TbLAF10, whereas the other forms a helical bundle with TbLAF11 (Fig EV3). TbLAF10 is one of the largest mitoribosomal assembly factors, which spans 110 Å through the rRNA core to the CP (Fig 3, Fig 5). It has an rRNA contact area of ~8400 Å and keeps the nucleotides U904-A917 as well as U953-A965 (putative H80-83) in a premature conformation (Fig 4).

Secondly, the N-terminal residues 72-75 of EngA stabilize a short helix (residues 367-374), which is buried within rRNA groove via Arg367 and Arg369 (Fig 3b). It disrupts the local structure of H75 and stabilizes the flipped nucleotide A894. This loop is also highly charged in the corresponding E.coli structure, but does not adopt the helical conformation observed here.

Finally, the N-terminus forms additional contacts with five mitoribosomal proteins (bL28m, bL35m, bL19m, mL64, mL74), a stabilizing protein mass that compensates for the missing rRNA in this region. Overall, while the N-terminal GTPase domain aligns well with the bacterial EngA, its interacting partners in our structure are proteinaceous and specific to mitochondria.

The conserved globular domains of mt-EngA are associated with the pre-mtLSU core via TbLAF9. Its three helices from the N-terminus encloses the N-terminal GTPase domain helix 230-242 (Fig EV3). Here, TbLAF9 replaces the missing rRNA H82, H88 and protein L1, which binds the EngA N-terminal GTPase domain in bacteria. TbLAF9 spans over 100 Å to the top of the CP, where it also stabilizes unwound rRNA (Fig 4, Fig 5). Thus, mt-EngA is stably bound via protein extension and also associated with the protein-based scaffold of assembly factors, including the high molecular weight TbLAF9 and TbLAF10, which are connected to the CP.

**The module mt-RbgA:mt-EngA coordinates maturation of interfacial rRNA**

The process of the LSU assembly is dynamic with cooperative action of different assembly factors (Davis et al 2016; Davis and Williamson 2017). Although RbgA and EngA have previously been visualized separately on the bacterial LSU though deletion and reconstitution experiments, our cryo-EM structure shows both factors simultaneously associated with the mt-LSU and with each other. The presence of both factors rationalizes why rRNA domain V is better resolved than in the mature mtLSU (Fig 4, Fig 6). We were able to model 33% more nucleotides relative to the mature LSU, thereby showing that the H89-93 region does not occupy the expected bacterial position and highlighting a need for prominent remodeling during maturation (Fig 6, Appendix Fig S5).

The contacts between mt-RbgA and mt-EngA are formed between the N-terminal domain and KH domains, respectively (Fig 3b). The shared surface area is ~540 Å, and each of the domains is also associated with conserved rRNA. The formed contacts between mt-RbgA and mt-EngA include electrostatic interactions, as well as hydrophobic residues (Fig 3b). Since the structures...
and positions of both factors are conserved with bacteria, and we identified homologs in representative eukaryotic species, these results indicate that the simultaneous binding and overall mode of interaction might be conserved in mitochondria of different organisms, including humans.

Progressive maturation of the L7/L12 stalk
The L7/L12 stalk is a universal mobile element that associates translational protein factors with the A-site. It typically consists of the rRNA platform that connects to the flexible factor-recruiting series of bL12m copies. The ubiquitous ribosomal proteins uL10m, uL11m and mitochondria-specific mL43 link the different components together. In our structure, most of the protein constituents of the stalk are missing and others adopted conformational changes (Fig 7A).

In the region of the platform, at least three proteins (uL16m, bL36m, mL88) associated with the rRNA in the mature mtLSU are absent. Consistently, the rRNA platform is not folded, as the folding relies on the missing mitoribosomal proteins. Instead, the N-terminal domain of TbLAF4 extends from the subunit interface to occupy part of the space left by uL16m absence (Fig 7A and B). It binds two specific assembly factors TbLAF2 and TbLAF7. TbLAF2 mediates further contacts with the core of the pre-mtLSU. TbLAF7 consists of 7-stranded beta-barrel, 12 α-helices, and 63-residue tail inserted into the mitoribosomal core. The structure suggests that both TbLAF2 and TbLAF7 need to dissociate for the missing mitoribosomal proteins to assemble (Fig 7A).

In the factor-recruiting region, instead of the terminal bL12m copies, TbLAF3 and density corresponding to UNK6 form a protrusion (Fig 7A). They comprise a protein continuum of at least 13 helices associated with each other. This assembly is the attached to the platform region through a 30-residue C-terminal tail of TbLAF3, forming a helical bundle with mL75 (Fig 7A, 7C). Overall, this protein module extends ~70 Å from the core in a similar fashion to the L7/L12 stalk, and both appear to be mutually exclusive.

The position of the uL10m N-terminus, which links the stalk to the body in the mature LSU, is occupied by TbLAF3 C-terminus. It interacts with mL43, resulting in its helix being bent by 90° (Fig 7B). This conformational change and the lack of uL10m is correlated with ~15 Å shift of uL11m to occupy the formed void (Fig 7B). Nevertheless, TbLAF3 is only peripherally associated with mL43, and it cannot be excluded that the protrusion is independently replaced by the mature L7/L12 stalk.

Together, our structure proposes a working model for the L7/L12 stalk maturation via five steps which include dismantling, remodeling and assembly (Fig EV5): 1) TbLAF4, which is extended from the subunit interface anchoring mt-RgbA, has to be removed; 2) TbLAF2:TbLAF7 is released from the ribosomal core; 3) rRNA platform is folded, and mitoribosomal proteins uL16m, bL36m, and mL88 are recruited to the rRNA platform; 4) TbLAF3:TbLAF5 is removed, and uL11m, mL43 adopt their mature conformations; 5) bL10m and bL12m stalk proteins are associated to form the functional L7/L12 stalk.
Below the L7/L12 stalk we identified three additional proteins: an ortholog of the bacterial ribosome silencing factor mt-Rsf, a LYT (leucine-tyrosine-arginine) motif containing protein TbLAF1, as well as an associated mt-ACP (Fig 1, Fig 5). The human and fungi mitochondrial counterparts of these three proteins have been shown to be involved in late assembly stages of the mitoribosome, preventing association of the mtSSU (Brown et al 2017; Itoh et al 2020). In our structure, the module is further stabilized by mL85, however it does not appear to obstruct the binding of the mature mtSSU, likely because of the reduced rRNA and different overall shape. This suggests that in order for the mtACP:TbLAF1 protein module to act according to the previously suggested mechanism, a complementary structural domain from pre-mtSSU would be required for a steric hindrance.

Assembly of the CP is linked to the subunit interface and L1 maturation via mt-ACP

The most prominent architectural feature of the pre-mtLSU complex is the absence of the CP. It is a universal ribosomal element that defines the shape of the LSU and forms bridges with the SSU head. In mitoribosomes, the CP is particularly protein-rich (Amunts et 2014; Greber et al 2014; Amunts et 2015; Greber et al 2015; Waltz et al 2020; Tobiasson and Amunts 2020). The acquisition of the CP mitochondria-specific proteins took place in the early evolution of the mitoribosome and therefore expected to be conserved (Petrov et al 2019).

In the pre-mtLSU, all the CP mitoribosomal proteins are missing and the high molecular weight assembly factors TbLAF6 (69 kDa) and TbLAF9 (67 kDa) are present (Fig 1, Fig 8A). TbLAF6 binds on the solvent side of the CP covering the otherwise exposed rRNA, which only engages in limited base pairing. This assembly factor is annotated as a putative TruD family pseudouridine synthase. However, in our structure, it displays a two-strand antiparallel β-sheet near the catalytic site protruding into the ribosomal core interacting with four mitoribosomal proteins (uL15m, bL28m, bL35m, mL74) (Fig 8B). TbLAF9 is an exclusively helical protein, comprised of at least 29 helices. It binds on top of the rRNA, providing an additional protective protein cap (Fig 8A).

Two of the TbLAF9 helices interface with a four-helix bundle, which we identified as mt-ACP (mt-ACP2) with a local resolution of 3.5 Å (Fig 2). Since mt-ACP proteins are known to form interactions with Leu-Tyr-Arg (LYR)-motif proteins, we compared the mt-ACP1-TbLAF1 module from the subunit interface with the CP mt-ACP2 region (Fig 8C). The helices of the LYR-motif protein TbLAF1 aligned well with a density corresponding to three helices associated with the mt-ACP2. The interactions in both cases are mediated by the 4′-phosphopantetheine (4′-PP) modification of mt-ACP, resembling the canonical interactions between mt-ACP and the LYR-motif proteins. The 4′-PP appears to be acylated as indicated by the density but the exact length of the acyl chain cannot be unambiguously determined from the density (Fig 8C). The presence of the 4′-PP modification, previous structural data (Zhu et al 2015; Fiedorczuk et al 2016; Brown et al 2017), and the overall shape of the associated density suggest that the interacting partner of mtACP2 is a protein from the LYR-motif family containing at least three helices. Therefore, we searched our current and previously published (Zikova et al 2008) mass
spectrometry data using ScanProsite (de Castro et al 2006). The hits were subjected to
secondary structure prediction and fitting to the density map. The analysis singled out the protein
Tb927.9.14050 (UniProt ID Q38D50), which we named consistently with the adopted
nomenclature TbLAF11. The local resolution of 3.5–4.0 Å in this region (Fig 2) allowed for 164
N-terminal residues to be built (Supplementary File 2), which includes the three helices
associated with the mACP2 in proximity to the L1 stalk, and two helices interacting with EngA.
The importance of the mt-ACP2:TbLAF11 protein module in our structure is of twofold. First, it
directly binds the L1 stalk and locks it in an inward facing nonfunctional conformation (Fig 1).
Second, it is involved in mt-EngA stabilization by forming a U-shaped continuum from TbLAF9
on the solvent side of the CP to the subunit interface (Fig 1, Fig 5). Therefore, it contributes to
the protein network connecting between the various functional sites. Our data suggests that mt-
ACP2 is a principal partner in the mitoribosomal assembly pathway. In the pre-mtSSU, mt-ACP
was also identified as one of the assembly factors (Saurer et al 2019). In addition, ACPs in
mitochondria act as subunits of the electron transport chain (Zhu et al 2015; Fiedorczuk et al
2016) and Fe-S cluster assembly complexes (Van Vranken et al 2016). This further supports the
proposed concept that mt-ACPs could be signaling molecules in an intramitochondrial metabolic
state sensing circuit (Masud et al 2019).
At the CP, the assembly factors cooperatively bind unwound rRNA nucleotides U934-U953
(H83) (Fig 5, Fig 8A). Remarkably, the rRNA forms a loop 25 Å in diameter that contains the
TbLAF6 β-sheet and mL64 C-terminal helix, both inserted from the opposite directions (Fig 8B).
The conserved helix of mL64 is shifted in our pre-mtLSU structure ~30 Å from the final location
on the mature LSU, where it aligns the E-site. Interestingly, this is also one of the most
conserved mitoribosomal proteins across species (Petrov et al 2019). To switch to the conserved
and mature position, the extended C-tail of mL64 has to liberate from the rRNA loop and then
undergo a large conformational shift towards the L1 stalk. Subsequently, the C-tail is inserted to
its mature position, where it contacts CP components absent from the assembly intermediate.
Since the L1 stalk is also shifted, the maturation towards a mature LSU is likely to occur in a
concerted manner upon the release of the mt-ACP2:TbLAF11 module.

**Conclusions**
Our structure of pre-mtLSU in complex with 16 assembly factors reveals that high molecular
weight assembly factors shield the rRNA and form a network that spans over 180 Å, which
connects the subunit interface with the progressive maturation of the L7/L12 stalk, L1 stalk, and
the assembly of the CP. The tight binding of the mt-ACP2 with partner proteins emphasizes its
role in forming the connections. The factors mt-RbgA and mt-EngA are held together by protein
linkers and interact with each other. The analysis shows that most of the assembly factors are
also found in mammals. Overall, the protein-based communication allows the formation of the
defined pre-mitoribosomal complex with newly identified factors that provides a conceptual
model how mtLSU can progress through the biogenesis pathway.
Figure 1. Structure of *T. brucei* pre-mtLSU with 16 assembly factors. Left, the overall modeled structure of the pre-mtLSU (rRNA blue) with models of assembly factors (shades of purple and white) covering the subunit interface, CP, L7/L12 stalk and connecting to the L1 stalk. Right, structure of the mature mtLSU (PDB ID 6HIX) with 18 additional mitoribosomal proteins (shades of orange) absent from pre-mtLSU.
Figure 2. Cryo-EM data quality. (A) Final map colored by local resolution. (B) Models for individual assembly factors and newly identified proteins colored by local resolution of the density in the corresponding regions.
Figure 3. Binding of the mt-RgbA and mt-EngA to the subunit interface. (A) mt-RgbA is bound to TbLAF4 and TbLAF5, which are connected to the L7/L12 stalk; mt-EngA is associated with TbLAF9 and TbLAF10, which are connected to the CP. (B) A short helix of mt-EngA (yellow) interacts with a flipped A894 nucleotide from H75 (white). Two GTPs in their binding sites on mt-EngA are shown as sticks. A superimposed GTP in its binding site on mt-RgbA is shown in as white sticks. The residues forming interactions between mt-EngA and mt-RbgA are shown in the top right inset. (C) Schematic representation of mt-EngA and mt-RbgA indicating the positions of the conserved GTP binding motifs.
Figure 4. Schematic representation of assembly factors’ binding to rRNA mapped on the secondary structure diagram. The rRNA regions contacting individual assembly factors are represented by different colors. Bound regions of at least 3 nucleotides are shown. For regions where more than one factor is bound, only a factor with higher local binding is shown. Unbound rRNA is white, unmodeled rRNA is grey.
Figure 5. Network of interactions between the assembly factors in pre-mtLSU. (A) Assembly factors shown on the background of the pre-mtLSU density map, featuring the interconnection. (B) Schematic of protein-protein network. The node size represents the relative molecular mass of the protein. All the assembly factors are interconnected with each other. (C) Homology search of the assembly factors. Colored squares indicate identified homologs/orthologs using *T. brucei* (green) or human (purple) assembly factor as queries. White squares indicate not-identified homologs/orthologs. The stars mark proteins, for which experimental data has been reported.
Figure 6. Tertiary structure of rRNA in pre-mtLSU (A) and mature mtLSU (B). Shown from the subunit interface (left) and sideview (right). Two views of rRNA related by 90° are shown with each domain in a different color. Domain V is more structured in pre-mtLSU, and H89-93 adopt a different conformation. Domain II that is responsible for L7/L12 stalk is largely disordered.
Figure 7. Assembly of the L7/L12 stalk. (A) In pre-mtLSU, TbLAF4 extends from the subunit interface to occupy the position of uL16m in the mature mtLSU. TbLAF2 and TbLAF7 are bound at the stalk base to the unfolded tRNA H41/42. TbLAF3 and additional protein form a protrusion similar to bL10m:bL12m. Other mitoribosomal protein removed for clarity. (B) Conformational changes from pre-mtLSU (green) to mature mtLSU (white) include mL43 and uL11m. (C) TbLAF3, mL75, and UNK protein form continuum of at least 13 helices that is peripherally associated. (D) Model for the L7/L12 stalk maturation.
Figure 8. The CP assembly intermediate. (A) TbLAF6 and TbLAF9 form the CP in the pre-mtLSU. (B) TbLAF6 and mL64 elements are inserted through rRNA loop corresponding to H83. Conformational change of mL64 from pre-mtLSU to mature mtLSU (white) is indicated. (C) Comparison between the mt-ACP1:TbLAF1 (left) and the CP mt-ACP2:TbLAF11 region (right). The density (white) for acylated 4’-PP is indicated. Bottom panel, comparison with mt-ACP and associated LYR-motif proteins from complex I (PDB ID 5LNK) and human mitoribosome (PDB ID 5OOM) shows the canonical interactions.
**Figure EV1.** The binding of mt-RbgA and mt-EngA at the mtLSU interface. (A) Comparison between pre-mtLSU and bacterial counterparts *E. coli* 50S:EngA (PDB ID 3J8G) and *B. subtilis* 45S:RbgA (PDB ID 6PPK) shows nearly identical positions of the factors on their ribosomal complexes. (B) Comparison between mt-RbgA:mt-EngA module from the pre-mtLSU and superimposed bacterial counterparts combined from the two structures from (A) shows nearly identical conformations of the factors. The N-terminal extension of mt-EngA (orange) is buried in the mitoribosomal core and stabilizes the binding, as well as 275-305 region (orange).
Figure EV2. (A) The active site of TbLAF4 (yellow) is occupied by uridine 1048. (B) The factor TbLAF4 (yellow) binds mt-RbgA (pale-yellow) via a shared β-sheet (circled). The methyltransferase site of TbLAF5 does not allow for the binding of S-adenosyl methionine cofactor (white sticks and surface) due to clashes with the protein residues (red).
Figure EV3. (A) N-terminal extension (yellow) of mt-EngA stabilizes helix-turn-helix (275-305), which forms interaction with TblAF10 on the other side (bottom right panel), and a helical bundle with TblAF11 that is in contact with TblAF9. (B) Sequence alignment of the N-terminus of mt-EngA shows presence of the extension in different organisms.
Figure EV4. Binding of assembly factors to rRNA. For each panel, rRNA is shown with an individual protein characterized in the structure, which have not been reported in the mature LSU. Bottom right panel illustrates the total RNA that is involved in the interactions (yellow) with the assembly factors. Regions and nucleotides of respective rRNA domains are also presented in Supplementary File 3.
Figure EV5. Proposed model for the L7/L12 stalk maturation. The series of steps starts with dismantling the assembly factors from the unfolded rRNA (white dashes) that triggers rRNA folding (white line), binding of the mitoribosomal proteins (grey) and conformational changes (arrows).
Materials and Methods

Strains and growth conditions

*T. brucei* procyclic Lister strain 427 was grown in SDM-80 medium supplemented with 10% fetal bovine serum. Mitochondria were isolated as described earlier Schneider (2007). 1.5x10^{11} cells were harvested, washed in 20 mM sodium phosphate buffer pH 7.9 with 150 mM NaCl and 20 mM glucose, resuspended in 1 mM Tris-HCl pH 8.0, 1 mM EDTA, and disrupted by 10 strokes in 40 ml Dounce homogenizer. The hypotonic lysis was stopped by immediate addition of 1/6 volume of 1.75 M sucrose. Crude mitochondria were pelleted (15 min at 16000 xg, 4°C), resuspended in 20 mM Tris-HCl pH 8.0, 250 mM sucrose, 2 mM MgCl₂, 0.3 mM CaCl₂ and treated with 5 μg/ml DNase I for 60 min on ice. DNase I treatment was stopped by addition of one volume of the STE buffer (20 mM Tris-HCl pH 8.0, 250 mM sucrose, 2 mM EDTA) followed by centrifugation (15 min at 16000 xg, 4°C). The pellet was resuspended in 60% Percoll in STE and loaded on the bottom of six 10-35% Percoll gradient in STE in polycarbonate tubes for SW28 rotor (Beckman). Gradients were centrifuged for 1 hour at 24000 rpm, 4°C. The middle-diffused phase containing mitochondrial vesicles (15-20 ml per tube) was collected, washed twice in the STE buffer, snap-frozen in liquid nitrogen and stored at -80°C.

Purification of mitoribosomes

Mitochondria were further purified further using a stepped sucrose gradient (60 %, 32 %, 23 %, 15%) in a low ionic strength buffer (50 mM HEPES/KOH pH 7.5, 5 mM MgOAc, 2 mM EDTA). A thick pellet at the 60-32% interface was collected and lysed by mixing with 5 volumes of detergent containing lysis buffer (25 mM HEPES/KOH pH 7.5, 100 mM KCl, 15 mM MgOAc, 1.7 % Triton X-100, 2 mM DTT, Complete-EDTA Free Protease Inhibitor). The lysate was centrifuged at 30,000 xg twice, saving the supernatant after each spin. The supernatant was then subjected to differential PEG precipitation; PEG 10,000 was added to reach a concentration of 1.5 % (w/v) and incubated on ice for 10 mins, followed by a spin at 30,000 xg. The supernatant was transferred to a fresh tube, and PEG 10,000 was added to reach a concentration of 8 % (w/v) then incubated on ice for 10 mins, followed by a spin at 30,000 xg. The pellet was then resuspended in 800 µl of lysis buffer and then layered onto a 34% sucrose cushion (25 mM HEPES/KOH pH 7.5, 100 mM KCl, 15 mM MgOAc, 1.0 % Triton X-100, 2 mM DTT, Complete-EDTA Free Protease Inhibitor) in a TLA120.2 centrifuge tube (0.4 ml of cushion per tube). Mitoribosomes were pelleted through the cushion by centrifugation at 231,550 xg for 45 min. Pelleted mitoribosomes were resuspended using a total of 100 µl of resuspension buffer (25 mM HEPES/KOH pH 7.5, 100 mM KCl, 15 mM MgOAc, 0.01 % β-DDM, 2 mM DTT). The resuspended mitoribosomes were then layered onto a continuous 15-30 % sucrose gradient and centrifuged in a TLS55 rotor for 120 min at 213,626 xg. The gradient was fractionated manually, and fractions containing mitoribosome as judged by the 260 nm absorbance were pooled and buffer exchanged in a centrifugal concentrator.

Cryo-EM and model building
For cryo-EM analysis, 3 μL of the sample at a concentration of OD260 3.5, was applied onto a glow-discharged (20 mA for 30 seconds) holey-carbon grid (Quantifoil R2/2, copper, mesh 300) coated with continuous carbon (of ~3 nm thickness) and incubated for 30 seconds in a controlled environment of 100% humidity and 4 ºC temperature. The grids were blotted for 3 seconds, followed by plunge-freezing in liquid ethane, using a Vitrobot MKIV (FEI/Thermofischer). The data was collected on a FEI Titan Krios (FEI/Thermofischer; Scilifelab, Stockholm, Sweden, and ESRF, Grenoble, France) transmission electron microscope operated at 300 keV, using C2 aperture of 70 μm; slit width of 20 eV on a GIF quantum energy filter (Gatan). A K2 Summit detector (Gatan) was used to collect images at a pixel size of 1.05 Å (magnification of 130,000X) with a dose of ~35 electrons/Å2 fractionated over 20 frames. A defocus range of 0.8 to 3.5 μm was applied.

19,158 micrographs (after bad images were removed based on real and reciprocal space features) were collected across 5 non-consecutive data acquisition sessions and processed together using RELION. 896,263 particles were picked using Warp and coordinates were imported into RELION for particle extraction at an initial binning factor of two. The particles were subjected to supervised 3D classification using references generated previously in a screening dataset, which was started based on the T. brucei cytosolic ribosome as an initial model. This crude separation classified the 207,788 particles as mtLSU-like, and the remaining as mature mtLSU-like, SSU-like or monosomes. This subset was subjected to auto-refinement separately to improve the angular assignments and then classified further using fine-angular searches with a solvent mask applied. From the mtLSU-like particles, 32,339 particles were retained as pre-mtLSU of good quality and the rest were discarded as non-particles. The retained pre-mtLSUs were then subjected to auto-refinement once more to improve the angles further, this time applying a solvent mask during the refinement procedure, and then the 3D reconstructions obtained were used as a reference for CTF refinement to improve the reconstruction. The final map was then estimated for local resolution using RELION and sharpened with a B-factor appropriate for the reconstruction as estimated automatically using the postprocessing procedure.

Model building was done using Coot 0.9 (Emsley et al 2010). First the model of the mature mtLSU (PDB ID:6HIX) was fitted to the density. Chains present in the pre-mtLSU were then individually fitted and locally refined. Additional chains were first identified using information from sidechain densities. First the map density, chemical environment and sidechain interactions were used to create probable sequences. Those sequences were then queried against T. brucei specific databases; potential hits were evaluated individually and finally assigned. Models were modeled de-novo. All models were refined iteratively using PHENIX (Liebschner et al 2019) realspace refinement and validated using MolProbity (Williams et al 2018). The data collection, model refinement and validation statistics are presented in Supplementary data file 1. All figures were prepared either in Chimera (Pettersen et al 2004) or ChimeraX (Goddard et al 2018) with additional graphical elements created using Inkscape.

Search for homologs of assembly factors and sequence alignments
Homologs of assembly factors found in our pre-mtLSU and identified by cryo-EM were searched in the NCBI protein database with Position-Specific Iterated BLAST (Altschul et al 1997) using sequences of individual factors from *T. brucei* as queries. The searches were targeted against selected genera. Sequence alignments were generated with the MUSCLE (Larkin et al 2007) algorithm in Geneious (Biomatters Ltd., New Zealand) and corrected manually.

**Data availability**

The electron density map has been deposited in EMDB under accession code EMD-XXX. The model has been deposited in PDB under accession code XXXX. All data is available in the paper or Supplementary Information.

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**Author contributions**

Project conceptualization: OG, AZ, AA; Sample preparation for cryo-EM: OG, SA, AA; Data acquisition and processing: SA; Model building and validation: VT, OG, SA, RB; Structural data interpretation: VT, OG, AA; Manuscript writing and figure preparation: VT, OG, SA, RB, AZ, AA.

**References**

Achila, D., Gulati, M., Jain, N. & Britton, R. A. (2012) Biochemical characterization of ribosome assembly GTPase RbgA in Bacillus subtilis, *J Biol Chem.* 287, 8417-23

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research,* 25(17), 3389-3402

Amunts, A., Brown, A., Bai, X. C., Llacer, J. L., Hussain, T., Emsley, P., Long, F., Murshudov, G., Scheres, S. H. & Ramakrishnan, V. (2014) Structure of the yeast mitochondrial large ribosomal subunit, *Science.* 343, 1485-9
Amunts, A., Brown, A., Toots, J., Scheres, S. H. W. & Ramakrishnan, V. (2015) Ribosome. The structure of the human mitochondrial ribosome, *Science*. 348, 95-98

Antonicka, H. and Shoubridge, E.A., 2015. Mitochondrial RNA granules are centers for posttranscriptional RNA processing and ribosome biogenesis. *Cell reports*, 10(6), pp.920-932

Barrientos, A., Korr, D., Barwell, K. J., Sjulsen, C., Gajewski, C. D., Manfredi, G., Ackerman, S. & Tzagoloff, A. (2003) MTG1 codes for a conserved protein required for mitochondrial translation, *Mol Biol Cell*. 14, 2292-302

Bogenhagen, D.F., Martin, D.W. and Koller, A., 2014. Initial steps in RNA metabolism occur at mitochondrial DNA nucleoids. *Cell metabolism*, 19(4), pp.618-629

Brown, A., Rathore, S., Kimanius, D., Aibara, S., Bai, X. C., Rorbach, J., Amunts, A. & Ramakrishnan, V. (2017) Structures of the human mitochondrial ribosome in native states of assembly, *Nat Struct Mol Biol*. 24, 866-9

Couvillion MT, Soto IC, Shipkovenksa G, Churchman LS., 2016. Synchronized mitochondrial and cytosolic translation programs. *Nature*, 533(7604):499–503

Davis, J. H., Tan, Y. Z., Carragher, B., Potter, C. S., Lyumkis, D. & Williamson, J. R. (2016) Modular Assembly of the Bacterial Large Ribosomal Subunit, *Cell*. 167, 1610-1622 e15

Davis, J. H. & Williamson, J. R. (2017) Structure and dynamics of bacterial ribosome biogenesis, *Philos Trans R Soc Lond B Biol Sci*. 372

De Casto, E., Sigrist, C. J., Gattiker, A., Bulliard, V., Langendijk-Genevaux, P. S., Gasteiger, E., ... & Hulo, N. (2006). ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic acids research*, 34(suppl_2), W362-W365

De Silva, D., Tu, Y.T., Amunts, A., Fontanesi, F. and Barrientos, A., 2015. Mitochondrial ribosome assembly in health and disease. *Cell Cycle*, 14(14), pp.2226-2250

Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr*. 66, 486–501

Fiedorczuk, K., Letts, J. A., Degliesposti, G., Kaszuba, K., Skehel, M., & Sazanov, L. A. (2016). Atomic structure of the entire mammalian mitochondrial complex I. *Nature*, 538(7625), 406-410

Greber, B. J., Boehringer, D., Leibundgut, M., Bieri, P., Leitner, A., Schmitz, N., Aebersold, R. & Ban, N. (2014) The complete structure of the large subunit of the mammalian mitochondrial ribosome, *Nature*. 515, 283-6

Greber, B. J., Bieri, P., Leibundgut, M., Leitner, A., Aebersold, R., Boehringer, D. & Ban, N. (2015) Ribosome. The complete structure of the 55S mammalian mitochondrial ribosome, *Science*. 348, 303-8

Greber, B. J. & Ban, N. (2016) Structure and Function of the Mitochondrial Ribosome, *Annu Rev Biochem*. 85, 103-32.

Goddard, T.D., Huang, C.C., Meng, E.C., Pettersen, E.F., Couch, G.S., Morris, J.H. and Ferrin, T.E. (2018) UCSF ChimeraX: Meeting modern challenges in visualization and analysis. *Protein Science* 27, 14–25.
Gutgsell, N. S., Deutscher, M. P. & Ofengand, J. (2005) The pseudouridine synthase RluD is required for normal ribosome assembly and function in Escherichia coli, *RNA*. 11, 1141-52.

Gutgsell, N. S., Del Campo, M., Raychaudhuri, S. & Ofengand, J. (2001) A second function for pseudouridine synthases: A point mutant of RluD unable to form pseudouridines 1911, 1915, and 1917 in Escherichia coli 23S ribosomal RNA restores normal growth to an RluD-minus strain, *RNA*. 7, 990-8.

Jomaa, A., Jain, N., Davis, J. H., Williamson, J. R., Britton, R. A. & Ortega, J. (2014) Functional domains of the 50S subunit mature late in the assembly process, *Nucleic Acids Res.* 42, 3419-35

Kimanius, D., Forsberg, B. O., Scheres, S. H. & Lindahl, E. (2016) Accelerated cryo-EM structure determination with parallelisation using GPUs in RELION-2, *Elife*. 5

Itoh, Y., Naschberger, A., Mortezaei, N., Herrmann, J. & Amunts, A. (2020) Analysis of translating mitoribosome reveals functional characteristics of protein synthesis in mitochondria of fungi, *bioRxiv* 2020.01.31.929331

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., ... & Thompson, J. D. (2007). Clustal W and Clustal X version 2.0. *bioinformatics*, 23(21), 2947-2948

Ni, X., Davis, J. H., Jain, N., Razi, A., Benlekbir, S., McArthur, A. G., Rubinstein, J. L., Britton, R. A., Williamson, J. R. & Ortega, J. (2016) YphC and YsxC GTPases assist the maturation of the central protuberance, GTPase associated region and functional core of the 50S ribosomal subunit, *Nucleic Acids Res*. 44, 7073-83

Nikolay, R., Hilal, T., Qin, B., Mielke, T., Burger, J., Loerke, J., Textoris-Taube, K., Nierhaus, K. H. & Spahn, C. M. T. (2018) Structural Visualization of the Formation and Activation of the 50S Ribosomal Subunit during In Vitro Reconstitution, *Mol Cell*. 70, 881-893 e3

Ott, M., Amunts, A., & Brown, A., 2016. Organization and regulation of mitochondrial protein synthesis. *Annual Review of Biochemistry*, 85, 77-101.

Pausch, P., Steinchen, W., Wieland, M., Klaus, T., Freibert, S. A., Altegoer, F., Wilson, D. N. & Bange, G. (2018) Structural basis for (p)ppGpp-mediated inhibition of the GTPase RbgA, *J Biol Chem*. 293, 19699-19709

Pearce, S. F., Rebelo-Guimaraes, P., D’Souza, A. R., Powell, C. A., Van Haute, L., & Minczuk, M. (2017). Regulation of mammalian mitochondrial gene expression: recent advances. *Trends in biochemical sciences*, 42(8), 625-639.
Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C. and Ferrin, T.E. (2004) UCSF Chimera – a visualization system for exploratory research and analysis. Journal of Computational Chemistry 25, 1605–1612

Petrov, A. S., Wood, E. C., Bernier, C. R., Norris, A. M., Brown, A. & Amunts, A. (2019) Structural Patching Fosters Divergence of Mitochondrial Ribosomes, Mol Biol Evol. 36, 207-219

Ramrath, D. J. F., Niemann, M., Leibundgut, M., Bieri, P., Prange, C., Horn, E. K., Leitner, A., Boehringer, D., Schneider, A. & Ban, N. (2018) Evolutionary shift toward protein-based architecture in trypanosomal mitochondrial ribosomes, Science. 362

Saurer, M., Ramrath, D. J. F., Niemann, M., Calderaro, S., Mattei, S., Sciola, A., Leitner, A., Bieri, P., Horn, E. K., Leibundgut, M., Boehringer, D., Schneider, A. & Ban, N. (2019) Mitoribosomal small subunit biogenesis in trypanosomes involves an extensive assembly machinery, Science. 365, 1144-1149.

Seffouh, A., Jain, N., Jahagirdar, D., Basu, K., Razi, A., Ni, X., Guarne, A., Britton, R. A. & Ortega, J. (2019) Structural consequences of the interaction of RbgA with a 50S ribosomal subunit assembly intermediate, Nucleic Acids Res. 47, 10414-10425

Schneider, A., Charriere, F., Pusnik, M. & Horn, E. K. (2007) Isolation of mitochondria from procyclic Trypanosoma brucei, Methods Mol Biol. 372, 67-80

Tegunov, D. & Cramer, P. (2019) Real-time cryo-electron microscopy data preprocessing with Warp, Nat Methods. 16, 1146-1152

Tobiasson, V. & Amunts, A. (2020) Ciliate mitoribosome illuminates evolutionary steps of mitochondrial translation, Elife. 9, e59264

Van Vranken, J.G., Jeong, M.Y., Wei, P., Chen, Y.C., Gygi, S.P., Winge, D.R. and Rutter, J. (2016). The mitochondrial acyl carrier protein (ACP) coordinates mitochondrial fatty acid synthesis with iron sulfur cluster biogenesis. Elife, 5, e17828.

Waltz, F., Soufari, H., Bochler, A., Giege, P. & Hashem, Y. (2020) Cryo-EM structure of the RNA-rich plant mitochondrial ribosome, Nat Plants. 6, 377-383

Williams, JS. et al. (2018) MolProbity: More and better reference data for improved all-atom structure validation. Protein Science 27, 293-315

Zikova, A., Panigrahi, A. K., Dalley, R. A., Acestor, N., Anupama, A., Ogata, Y., Myler, P. J. & Stuart, K. (2008) Trypanosoma brucei mitochondrial ribosomes: affinity purification and component identification by mass spectrometry, Mol Cell Proteomics. 7, 1286-96.

Zhang, X., Yan, K., Zhang, Y., Li, N., Ma, C., Li, Z., Zhang, Y., Feng, B., Liu, J., Sun, Y., Xu, Y., Lei, J. & Gao, N. (2014) Structural insights into the function of a unique tandem GTPase EngA in bacterial ribosome assembly, Nucleic Acids Res. 42, 13430-9

Zivanov, J., Nakane, T., Forsberg, B. O., Kimanusi, D., Hagen, W. J., Lindahl, E., Scheres, S. H. (2018) New tools for automated high-resolution cryo-EM structure determination in RELION-3, Elife 7

Zhu, J., King, M.S., Yu, M., Klipcan, L., Leslie, A.G. and Hirst, J. (2015) Structure of subcomplex Iδ of mammalian respiratory complex I leads to new supernumerary subunit assignments. Proceedings of the National Academy of Sciences, 112, 12087-12092
Appendix Figure S1. Cryo-EM data processing. (A) Representative micrograph. (B) Processing workflow. (C) Fourier shell correlation (FSC) curves. Resolution is estimated based on the 0.143 FSC cut-off criterion (red line).
Appendix Figure S2. Examples of densities and models for individual assembly factors and newly identified proteins colored by local resolution of the density in the corresponding regions.
Appendix Figure S3. Cryo-EM density map of the pre-mtLSU showing distribution of the assembly factors.
Appendix Figure S4. Sequence alignment of EngA homologs from representative bacterial and eukaryotic species. The yellow, green, blue and orange horizontal bars mark the N-terminal extension, GTPase domain (GD) 1, GD2, and the KH domain, respectively. The white asterisks and crosses mark side chains in *T. brucei* mt-RbgA that coordinate GTP and interact with mt-RbgA, respectively. The green, yellow, and red vertical bars above the alignment correspond to 100%, <100% and ≥30%, and <30% identities at the respective position.
Appendix Figure S5. Secondary structure rRNA diagram derived from the model and colored by domain. Unmodeled sections that appear in the mature mtLSU are shown in gray. Domains in Roman numerals.

Supplementary File 1. Cryo-EM data collection, refinement and validation statistics

| Consensus map |
|---------------|
| Data collection and processing |
| Magnification | 130000x |
| Voltage (kV)  | 300    |
| Electron exposure (e-/Å²)       | 35 |
|--------------------------------|----|
| Defocus range (µm)             | -0.8 ~ -3.5 |
| Pixel size (Å)                 | 1.05 |
| Symmetry imposed               | C1 |
| Initial particle images (no.)  | 896,263 |
| Final particle images (no.)    | 32,339 |
| Map resolution (Å)             | 3.50 |
| FSC threshold 0.143            |    |
| Map resolution range (Å)       | 3.0~10 |

**Refinement**

Map sharpening $B$ factor (Å²)  -70

Model composition

|                     |       |
|---------------------|-------|
| Non-hydrogen atoms  | 146831|
| Protein residues    | 17415 |
| Ligands             | 10    |

$B$ factors min/max/avg (Å²)

|       |       |       |       |
|-------|-------|-------|-------|
| Protein| 17/172/68 |
| Nucleotide| 22/281/57 |
| Ligand  | 35/194/61 |

R.m.s. deviations

|                     | 0.002 | 0.46  |
|---------------------|-------|-------|
| Bond lengths (Å)     |       |       |
| Bond angles (°)      |       |       |

Validation

|                     |       |
|---------------------|-------|
| MolProbity score    | 1.65  |
| Clashscore          | 6.7   |
| Poor rotamers (%)   | 0.35  |

Ramachandran plot

|                     |       |       |       |
|---------------------|-------|-------|-------|
| Favored (%)         | 95.6  |
| Allowed (%)         | 4.10  |
| Disallowed (%)      | 0.03  |
## Supplementary File 2. Summary of pre-mtLSU components

| Alias | Chain ID | TriTrypDB Gene ID (Lister strain 427) | TriTrypDB Gene ID (reference strain TREU927) | Uniprot ID (reference strain TREU927) | Full size | Modeled residues | Comment |
|-------|----------|--------------------------------------|---------------------------------------------|-------------------------------------|-----------|-----------------|---------|
| 12S rRNA | AA | rRNA | rRNA | N/A | 1176 | 1-205, 254-264, 270-356, 369-380, 404-413, 445-450, 456-534, 541-582, 591-594, 796-883, 887-967, 980-999, 1004-1008, 1012-1021, 1071-1090, 1095-1176 |
| uL3m | AE | Tb427.03.5610 | Tb927.3.5610 | Q580R4 | 473 | 38-265, 272-404 |
| uL4m | AF | Tb427tmp.02.3810 | Tb927.11.6000 | Q385G8 | 351 | 18-459 |
| bL9m | AI | Tb427.05.3410 | Tb927.5.3410 | Q57UC5 | 263 | 9-220 |
| uL11m | AK | Tb427.02.4740 | Tb927.2.4740 | N/A | 342 | 26-200, 207-235, 239-306 |
| uL13m | AN | Tb427.04.1070 | Tb927.4.1070 | Q580D5 | 202 | 10-180 |
| uL14m | XG | Tb427.04.930 | Tb927.4.930 | Q580C1 | 217 | 20-107, 114-189 |
| uL15m | AP | Tb427.05.3980 | Tb927.5.3980 | Q57U68 | 374 | 10-136, 150-322, 354-363 |
| bL17m | AR | Tb427.08.5860 | Tb927.8.5860 | Q57Y17 | 301 | 11-266 |
| bL19m | AT | Tb427.01.1210 | Tb927.1.1210 | Q4GZ98 | 144 | 2-139 |
| bL20m | AU | Tb427tmp.01.1930 | Tb927.11.10170 | Q383R2 | 213 | 10-140, 162-205 |
| bL21m | AV | Tb427.07.4140 | Tb927.7.4140 | Q57UP4 | 188 | 6-185 |
| uL22m | AW | Tb427.07.2760 | Tb927.7.2760 | Q57Y86 | 278 | 2-278 |
| uL23m | AX | Tb427tmp.03.0260 | Tb927.11.870 | Q387G3 | 246 | 64-228 |
| uL24m | AY | Tb427.03.1710 | Tb927.3.1710 | Q57ZE0 | 378 | 1-311, 318-340 |
| bL28m | A1 | Tb427.06.4040 | Tb927.6.4040 | Q586A2 | 241 | 10-226 |
| uL29m | A2 | Tb427tmp.160.5240 | Tb927.9.7170 | Q38EM7 | 471 | 9-233, 248-471 |
| uL30m | A3 | Tb427tmp.211.0230 | Tb927.9.8290 | Q38ED8 | 218 | 51-200 |
| bL32m | A5 | Tb427.04.2330 | Tb927.4.2330 | Q584F4 | 80 | 26-80 |
| bL35m | A8 | Tb427.10.1870 | Tb927.10.1870 | Q38C55 | 181 | 40-181 |
| mL41 | Ae | Tb427tmp.01.1600 | Tb927.11.9830 | Q383U6 | 197 | 47-161 |

**Comment:**
- 2Fe-2S cluster binding
- Built as UNK
| mL | Amino Acid | Accession | Accession | QID | Length (p) | Width (f) |
|----|------------|-----------|-----------|-----|-----------|-----------|
| mL42 | Af | Tb427tmp.01.1840 | Tb927.11.10080 | Q383S1 | 189 | 41-173 |
| mL43 | Ag | Tb427.04.4600 | Tb927.4.4600 | Q583E5 | 260 | 2-186 |
| mL49 | Al | Tb427.05.3110 | Tb927.5.3110 | Q57Z82 | 218 | 37-101, 114-218 |
| mL52 | Ao | Tb427tmp.02.2250 | Tb927.11.11630 | Q383B7 | 242 | 27-222 NAD binding |
| mL53 | Ap | Tb427.07.2990 | Tb927.7.2990 | N/A | 309 | 16-303 |
| mL63 | At | Tb427.07.7010 | Tb927.7.7010 | Q57XS1 | 154 | 10-154 |
| mL64 | Av | Tb427tmp.01.3500 | Tb927.11.11630 | Q383B7 | 242 | 27-222 |
| mL67 | BA | Tb427tmp.55.0016 | Tb927.11.1630 | Q386Z1 | 831 | 27-83, 130-328, 335-542, 562-824 |
| mL68 | BB | Tb427.10.600 | Tb927.10.600 | Q38CI0 | 541 | 62-258, 264-294, 304-341, 346-450 |
| mL70 | BD | Tb427.06.4200 | Tb927.6.4200 | Q586Y7 | 547 | 105-521 |
| mL71 | BE | Tb427.07.3460 | Tb927.7.3460 | Q57WG1 | 449 | 11-190, 228-448 |
| mL72 | BF | Tb427.06.3930 | Tb927.6.3930 | Q58SZ1 | 426 | 26-62, 118-421 |
| mL74 | BH | Tb427.10.7380 | Tb927.10.7380 | Q38AM5 | 349 | 89-314 |
| mL75 | BI | Tb427.10.380 | Tb927.10.380 | Q38CK0 | 342 | 20-342 |
| mL76 | BJ | Tb427tmp.01.2340 | Tb927.11.110570 | Q383M2 | 333 | 173-333 |
| mL77 | BK | Tb427.06.2480 | Tb927.6.2480 | Q584Q8 | 386 | 84-156, 188-233, 254-269, 280-386 |
| mL78 | BL | Tb427.10.11050 | Tb927.10.11050 | Q389N4 | 312 | 31-130, 141-197, 216-265, 281-306 |
| mL80 | BN | Tb427.06.1440 | Tb927.6.1440 | Q585A3 | 302 | 53-266 |
| mL81 | BO | Tb427tmp.02.3230 | Tb927.11.5530 | Q38SL5 | 262 | 36-193, 210-262 |
| mL83 | BQ | Tb427.07.3430 | Tb927.7.3430 | Q57WF8 | 231 | 16-200 |
| mL84 | BR | Tb427.06.4080 | Tb927.6.4080 | Q586A6 | 205 | 11-205 |
| mL85 | BS | Tb427tmp.160.2250 | Tb927.9.3640 | Q38FG8 | 198 | 20-163 |
| mL86 | BT | Tb427.05.4120 | Tb927.5.4120 | Q57Z37 | 191 | 10-176 |
| ml.87 | BU | Tb427tmp.01.0500 | Tb927.11.8040 | Q384L5 | 185 | 104-185 |
| ml.89 | BW | Tb427.03.820 | Tb927.3.820 | Q57WW5 | 188 | 2-188 |
| ml.90 | BX | Tb427.06.1700 | Tb927.6.1700 | Q585P1 | 190 | 61-100, 108-174 |
| ml.91 | BZ | Tb427tmp.01.1215 | Tb927.11.9450 | Q383Y4 | 190 | 2-190 |
| ml.92 | Ba | Tb427.10.11350 | Tb927.10.11350 | Q57WW9 | 148 | 64-146 |
| ml.93 | Bb | Tb427.10.11370 | Tb927.10.11370 | Q589K3 | 146 | 10-146 |
| ml.94 | Bc | Tb427.10.11370 | Tb927.10.11370 | Q589K3 | 146 | 10-146 |
| ml.95 | Bf | Tb427.10.13777 | Tb927.10.13777 | Q588M2 | 113 | 27-68, 75-112 |
| ml.96 | Bg | Tb427.02.2590 | Tb927.2.2590 | Q587H8 | 105 | 24-105 |
| ml.97 | Bh | N/A | Tb927.9.8905 | N/A | 92 | 2-91 |
| ml.98 | XR | Tb427.01.1390 | Tb927.1.1390 | Q4GZ80 | 245 | 22-203 |
| ml.99 | XS | Tb427tmp.01.1810 | Tb927.11.10050 | Q583S4 | 102 | 6-102 |
| mt-ACP1 | XD | Tb427.03.860 | Tb927.3.860 | Q57WW9 | 148 | 64-146 |
| mt-ACP2 |XE | Tb427.03.860 | Tb927.3.860 | Q57WW9 | 148 | 64-146 |
| mt-EngA | XL | Tb427.07.1640 | Tb927.7.1640 | Q57TZ4 | 576 | 45-504, 514-574 |
| mt-RbgA | XQ | Tb427tmp.211.0810 | Tb927.9.9150 | Q38E75 | 451 | 44-202,215-338 |
| mt-RsfS | XJ | Tb427.06.3420 | Tb927.6.3420 | Q584Y2 | 349 | 163-312 |
| TbLAF1 | XM | Tb427.07.4210 | Tb927.7.4210 | Q57UQ1 | 116 | 25-115 |
| TbLAF2 | XA | Tb427.10.15860 | Tb927.10.15860 | Q387S8 | 156 | 3-156 |
| TbLAF3 | XF | Tb427.04.4610 | Tb927.4.4610 | Q583E6 | 319 | 120-170, 194-245, 260-317 |
| TbLAF4 | XP | Tb427tmp.160.2000 | Tb927.9.3350 | Q38FJ3 | 406 | 35-405 |
| TbLAF5 | XO | Tb427tmp.211.3800 | Tb927.9.12850 | Q38DC9 | 586 | 78-344, 363-440, 453-505 |
| TbLAF6 | XC | Tb427tmp.02.3800 | Tb927.11.5990 | Q385G9 | 616 | 1-414, 444-616 |
| TbLAF7  | XH  | Tb427.05.2070 | Tb927.5.2070 | Q57ZS6 | 634 | 2-55, 86-113, 184-276, 300-336, 367-412, 471-577, 587-623 |
|---------|-----|--------------|--------------|--------|-----|----------------------------------------------------------|
| TbLAF8  | XN  | Tb427.08.3300 | Tb927.8.3300 | Q57YY3 | 691 | 58-101, 123-150, 200-668 |
| TbLAF9  | XI  | Tb427.05.3870 | Tb927.5.3870 | Q57U79 | 731 | 25-95, 156-203, 213-289, 319-647, 656-727 |
| TbLAF10 | XB  | Tb427tmp.52.0011 | Tb927.11.12930 | N/A     | 754 | 47-391, 443-711 | MgADP binding |
| TbLAF11 | XR  | Tb427tmp.211.4580 | Tb927.9.14050 | Q38D50 | 524 | 2-165 |
**Supplementary File 3. Contacts of assembly factors with rRNA.** Regions and nucleotides of respective rRNA domains are color-coded as in Fig 4 and Fig EV4.

| Assembly factor | Region              | Contacts with rRNA                                                                 |
|-----------------|---------------------|-----------------------------------------------------------------------------------|
| mt-EngA         | H74, H75, H88, H90-91 | 868-874, 891-898, 958-967, 994-999, 1004                                          |
| mt-RbgA         | H90-91              | 1021, 1071                                                                        |
| mt-RsfS         | H95                 | 1126-1132, 1138-1141                                                              |
| TblAF2          | H39, H43, H72, H74, H88, H93 | 132-135 347-351, 404, 821-829, 832-835, 860-862, 904, 965-677, 985-992, 1079-1082, 1119 |
| TblAF3          | H43                 | 411-412, 446                                                                      |
| TblAF4          | H39, H90-93         | 351-353, 857-859, 992-994, 1008, 1012-1021, 1071-1087, 1122-1124                  |
| TblAF5          | H43, H90, H95       | 409-413, 1020-1021, 1136-1137                                                     |
| TblAF6          | H13, H28, H37, H80-81, H88 | 65-66, 164-166, 313, 328, 908, 917-919, 935-939, 944-953                         |
| TblAF7          | H41, H43            | 407, 448-450                                                                      |
| TblAF8          | H95                 | 1131-1140                                                                        |
| TblAF9          | H80-81, H88         | 908-909, 915-916, 919-924, 930-940, 952-957, 961                                  |
| TblAF10         | H26, H32, H33, H35a, H37, H39, H51, H72, H73-75, H80-81, H93 | 127-139, 199-200, 270-276, 289-293, 306-316, 344-355, 497, 550, 555, 826-828, 847-848, 850-855, 860-862, 870, 903-922, 930-933, 947-980, 955-965, 983-992, 1085-1089 |