Trends in Parasitology

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Review

Mechanisms and players of mitoribosomal biogenesis revealed in trypanosomatids

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Translation in mitochondria is mediated by mitochondrial ribosomes, or mitoribosomes, complex ribonucleoprotein machines with dual genetic origin. Mitoribosomes in trypanosomatid parasites diverged markedly from their bacterial ancestors and other eukaryotic lineages in terms of protein composition, rRNA content, and overall architecture, yet their core functional elements remained conserved. Recent cryo-electron microscopy studies provided atomic models of trypanosomatid large and small mitoribosomal subunits and their precursors, making these parasites the organisms with the best-understood biogenesis of mitoribosomes. The structures revealed molecular mechanisms and players involved in the assembly of mitoribosomes not only in the parasites, but also in eukaryotes in general.

Highlights

Trypanosoma brucei and related parasites have divergent mitochondrial ribosomes with a low RNA content and an expanded proteome. Conserved functional cores of the mitoribosomes are cradled in robust protein shells.

Atomic models of several precursors of large and small trypanosomal mitoribosomal subunits have been obtained by cryo-electron microscopy (cryoEM).

Biogenesis of trypanosomal mitoribosomes includes a combination of conserved and lineage-specific mechanisms, and a network of numerous assembly factors.

Several conserved assembly factors were visualized for the first time in the precursors of the trypanosomatid small mitoribosomal subunit (mtSSU) and large mitoribosomal subunit (mtLSU), providing mechanistic insight into their roles.

Protein synthesis in mitochondria is catalyzed by machinery that diverged from a bacterial ancestor

Mitochondria are endosymbiotic organelles which retained vestigial genomes, encoding predominantly components of oxidative phosphorylation complexes. Synthesis of proteins encoded by mitochondrial genomes is catalyzed by mitochondrial ribosomes, or **mitoribosomes** (see Glossary), which evolved from the ribosomes of the alphaproteobacterial endosymbiont. Mitochondrial ribosomal RNA (rRNA) – and, in most species, also some **mitoribosomal proteins** (**mt-RPs**) – are expressed from mitochondrial genomes [1], while the remaining mt-RPs are found in nuclear genomes and are imported from the cytosol. The ribonucleoprotein nature and the dual genetic origin of mitoribosomes place specific demands on their assembly, a fundamental process that is poorly understood.

Recent advances in cryo-electron microscopy (cryoEM) techniques allowed determination of high-resolution structures of mitoribosomes from four eukaryotic supergroups (Obazoa: fungi [2,3] and mammals [4,5]; Archaeplastida: spinach [6] and *Chlamydomonas* [7]; SAR: *Tetrahymena* [8]; and Discoba, *Trypanosoma* [9] and *Leishmania* [10]). The structures revealed that mitoribosomes diverged in composition and architecture significantly not only from their bacterial ancestors but also between eukaryotic lineages (Figure 1). In many aspects, the most pronounced compositional and architectural deviations have been reported in trypanosomatid parasites. In addition to the structures of the small and large mitoribosomal subunits (**mtSSU** and **mtLSU**, respectively) from ***Trypanosoma brucei*** [9], *Trypanosoma cruzi* and *Leishmania* sp. [10], atomic models of three mtSSU [11,12] and two mtLSU [10,13,14] precursors have been obtained. Structural characterization of assembly intermediates is essential for an understanding of mitoribosomal biogenesis. The only mitoribosomal precursors with known structures outside the **trypanosomatids** are late human pre-mtLSU complexes [15–21] and a series of pre-mtSSU complexes from mammals [22]. Thus, trypanosomatids are currently one of two groups with the best-described mechanisms of mitoribosome assembly, providing an unparalleled opportunity to define principles of **mitoribosomal biogenesis** in general.

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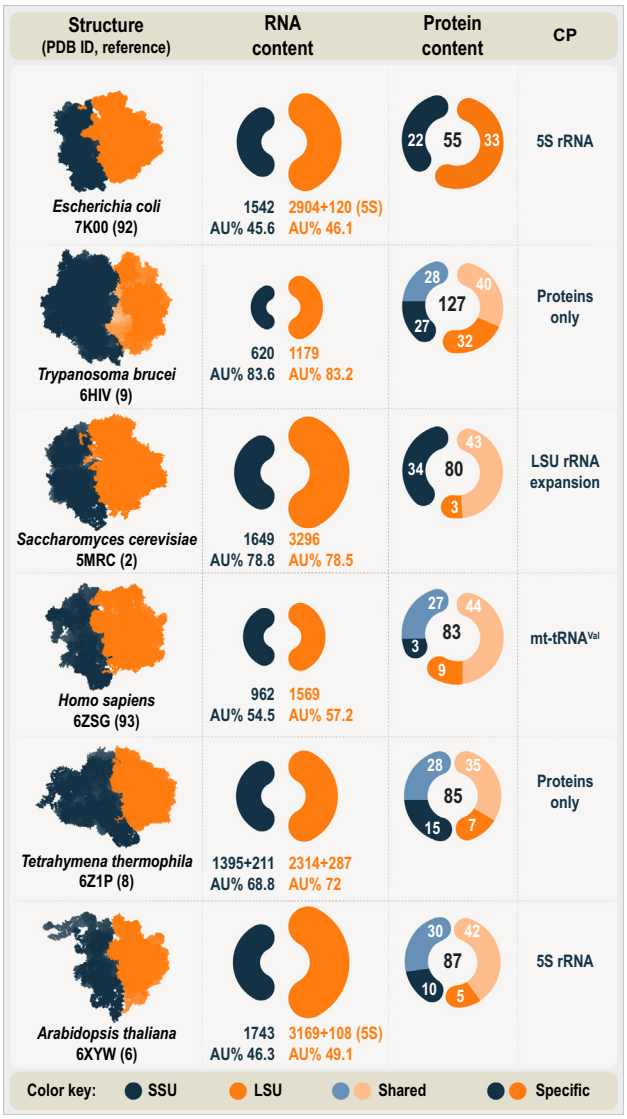

Figure 1. Architectural and compositional diversity of mitoribosomes. The shape, size, and the RNA and protein content of mitoribosomal small subunits (mtSSUs) and mitoribosomal large subunits (mtLSUs) from selected species representing eukaryotic groups with structurally characterized mitoribosomes are depicted in comparison with bacterial ribosomes. The size of individual rRNA molecules in nucleotides and their AU content (%) is shown. The numbers of ribosomal proteins shared with at least one other ribosome and specific to the organism are shown for SSUs and LSUs individually. The main component of the central protuberance (CP) is shown in the last column. See also [2,6,8,9,92,93].

Mitoribosomes of trypanosomatids are highly divergent and exhibit unique features

Early proteomic and electron microscopy characterization of *T. brucei* [23] and *Leishmania tarentolae* [24,25] mitoribosomal complexes, together with presence of short rRNA genes in mitochondrial genomes [26], indicated that the translation machineries in mitochondria of these organisms differ substantially from those of canonical ribosomes. Atypical structural features were also proposed to underlie insensitivity of *T. brucei* mitoribosomes to tetracycline [27]. However, only single-particle cryoEM analysis allowed us to uncover details of architectural divergence of mitoribosomes in trypanosomatids and define their exact composition [9]. The overall shape of trypanosomal mitoribosomes is largely different from that of any other ribosomes with known structure. The most striking feature is the size of the mtSSU, which is larger than the mtLSU. The mtSSU corresponds to the entity termed 45S SSU*, separated from other

Glossary

5S rRNA: a small RNA molecule forming the central protuberance in bacterial and eukaryotic cytosolic ribosomes, and in some mitoribosomes.
Assembly factor: a protein acting in the biogenesis of a macromolecular complex, but typically absent from its mature version. Here used in the context of mitochondrial or other ribosomes.
Central protuberance (CP): a prominent structural feature of the large ribosomal subunit involved in interaction with the small subunit.
Decoding center: the universally conserved site of codon–anticodon interaction between mRNA and tRNA on small ribosomal subunits.
Intersubunit side: the side of the large or small ribosomal subunit that faces the opposite subunit. Intersubunit sides contain the functional sites of ribosomes.
Kinetoplast: in Kinetoplastids, an eponymous structure of mitochondrial DNA which is composed of a concatenated network of tens of copies of identical maxicircles and thousands of minicircles. The former contain protein-coding and rRNA genes, and the latter encode gRNA.
Kinetoplast ribosomal PPR-repeat-containing proteins (KRIPPs): PPR proteins found to be associated with protein synthesis machinery in the mitochondria of Kinetoplastids.
L1 stalk: a conserved mobile structure of the LSU required for tRNA movement and release.
L7/12 stalk: a conserved mobile element of the LSU involved in delivery of initiation and elongation factors to the A-site of the PTC.
Last eukaryotic common ancestor (LECA): a hypothetical eukaryotic organism (or ancestral state) that gave rise to all extant eukaryotic lineages. LECA can be viewed also as a node on the phylogenetic tree, from which all extant eukaryotic lineages branched.
Mitochondrial acyl carrier protein (mt-ACP): a nuclear-encoded protein that has multiple roles in mitochondria, including the assembly of mitoribosomes.
Mitochondrial initiation factors (mt-IFs): protein factors required for translation initiation in mitochondria.
Mitoribosomal biogenesis: the assembly processes that result in the mature subunits of mitoribosomes.
Mitoribosomal proteins (mt-RPs): protein subunits of mature mitochondrial ribosomes.

mitoribosomal complexes in mitochondrial lysates of *T. brucei* and *L. tarentolae* by gradient centrifugation and characterized by mass spectrometry and electron microscopy, previously interpreted as an alternative mtSSU particle [24,28].

The entire trypanosomal mitoribosome is a 4.5 MDa complex comprised of at least 127 mt-RPs and two molecules of rRNA (9S and 12S rRNA of mtSSU and mtLSU, respectively) with an aggregated length of 1796 nt (Figure 2). Thus, compared to bacterial ribosomes and mitoribosomes from most other organisms, trypanosomatid mitoribosomes feature highly reduced RNA content and a markedly expanded repertoire of proteins (Figure 1). Trypanosomal rRNA is not only reduced but is also extremely AU-rich, which limits its base-pairing capacity (Figure 1). Consequently, many rRNA helices observed in canonical bacterial ribosomes and other mitoribosomes are either completely missing or replaced by regions with two parallel single strands, connected by only a few, or no, base pairs (Figure 2B,C). Despite the overall reduction and decreased content of double-stranded regions, rRNA elements that form the **decoding center** of mtSSU and **peptidyl transferase center** (**PTC)** of mtLSU, two key regions responsible for codon recognition and peptide bond synthesis, respectively, are structurally conserved. Thus, the functional centers do not differ from any other characterized ribosome, consistent with the assumption that they represent irreplaceable elements fundamental for protein synthesis. Conversely, universally present ribosomal substructures that display evolutionary plasticity in architecture are compositionally highly divergent in trypanosomatids. The most striking example is the **central protuberance** (**CP**) of mtLSU, a structure responsible for interaction with the small subunit and tRNAs. The main component of CP differs between mitoribosomes and their ancestors (Figure 1). The CP is based on **5S rRNA** in bacterial ribosomes [29] and mitoribosomes of plants [6] and several other eukaryotic groups [30], expanded mtLSU rRNA in yeast mitoribosomes [3,31], or a repurposed mitochondrial tRNA (mt-tRNA) in mammalian mitoribosomes [32]. However, the CP in trypanosomatids lacks any rRNA element and is purely proteinaceous. Similarly, the base of the **L1 stalk** of mtLSU, involved in conformational changes associated with E-site mt-tRNA binding and release, is RNA-free in trypanosomes [9].

The structure of the *T. brucei* mitoribosome revealed that the **polypeptide tunnel**, a path for nascent polypeptides from the PTC to the surface of mtLSU, is elongated, constricted, and branched due to the presence of trypanosomatid-specific mt-RPs. The branching results in two exits from the tunnel. Conceivably, the canonical exit might be used by translation products that require cotranslational insertion into the membrane, and the alternative exit by matrix proteins [9]. However, in *L. tarentolae* five mt-RPs causing the branching (mL67, mL71, mL77, mL78, and mL81, Figure 2A) were found only in a precursor of mtLSU, but not in the mature mtLSU or complete mitoribosome. In addition, the narrowing of the tunnel was proposed to be incompatible with the passage of nascent polypeptides. Therefore, the five proteins were claimed to be **assembly factors**, and the branching of the tunnel a feature which is not retained in the mature mtLSU of trypanosomatids [10]. However, the cryoEM map of the entire *T. brucei* mitoribosome contains a density corresponding to the five proteins (EMD-0229; [9]), indicating that they remain associated with mtLSU at least until translation is initiated in this species. Further investigation is needed to resolve whether these proteins are mt-RPs or assembly factors released prior the beginning of the elongation process, and consequently whether the polypeptide tunnel features two exits or just one.

Extraordinary complexity of the trypanosomal mitoribosomal proteome

In consequence of high selection pressure on the conservation of the central rRNA elements and associated mt-RPs, and rather divergent evolution of surrounding and peripheral regions, the conserved cores of both trypanosomatid mitoribosomal subunits are molded in partially

Mitoribosome: a ribosome in mitochondria that is responsible for the synthesis of proteins encoded by the mitochondrial genome.
mtLSU: the large mitoribosomal subunit; it contains the peptidyl transferase center and polypeptide tunnel, and it catalyzes the synthesis of peptides bonds.
mtSSU: the small mitoribosomal subunit; it binds mRNA and contains the decoding center.
mtSSU assemblosome: the earliest and the biggest characterized mtSSU assembly intermediate.
Pentatricopeptide repeat (PPR) motif: a 35-amino-acid sequence motif that forms a helix-turn-helix; it is typically involved in the binding of single-stranded RNA.
Peptidyl transferase center (PTC): a universally conserved site on the LSU that catalyzes peptide bond synthesis.
Polypeptide tunnel: a channel connecting the PTC with the surface of the LSU; it serves as a path for nascent polypeptides.
Procyclic form: a life cycle stage of *Trypanosoma brucei* with trypomastigote morphology that resides in the tsetse fly midgut. This form can be easily cultivated *in vitro*.
Trypanosomatids: a group of exclusively parasitic flagellated protists from the eukaryotic supergroup Discoba.
***Trypanosoma brucei*:** the best-studied representative of trypanosomatids; it causes sleeping sickness.

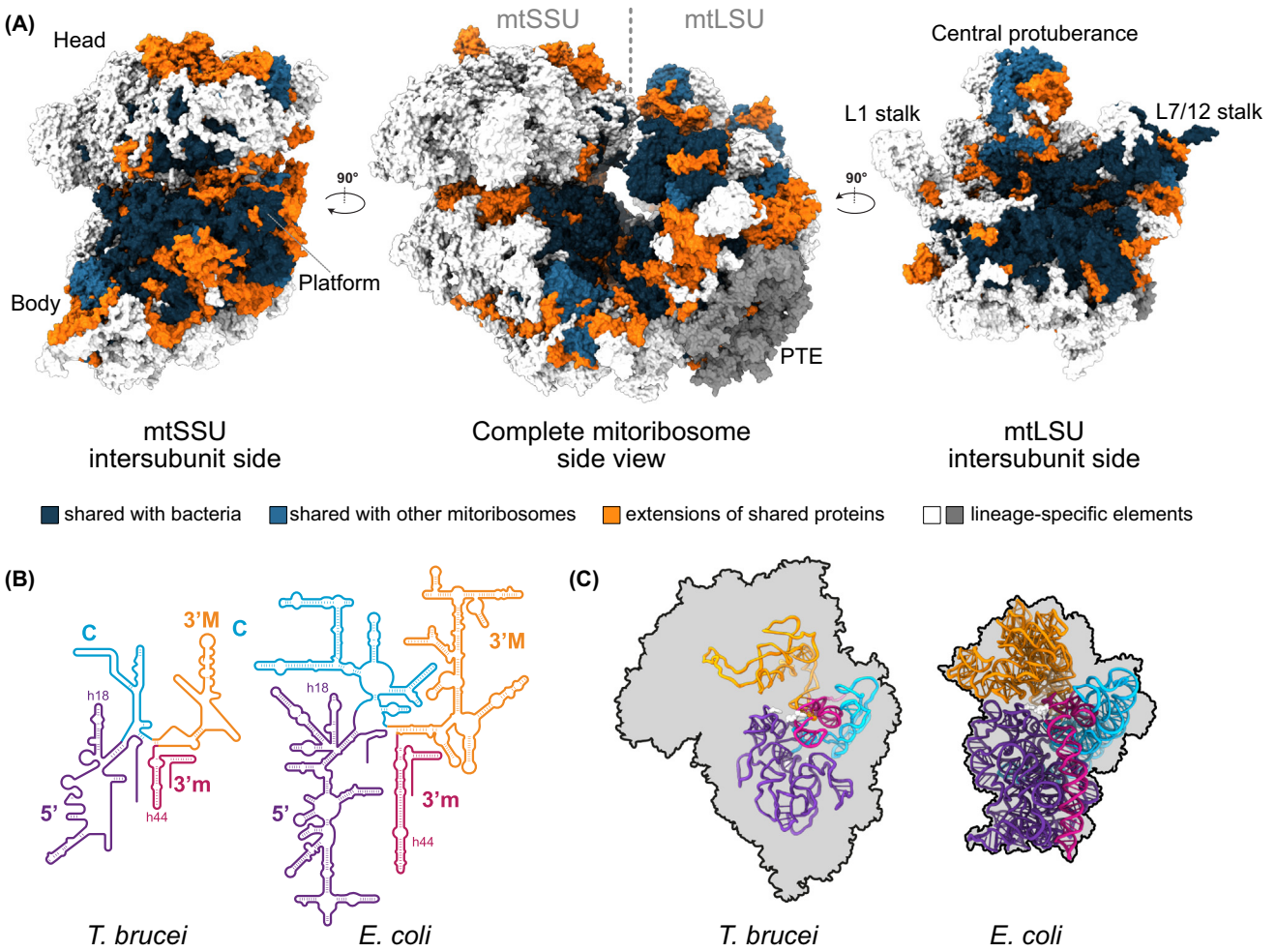

Figure 2. Overview of the structure of mitoribosomes in *Trypanosoma*. (A) Surface representation of atomic model of *Trypanosoma brucei* ribosomes showing components colored according to their conservancy. Regions colored in white, gray, and orange are lineage-specific. The gray region corresponds to five proteins around polypeptide tunnel exit (PTE), absent in the mature form of the mitoribosome large subunit (mtLSU) from *Leishmania tarentolae*. (B,C) Secondary structures (B) and 3D structures of rRNA viewed from intersubunit sides (C) of bacterial small subunit (SSU) and mitoribosomal small subunit (mtSSU) rRNA from *T. brucei*. Domains of rRNA (5′,5′ domain; C, central domain; 3′M, 3′major domain; 3′m, 3′minor domain) are colored individually, and base pairs are indicated. The white balls in the panel (C) represent key conserved decoding nucleotides in helices h18 and h44. *E. coli, Escherichia coli.*

autonomous outer shells (Figure 2A). The shells are formed by lineage-specific mt-RPs and lineage-specific extensions of conserved mt-RPs. *T. brucei* mtSSU and mtLSU contain 15 and 27 mt-RPs, respectively, with homologs in bacterial ribosomes, and an additional 13 and 15 mt-RPs shared with other structurally characterized eukaryotic mitoribosomes. Identification of some trypanosomal mt-RPs as homologs of conserved ribosomal proteins was possible only based on their structural similarity (Figure 3B) and positional context in the complex. The remaining 27 mtSSU and 30 mtLSU mt-RPs have not been identified outside the eukaryotic supergroup Discoba [9,33]. A substantial number of the lineage-specific mt-RPs replace missing rRNA secondary structure elements or stabilize the parts of rRNA, which lost ability to base-pair. Some of the lineage-specific mt-RPs are homologous to enzymes or other proteins with function unrelated to protein synthesis, such as proline isomerases mL83 and mL91, sulfur transferases mS67 and mL93, AP1 clathrin adaptor mL67, or A-kinase anchoring protein mS64. However, active sites or

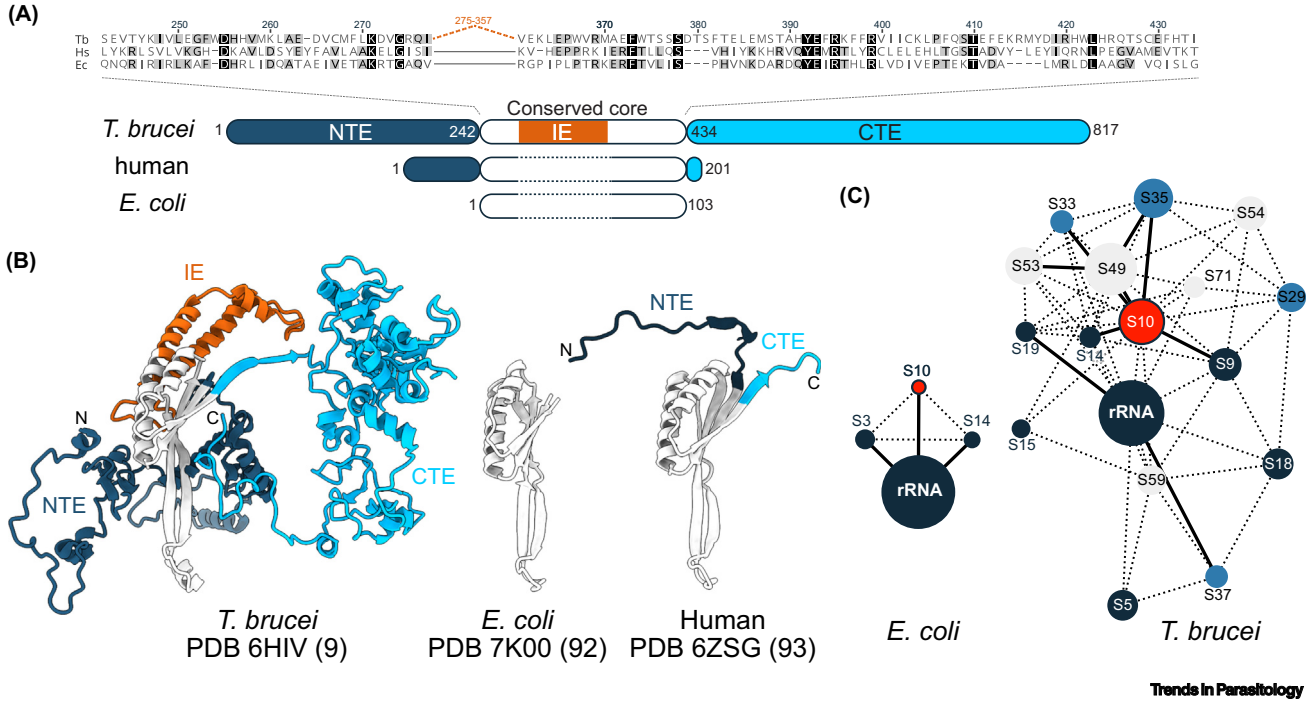

Figure 3. Conserved ribosomal protein uS10m as an example of structure-based identification, massive expansion, and intricate interaction network of trypanosomal mitoribosomal proteins (mt-RPs). (A) Schematic depiction of *Trypanosoma brucei* and human uS10m and uS10 from *Escherichia coli*, and sequence alignment of the conserved core. NTE, N-terminal extension; IE, internal extension; CTE, C-terminal extension. (B) Structures of proteins shown in (A). (C) Interaction of uS10 in *E. coli* and mitochondria of *T. brucei*. The sizes of nodes are proportional to the MW of individual components. Universally conserved elements are dark blue, mt-RPs shared with other mitoribosomes are pale blue, and lineage specific mt-RPs are gray. See also [9,92,93].

critical residues of these proteins are degenerated, documenting frequent repurposing for architectural roles in the mitoribosome.

Six trypanosomal mt-RPs belong to **pentatricopeptide repeat** (**PPR**) **motif** proteins; they are especially abundant in organelles of plants [34], and their primary function is related to RNA binding and metabolism [35]. The genome of *T. brucei* contains at least 39 PPR proteins [36]. Several PPR motif proteins in trypanosomes were previously shown to be involved in the mitochondrial protein synthesis apparatus [36–38], and were thus termed **kinetoplast ribosomal PPR-repeat-containing proteins** (**KRIPPs**) [36]. Contrary to the conventional function of PPR proteins in RNA binding, all six KRIPPs found in mature mitoribosomes, and four of five additional KRIPPs identified in an mtSSU precursor [11], contact rRNA only marginally or not at all. Instead, they localize to the proteinaceous outer shell. Together with other helical repeat proteins, also predominantly occurring in the shell, KRIPPs contribute to unusually high abundance of α-helices in trypanosomal mitoribosomes [9].

Several features of trypanosomal mitoribosomes can be illustrated on uS10m. This protein is one of the mt-RPs that were earlier found enriched in mitoribosomal complexes of *T. brucei* [23] and *L. tarentolae* [24], but due to the absence of sequence similarity were categorized as lineage-specific. Only their characteristic conserved folds and interaction networks revealed by the cryoEM structure of the *T. brucei* mitoribosome allowed their correct assignment [9]. Trypanosomal uS10m has a mass of 94 kDa, while the masses of its orthologs in bacteria and human mitoribosomes are 12 and 23 kDa, respectively (Figure 3). Thus, the case of uS10m illustrates a general trend: the vast majority of trypanosomal mt-RPs are larger than their bacterial

counterparts [9,23]. The conserved core of uS10m contains a four-stranded β-sheet. In all reported mitoribosomes, the sheet contains an additional three β-strands contributed by mS35. Because mS35 is absent in bacteria, it belongs to a subset of mt-RPs recruited to mitoribosomes during eukaryogenesis prior the emergence of the **last eukaryotic common ancestor** (**LECA**). The massive expansion of uS10m in trypanosomes is contributed by an internal extension of the conserved core and by amino- (N-) and carboxy-terminal (C-terminal) extensions (NTE and CTE; Figure 3). All three extensions reach to the solvent-exposed surface of the mtSSU and coconstitute the outer shell characteristic for the mitoribosome in trypanosomatids (Figure 3). The distal parts of terminal extensions loop back from the shell to the core of the mtSSU head, and stabilize proteins, which, in bacterial ribosomes and other mitoribosomes, are bound predominantly by rRNA elements. In total, trypanosomal uS10m interacts with 15 mt-RPs (Figure 3C), while its bacterial ancestor makes contacts only with uS3 and uS14 [39] and its major role is to stabilize or connect rRNA elements missing in trypanosomal mitoribosomes. An extremely dense and entangled protein–protein interaction network is another hallmark of mitoribosomes in trypanosomatids. Extensive wrapping, intertwining, and hooking of proteins into RNA loops presents an obstacle to assembly and presumably depends on a high level of coordination in folding of individual components.

Structure-based identification also contributed to the annotation of the *T. brucei* mitochondrial genome, compacted in a characteristic structure termed a **kinetoplast**. A kinetoplast is a concatenate of thousands of minicircles with genes for guide RNAs, which act in uridine insertion/deletion RNA editing [40], and tens of copies of maxicircles encoding both mitoribosomal rRNAs and 18 proteins, including three of unknown function, but no tRNA. Protein synthesis in the mitochondria of trypanosomes thus relies on cytosolic tRNAs, some of which are modified after import into mitochondria to adapt to the organellar translation [41]. One of the unknown open reading frames, previously referred to as MURF-5, turned out to encode uS3m [9]. Trypanosomal uS3m and the only other mitochondrially encoded mt-RP uS12m contain a strikingly high proportion of hydrophobic amino acid residues, consistent with the fact that uS3m is entirely enclosed by other mt-RPs. This contrasts with typical ribosomal proteins, including bacterial uS3, which interact predominantly with rRNA and are therefore rich in positively charged residues.

Biogenesis of trypanosomatid mitoribosomes employs intricate machineries containing a variety of assembly factors

Bacterial ribosomes, which have a relatively simple interaction network among their components, are assembled by a combination of hierarchical and parallel recruitment of ribosomal proteins or their clusters to partially folded rRNA. *In vivo*, the process requires several rRNA modifiers and helicases, GTPases, and other chaperons [42,43], but translationally competent bacterial ribosomes can also be reconstituted from individual unmodified components *in vitro* [44,45]. The biogenesis of eukaryotic cytosolic ribosomes depends on a markedly more intricate machinery, consisting of a myriad of assembly factors. This is caused jointly by their increased complexity, the need to process large pre-rRNA precursors, and the compartmentalization of biogenetic processes between nucleus and cytoplasm [46,47]. The assembly of mitochondrial ribosomes is by far the least understood. Until recently, the most information has been obtained from biochemical and proteomic studies based on genetic depletion or modification of mt-RPs or assembly factors performed in *Saccharomyces cerevisiae* or in mammalian cell cultures [48–50]. Regardless of the type of ribosomes, cryoEM applications have been instrumental in discovering the principles of their biogenesis and in assigning roles for numerous assembly factors, including those previously unidentified [51–56].

In the case of trypanosomal mitoribosomal precursors, the cryoEM structures were obtained from a native pool of mixed mitoribosomal particles, minimizing the risk of characterizing aberrant

or nonphysiological complexes. The precursors represented significant proportions of all mitoribosomal particles in the samples, indicating that these complexes are abundant *in vivo* [10–14]. This is in stark contrast to bacterial or mammalian mitochondrial preribosomal complexes whose isolation for proteomic or structural studies typically requires a depletion of an assembly factor to stall or slow down the assembly process [15,16,22,57,58] or enrichment by affinity purification using a tagged component [15,17,19,59]. The abundance of trypanosomal precursors suggests slow kinetics of the following events in the assembly pathway. Conceivably, the isolated trypanosomal precursors might represent checkpoints in the biogenesis, or immature entities poised to maturation upon a triggering signal. In total, nearly complete atomic models have been obtained for three native precursors of mtSSU [11,12] and two precursors of mtLSU [10,13,14] from trypanosomatid parasites. All the precursors contain rRNA in immature conformation complexed with a subset of mt-RPs and numerous assembly factors. The common principle of biogenesis of both mtSSUs and mtLSUs is that the main functional elements, the decoding center and PTC, are kept immature by assembly factors until the late stage of assembly. Further, the latest identified precursors feature structures sterically hindering premature association of the two subunits.

The number of assembly factors found in individual assembly intermediates varies between ten and 34. Some of the factors have homologs involved in biogenesis of bacterial ribosomes or mitoribosomes in other eukaryotes, or both (Table 1). This category includes several putative methyl transferases, pseudouridine synthases, GTPases, or a DEAD-box RNA helicase. The remaining assembly factors lack any discernible homologs acting in the ribosome assembly outside

Table 1. Trypanosomal mitoribosomal assembly factors with homologs shown or proposed to act in biogenesis of other ribosomes

Name	Alias	Ortholog		Notes
		Bacteria	Humans/*S. cerevisiae*	
Small subunit assembly factors				
mt-SAF1	Rsm22	–	METTL17/Rsm22	MTase
mt-SAF14		RsmH	METTL15	MTase
mt-ACP	mt-SAF32	ACP	mt-ACP/ACP1	acyl carrier protein
mt-SAF39		IF-3	mt-IF-3	mt-IF-3 paralog
mt-IF-2		IF-2	mt-IF-2	
Large subunit assembly factors				
mt-EngA		EngA (Der)	–	GTPase
mt-EngB		EngB	GTPBP8[a]	GTPase
mt-LAF2		–	DDX28[a]	DEAD box helicase
mt-LAF3		–	Pus5[a]	RluA pseudouridine synthase
mt-LAF4	RPUSD4	–	RPUSD4	TruD pseudouridine synthase
mt-LAF5	MRM	–	MRM3[a]	SpoU MTase
mt-LAF6		–	MRM3[a]	SpoU MTase
MALSU1	mt-RsfS	RsfS	MALSU1	
L0R8F8		–	L0R8F8	
mt-ACP		ACP	mt-ACP/ACP1	Acyl carrier protein
Mtg1	GTPBP7	RbgA (YlqF)	GTPBP7/Mtg1	GTPase

[a]Putative ortholog.

of the group Kinetoplastida. Typically, individual factors are interconnected in a dense network linking immature functional substructures, suggesting coordination of maturation events. In total, 68 factors have been identified in trypanosomatids, heavily outnumbering assembly factors known to be involved in biogenesis of human or yeast mitoribosomes [47,60].

Biogenesis of trypanosomal mtSSU involves a large intermediate, the assemblosome

The order in which the three identified mtSSU precursors occur during the biogenetic pathway was unambiguously deduced from the level of maturation of their rRNA and progressively increasing number of bound mt-RPs (Figure 4A,B). The earliest precursor, termed the mtSSU 'assemblosome' [11], is followed by the 'middle' and 'late' mtSSU assembly intermediates [11,12]. The assemblosome is a huge complex with mass exceeding 4 MDa. Its head is nearly mature but is rotated with respect to the body, creating a wide furrow filled with assembly factors. The morphological landmark of the assemblosome is a conspicuous protrusion on the **intersubunit side** formed by a homopentameric lineage-specific assembly factor mt-SAF24, capped by a disc-shaped structure of unknown composition. In total, the complex contains 34 mtSSU assembly factors (mt-SAFs) which cover a large part of the solvent-exposed surface of the body and nearly completely covers the prospective intersubunit side. RNAi knockdowns showed that the tested mt-SAFs are essential for the growth of **procyclic form** trypanosomes in culture and are required for the stability of mtSSU rRNA [11,61]. Some identified mt-SAFs have known folds and can be assigned to protein families such as pseudouridine synthases mt-SAF11, metallo-β-lactamase mt-SAF20, tyrosine phosphates mt-SAF26, or six KRIPPs proteins. Only very few factors have homologs known to act in the assembly of other ribosomes. These include **mitochondrial acyl carrier protein** (**mt-ACP**), also referred to as mt-SAF32, which was shown to play a role in human mtLSU assembly [18] and also has a dual function in the assembly of the trypanosomal mtLSU (see subsequent text). Further, the assemblosome contains a heterodimer of conserved methyl transferases (MTases) mt-SAF1 and mt-SAF14. The former is homologous to METTL17 in humans and Rsm22 in *S. cerevisiae*, and the latter to bacterial RsmH and human METTL15. RsmH/METTL15 is an N4-methylcytidine MTase which modifies a conserved cytidine in h44 of the decoding center [62–64] and also structurally guides the mtSSU biogenesis, as revealed by the recent series of mammalian pre-mtSSU complexes [22]. METTL17/Rsm22 is a putative MTase with unknown substrate, but its loss results in a decrease in methylation of the METTL15 substrate [65], suggesting functional crosstalk. The mt-SAF1/mt-SAF14 heterodimer is localized at the immature decoding center; however, its arrangement in the assemblosome does not allow us to predict specific target nucleotides. Nevertheless, the active sites of both MTases bind *S*-adenosyl homocysteine or methionine, indicating that they are catalytically active.

The assemblosome contains 43 out of 55 mt-RPs of trypanosomal mtSSU (Figure 4A,C). Out of the conserved mt-RPs, only uS12m and bS21m are yet to be bound. According to the classical Nomura's assembly map based on *in vitro* reconstitution experiments [66] and the quantitative MS approach *in vivo* [67], bS21 gets recruited in late stages of bacterial SSU assembly. Therefore, although the assemblosome was referred to as an 'early' intermediate [11], the biogenesis of trypanosomal mtSSU likely involves multiple steps prior to this stage. All four domains of rRNA in the assemblosome exhibit several elements in immature conformation (Figure 4B), and extensive refolding is required, especially in the regions flanking the future mRNA channel and forming the decoding center. Crucial decoding components, rRNA helices h18 and h44, which are folded and juxtaposed in mature ribosomes, are stretched out and apart from each other in the assemblosome. The immature conformation of h18 and h44 is stabilized by the buried part of the mt-SAF24 pentamer, homodimer of putative pseudouridine synthase mt-SAF11, and pentatricopeptide repeat protein KRIPP17.

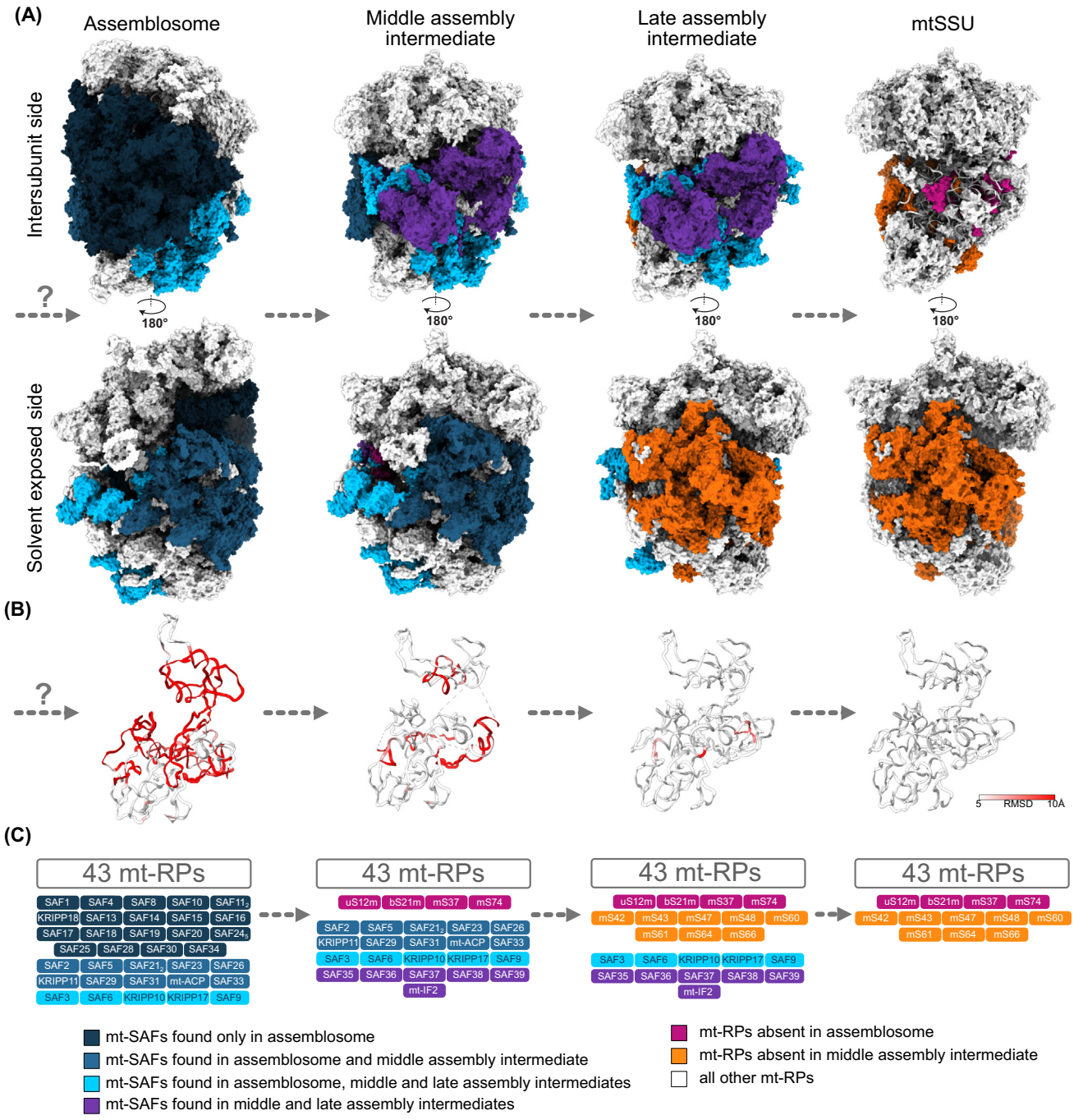

Figure 4. Assembly pathway of the trypanosomal mitoribosomal small subunit (mtSSU). (A) Atomic models of mtSSU precursors and the mature mtSSU. Proteins are shown as surfaces and rRNA as ribbon. Mitoribosomal proteins (mt-RPs) and mitoribosomal mtSSU assembly factors (mt-SAFs) are colored according to their presence in individual complexes as in (C). (B) Progress of mtSSU rRNA maturation. Structures of rRNA in individual complexes are colored by root-mean-square deviation (RMSD) of atomic positions of their sugar–phosphate backbones from the mature mtSSU. (C) Summary of protein components present in individual complexes. Two or more copies of the same protein are indicated by subscripts. mt-RPs present in all precursors are not listed.

Mitochondrial initiation factor 2 acts in the maturation of mtSSU

The transition from the assemblosome to the middle assembly intermediate requires dissociation of a large cluster of 19 mt-SAFs from the prospective intersubunit side. This results in the rotation of the head to its mature position. The closure of the head is accompanied by the binding of four mt-RPs, including conserved uS12m and bS21m, and five additional mt-SAFs and **mitochondrial initiation factor** 2 (mt-IF-2), which together keep the functional core protected and inaccessible (Figure 4A,C). Three of the factors share folds with various enzymes (DNA photolyase mt-SAF35, malonyl-CoA acyl carrier protein transacylase mtSAF36, and acyl-CoA thioesterase mt-SAF38). One factor, mt-SAF39, is a paralog of mt-IF-3. In the following step, ten mt-SAFs from the solvent-exposed side are exchanged for eight mitochondria-specific mt-RPs to form the late assembly intermediate. Thus, the late assembly intermediate contains a full complement of mt-RPs of trypanosomal mtSSU (Figure 4A,C; [11,12]). Interestingly, the largest mt-RP reported so far, mS48 (1788 amino acids), which contains five distinct domains spanning from the body to the head, contacts multiple mt-RPs and was therefore proposed to have a central organizational role in trypanosomal mtSSU [9], is recruited only in this stage of assembly.

Both mt-IF-2 and mt-IF-3-like factor mt-SAF39 remain bound until the late assembly intermediate. The function of mt-IF-2 in the mtSSU assembly requires its N-terminal domain [12], which is absent in the mammalian ortholog, and is likely independent from the protein's function in formylated $tRNA^{Met}$ binding and translation initiation [68]. The highly conserved G domain with the GTP binding site is presumably essential for both activities. In mammals, binding of mtLSU triggers GTP hydrolysis by mt-IF-2 during initiation, which leads to tRNA insertion into the P-site [69]. Analogous interaction of the mt-IF-2 G domain with factors mt-SAF9 and mt-SAF36 might induce GTP hydrolysis and subsequent conformational changes during mtSSU assembly in trypanosomes [12]. mt-SAF39 shares N-terminal and C-terminal domains with the genuine mt-IF-3 but is extended at both termini. The C-terminal domain (CTD) of mt-SAF39 occupies the same position as CTD of the canonical mt-IF-3 in the mature mtSSU in trypanosomes [9,10] or IF-3 in the bacterial ribosome [70]. The long C-terminal extension of mt-SAF39 contains several α-helices and snakes through the putative mRNA channel, blocking premature binding of the template. In mammals, mt-IF-3 was shown to be a component of an mtSSU precursor, indicating that it acts not only in translation initiation but also in the biogenesis of mtSSU [22]. This observation, together with early branching of the mammalian and trypanosomal lineages, suggests that the engagement of mt-IF-3 in mtSSU assembly is ancestral and that mt-SAF39 diverged from canonical mt-IF-3 after these two groups separated.

While rRNA in the middle assembly intermediate exhibits several immature regions interspersed in all domains, the vast majority of rRNA elements adopt mature conformation in the late precursor (Figure 4B). The only exceptions are two adjacent single-stranded regions in the body and helices h24 and h44, whose maturation requires dissociation of mt-SAF38 and mt-SAF22/KRIPP17, respectively [12]. The final maturation step(s) involves release of all remaining ten mt-SAFs and leads to exposure of the mRNA channel and the decoding center (Figure 4). The mature mtSSU complex characterized in trypanosomes contains mt-IF-3 and is devoid of mRNA and tRNAs, thus representing a preinitiation or a post-recycling complex [9,10].

Assembly of trypanosomal mtLSU shares mechanisms with bacterial ribosomes and mammalian mitoribosomes

Two precursors of trypanosomal mtLSU have been reported so far [10,13,14]. The earlier precursor, referred to as 'state A', contains 25 mitochondrial large-subunit assembly factors (mt-LAFs) and 50 out of 72 mt-RPs found in the trypanosomal mtLSU [9]. All nine factors that are specific to this state are clustered on the lower part of the intersubunit side, keeping several rRNA elements

immature. Transition to the later 'state B' requires dissociation of these factors, refolding of the proximal rRNA elements to mature or alternative conformations, and binding of an additional five mt-RPs and five mt-LAFs [13] (Figure 5).

The common features of both precursors are immature intersubunit side, including entire PTC, missing CP, and the **L7/12 stalk**, and an immature base of the L1 stalk. The intersubunit side of both states contains conserved GTPases, Mtg1/GTPBP7, and mt-EngA. The third GTPase, mt-EngB, is specific only to the state B [13]. Mtg1/GTPBP7 and mt-EngA locate to the same regions as their bacterial counterparts, EngA (aka Der) and RbgA (aka YlqF), when reconstituted with isolated bacterial LSU and its precursor, respectively [57,71]. The trypanosomal mtLSU assembly intermediates are the first visualized native complexes containing any of these GTPases. The simultaneous presence of the GTPases in an mtLSU precursor is in line with the view that they act in the same stage of LSU assembly in bacteria [58]. The bipartite module of GTPases mt-EngA and Mtg1/GTPBP7 make contacts with crucial functional rRNA elements and is presumably involved in their maturation. While Mtg1/GTPBP7 is widespread across all eukaryotes, mt-EngA has been lost in Opisthokonts, including mammals and fungi [14]. Accordingly, GTPBP7 does not contact any other GTPase and is bound mostly by interactions with rRNA and conserved proteins uL14m and uL19m in human pre-mtLSU complexes [15,20]. In contrast, trypanosomal Mtg1/GTPBP7 is stabilized in the complex by binding to factors mt-LAF6/MRM and mt-LAF3/RPUSD4. The former has a fold of SpoU MTase and the latter is a putative RluA/Pus5 pseudouridine synthase (Table 1), but neither of them appear to be catalytically active in the characterized precursors. Instead, they serve as a scaffold to stabilize GTPases and couple maturation of the PTC with the L7/12 stalk [14]. However, mt-LAF6 occurs in a heterodimer with another SpoU-like MTase mt-LAF5, whose interaction with the A-loop of the PTC helix

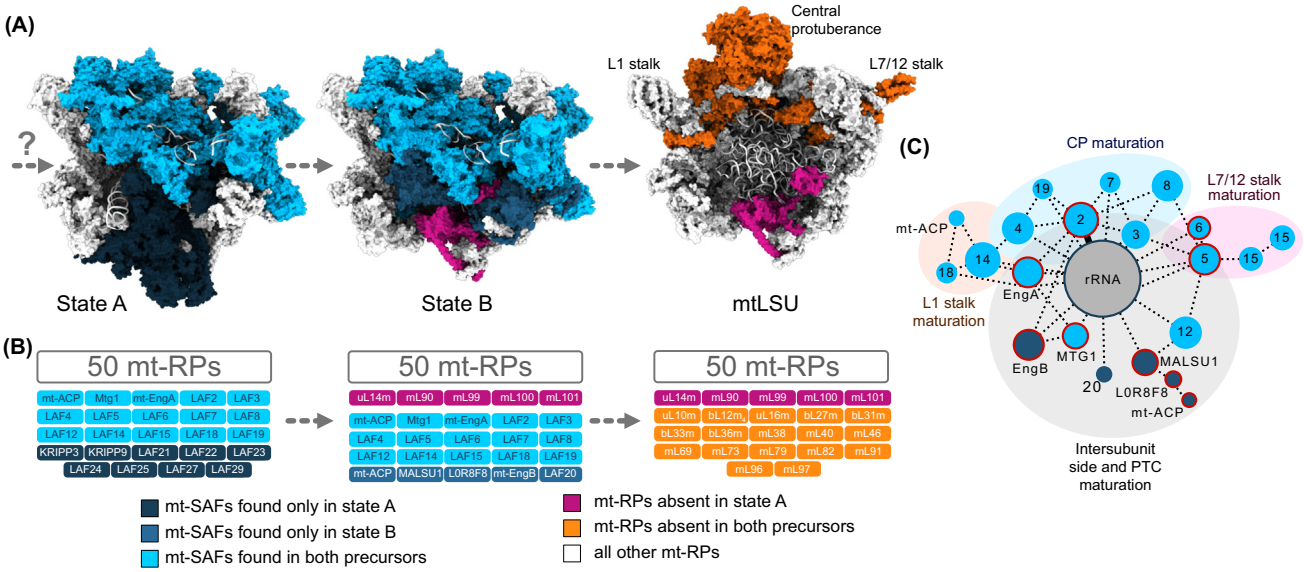

Figure 5. Assembly of the trypanosomal mitoribosomal large subunit (mtLSU). (A) Atomic models of mtLSU precursors and the mature mtLSU. Proteins are shown as surfaces and rRNA as ribbon. Mitoribosomal proteins (mt-RPs) and mitoribosomal large subunit assembly factors (mt-LAFs) are colored according to their presence in individual complexes as in (B). (B) Summary of protein components present in individual complexes. Two or more copies of the same protein are indicated by subscripts. mt-RPs present in all precursors are not listed. (C) Interaction network of mt-LAFs and rRNA in state B. mt-LAFs are colored as in (A) and (B). mt-LAFs in red circles are conserved. Overlapping subsets of mt-LAFs involved in maturation of individual functional regions of mtLSU are indicated. PTC, peptidyl transferase center.

H92 is compatible with the proposed methyltransferase enzymatic activity [13]. The mt-LAF5/6 dimer binds H92 in the same manner as a homodimer of human MTase MRM3 [19]. Thus, both mt-LAF5 and mt-LAF6 are presumably trypanosomal homologs of MRM3, and mt-LAF5 is the counterpart of the active MRM3 protomer, which methylates 2′-O-ribose of G1370 in human mtLSU rRNA [72]. Strikingly, the location of the H92/$(MRM3)_2$ complex dramatically differs between the trypanosomal [13] and mammalian precursors [19], exemplifying differences in the mtLSU assembly pathway between the two groups. Other remarkable difference between the two systems is that GTPase GTPBP5, GTPBP6 and GTPBP10 and a heterodimer NSUN4/MTERF4 acting in the late mtLSU biogenesis in humans [49,55] have not been identified in trypanosomatids.

Immature PTC in trypanosomal mtLSU precursors is linked also to the region of prospective CP, via interactions of mt-LAF3/RPUSD4 and mt-EngA with mt-LAF2. mt-LAF2 is a DEAD-box RNA helicase, which keeps helices H80-88 in immature conformation similarly to the human DDX28 [19], implying a conserved role for RNA helicase activity in the maturation of CP. Late LSU assembly intermediates with missing or immature CP have been also described in bacteria [73,74], human mitochondria [19,55] and yeast cytoplasm [75], indicating that late construction of CP is a universally conserved feature of LSU biogenesis.

However, due to high divergence of the CP composition (Figure 1) other mechanisms of CP construction are likely lineage specific. This includes tethering of the immature CP to the base of the L1 stalk in the trypanosomal pre-mtLSU complexes, which is mediated by mt-ACP. mt-ACP binds to a lineage specific factor mt-LAF18 at the CP and to a Leu-Tyr-Arg motif (LYRM) protein mt-LAF18 at the L1 stalk base, immobilizing the L1 stalk in a nonfunctional position. The latter interaction is mediated by a 4-phosphopantetheine (4′-PP) group covalently attached to mt-ACP. Another copy of mt-ACP-4'PP bound to the conserved LYRM protein L0R8F8 form together with another universally conserved factor MALSU1 (or mt-RsfS) a tripartite protrusion at the lower part of the intersubunit side of all characterized mtLSU precursors in trypanosomes and humans [10,13–20]. The conserved protrusion prevents premature binding of mtSSU to mtLSU assembly intermediates [18] or mtLSU particles undergoing quality control after stalling of translation [76]. Thus, including its engagement in the **mtSSU assemblosome**, mt-ACP has a triple role in the assembly of trypanosomal mitoribosomes. In addition, three other mt-ACP-4'-PP/LYRM protein modules have been described. Two of them are components of the mammalian respiratory chain complex I [77,78] and one is essential for the iron-sulfur cluster assembly [79]. Further, mt-ACP has a central role in fatty acid synthesis [79]. Due to its multifunctionality, mt-ACP was proposed to act as a molecule central to intramitochondrial metabolic state sensing [80].

Collectively, all crucial functional regions immature in the pre-mtLSU complexes are interconnected by a network of factors (Figure 4C), suggesting coordination of mtLSU maturation events [14]. These involve dissociation of numerous mt-LAFs from the intersubunit side and folding of PTC, replacement of some mt-LAFs in the CP and the L7/12 stalk by mt-RPs, and substantial refolding of some mt-RPs already present in the precursors. Notably, the findings from structural studies indicate conserved roles of three GTPases in maturation of mitochondrial and bacterial LSU. In addition, an ATP-dependent DEAD-box RNA helicase, a dimer of MRM3 Mtases, and the tripartite MALSU1/L0R8F8/mt-ACP module act in the assembly of divergent trypanosomal and mammalian mitoribosomes (Table 1). Therefore, based on the current knowledge, the biogenesis of mtLSU in trypanosomatids employs more conserved mechanisms than that of mtSSU.

Concluding remarks

The divergence of mitoribosomes implies that their biogenesis includes combination of conserved and lineage-specific mechanisms. Structures of precursors of both mitoribosomal subunits in

Outstanding questions

What are the mechanisms of earlier steps of assembly? Can genetic modification such as ablation of an assembly factor or tagging and biochemical enrichment be exploited to capture earlier precursors of trypanosomatid mitoribosomes?

Does assembly of mitoribosomes in trypanosomatids and in other eukaryotes occur cotranscriptionally? How are the early assembly processes affected by fragmentation of mitochondrial rRNA in some eukaryotic groups, represented by *Tetrahymena*, *Chlamydomonas*, or apicomplexan parasites?

Why are the precursors of both mitoribosomal subunits in trypanosomatids so abundant? Do they represent checkpoints in the assembly pathways?

What is the actual level of conservancy of biogenesis of extremely divergent trypanosomal mitoribosomes? To what extent can we extrapolate the understanding to other eukaryotic groups?

What are the mechanisms of translation processes and mitoribosomal recycling in trypanosomatids?

What did the mitoribosome in LECA look like? What allowed and/or drove diversification of mitochondrial ribosomes?

Microbial ribosomes are common targets of antibiotics. Is the trypanosomatid mitoribosome a good potential drug target to treat trypanosomiases and leishmaniases?

trypanosomatid parasites and their comparison with studies on bacterial ribosomes and mammalian mitoribosomes revealed that folding of highly conserved functional elements involves conserved events and players. Some factors previously proposed to participate in the assembly of mitoribosomes in various eukaryotes, based on biochemical studies, exemplified by GTPases Mtg1/GTPBP7 [81,82] and mt-EngA [83] or a DEAD box helicase [84,85], were for the first time captured in native complexes and shown to chaperone folding of functional cores. The conserved assembly factors are interconnected with numerous lineage-specific factors, which act as scaffolding elements, recruit other factors or mt-RPs, and stabilize and protect immature rRNA structures. Because the trypanosomal mitochondrial rRNA has low base-pairing potential and is likely prone to misfolding, some of the factors might prevent the rRNA from entering kinetic misfolding traps [86].

While structural studies provided insight into late steps of assembly of mitoribosomal subunits, the earlier stages remain obscure (see Outstanding questions). Characterization of hypothetical earlier precursors might require stalling of the assembly pathway by ablation of some of the identified assembly factors. It is currently unclear whether folding of mitochondrial rRNA and first assembly steps occur cotranscriptionally, as documented in bacteria [87]. The early processes might differ between lineages because, while mitochondrial rRNA molecules are processed from polycistronic transcripts in mammals [88,89], they are likely transcribed as individual units in trypanosomes [90]. In addition, the early steps are likely more complex in organisms such as *Tetrahymena* [8], *Chlamydomonas* [7], or Apicomplexa [91], whose rRNA is fragmented.

Beyond any doubt, structural studies on trypanosomal mitoribosomal complexes from *T. brucei* and related parasites reviewed in this article, together with studies in mammals, contributed the most to the current knowledge on mitoribosomal biogenesis. However, due to the marked divergence of trypanosomal and mammalian mitoribosomes, further investigation, preferably involving also other eukaryotic groups, is needed to sort out ancestral and derived assembly mechanisms in more detail.

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Declaration of interests

The authors declare no competing interests.

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