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Mitochondrial translation in trypanosomatids: a novel target for chemotherapy?

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Trypanosomatids cause widespread disease in humans and animals. Treatment of many of these diseases is hampered by the lack of efficient and safe drugs. New strategies for drug development are therefore urgently needed. It has long been known that the single mitochondrion of trypanosomatids exhibits many unique features. Recently, the mitochondrial translation machinery of trypanosomatids has been the focus of several studies, which revealed interesting variations to the mammalian system. It is the aim of this article to review these unique features and to discuss them in the larger biological context. It is our opinion that some of these features represent promising novel targets for chemotherapeutic intervention that should be studied in more detail.

Mitochondrial translation

Mitochondria are double membrane-bounded organelles whose main function is oxidative phosphorylation. They have their own genome, which encodes a small number of proteins (e.g. 13 in humans). Most of the more than 1000 mitochondrial proteins are encoded in the nucleus, synthesized in the cytosol and finally imported into the organelle. However, the few mitochondrially encoded proteins are essential for oxidative phosphorylation indicating the need for mitochondrial protein synthesis. In line with the evolutionary origin of the mitochondrion, its translation system is of the bacterial type. It requires a formylated initiator $tRNA^{Met}$, bacterial-type translation factors and ribosomes whose rRNAs and structure resemble bacterial ones. The mammalian mitochondrial genome encodes 22 tRNA genes of bacterial evolutionary origin, a set predicted to be sufficient for translation of all codons. All of these tRNAs are aminoacylated by imported aminoacyl-tRNA synthetases which are by and large of the bacterial type. Mitochondria from many organisms use a variant genetic code; the most frequent variation is the reassignment of the stop codon UGA to tryptophan (reviewed in [1]).

Mitochondrial translation in trypanosomatids

The insect stage or procyclic form of *Trypanosoma brucei* produces energy mainly by oxidative phosphorylation (reviewed in [2]). The bloodstream stage of *T. brucei*, however, lacks a functional respiratory chain and relies on glycolysis for energy production [2]. The mitochondrial genome of trypanosomatids encodes several proteins that are conventional for a mitochondrial genome (Box 1), as well as two rRNAs but no tRNAs. The lack of tRNAs is compensated for by import of a small fraction (1–10%) of cytosolic tRNAs [3]. Only two tRNAs are cytosol-specific, namely the initiator $tRNA^{Met}$ and $tRNA^{Sec}$ [3,4] (Figure 1). Unlike in mammals, all mitochondrial tRNAs of trypanosomatids are derived from nuclear-encoded tRNAs involved in cytosolic translation. Consequently, mitochondria of trypanosomatids have to function exclusively with eukaryotic-type tRNAs [5,6]. However, as in all eukaryotes, the mitochondrial translation system of trypanosomatids is of the bacterial type. Although many tRNAs might in principle be functionally interchangeable between the cytosol and mitochondria, this is not the case for the initiator $tRNA^{Met}$ and for tRNAs that read codons that differ from the standard genetic code. Thus, at least in some cases adaptations of the mitochondrial translation system are required to functionally integrate imported eukaryotic-type tRNAs. These adaptations mainly concern factors that directly interact with tRNAs, such as aminoacyl-tRNA synthetases (aaRSs), translation initiation and elongation factors, but can ultimately also affect the ribosome (Figure 2).

Mitochondrial translation initiation

In mitochondria of *T. brucei*, a nuclear-encoded elongator $tRNA^{Met}$ is used for both elongation and initiation [7]. After import, a fraction of the $tRNA^{Met}$ becomes formylated by an unusual methionyl-$tRNA^{Met}$ formyltransferase (MTF), which selectively recognizes elongator-type $tRNA^{Met}$ [7]. The trypanosomal MTF is still homologous to other MTFs even though at 71.3 kDa it is approximately twice the size of any of the other known MTFs [7]. Finally, the formylated elongator $tRNA^{Met}$ needs to bind to mitochondrial translation initiation factor 2 (mtIF2) before it can interact with the ribosome.

Ablation of mtIF2 leads to growth arrest in the procyclic form of *T. brucei*, whereas MTF, similar to what has been observed in yeast, seems dispensable [8]. However, these results were obtained in knockdown cell lines, thus it cannot be ruled out that the remaining amount of MTF suffices to sustain mitochondrial translation. Interestingly, cells with reduced levels of mtIF2 were affected by

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Box 1. Mitochondrially encoded proteins in trypanosomatids

The mitochondrial genome of *T. brucei* and most other trypanosomatids encodes three subunits of the cytochrome oxidase (Cox), cytochrome *b* (Cytb), several subunits of the NADH dehydrogenase, subunit 6 of the mitochondrial ATPase, a ribosomal protein and several as yet unidentified proteins [52]. Despite extensive efforts, mitochondrial translation has been notoriously difficult to study in trypanosomatids; one reason might be that most mitochondrially encoded proteins are highly hydrophobic which makes them difficult to resolve by gel electrophoresis and refractory to sequencing. Another reason is that mitochondria of trypanosomatids as of most eukaryotes cannot be genetically manipulated. However, using sophisticated two-dimensional gel electrophoresis in combination with *in organello* labeling and direct sequence analysis, it was possible to directly show that the open reading frames encoding Cox1, Cox2 and Cytb are indeed translated [46,47,53]. The processing steps required to produce the mature mRNAs encoding Cox2 and Cytb, unlike those for the Cox1 mRNA, include moderate U insertion/deletion-type RNA editing [54]. Interestingly, in contrast to the mitochondria of mammals, mitochondrial translation in *T. brucei* was resistant to chloramphenicol and many other translational inhibitors expected to block bacterial-type translation [47]. In the case of chloramphenicol the most probable explanation for this observation is the fact that the peptidyl transferase region in the mitochondrial rRNA, implicated in chloramphenicol binding, is partially missing [31].

reduced MTF activity, as cell growth was inhibited more severely in a combined knockdown cell line [8]. In *Leishmania major*, null mutants for the enzyme involved in the synthesis of 10-formyl tetrahydrofolate, a key intermediate for both purine synthesis and $tRNA^{Met}$ formylation [9], are not viable [10]. Because trypanosomatids are known to be purine auxotrophs, this provides circumstantial evidence that in *Leishmania* spp. the formylation of the imported $tRNA^{Met}$ might be essential.

In some cases, the formylmethionine needs to be removed for the newly synthesized proteins to be functional. This removal requires the sequential action of peptide deformylases (PDFs) and aminopeptidases [11]. Unlike human mitochondria, *T. brucei* has two distinct mitochondrial PDFs, which are highly divergent from other eukaryotic PDFs. One of the enzymes was shown to be essential for the procyclic form [12]. Bacterial PDFs have been proven to be excellent drug targets indicating that the same might be the case for trypanosomatids [13].

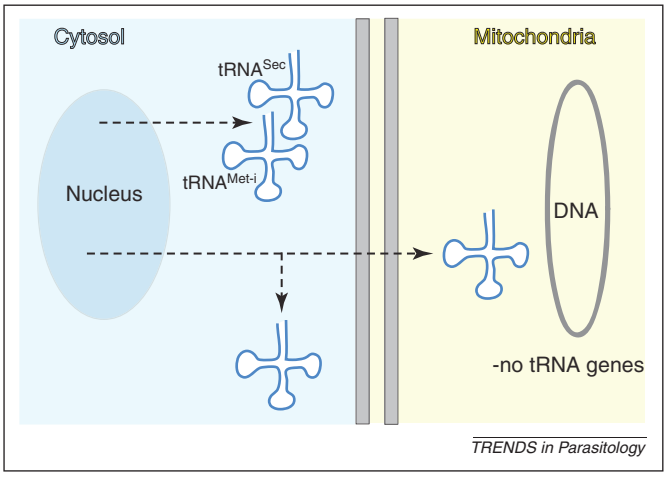

Figure 1. Genetic origin and localization of tRNAs in trypanosomatids. The mitochondrial genome of trypanosomatids does not encode any tRNAs, thus all tRNAs are encoded in the nucleus. Except for the cytosol-specific $tRNA^{Met-i}$ and $tRNA^{Sec}$ all cytosolic tRNAs are imported into the mitochondrion to variable extents (1–10%). As a consequence, all mitochondrial tRNAs of trypanosomatids are of the eukaryotic type and must function in the bacterial-type translation system of the mitochondrion.

Genetic code differences

In the mitochondria of trypanosomatids, as in most eukaryotes, the stop codon UGA has been reassigned to tryptophan. It is not obvious how the trypanosomal $tRNA^{Trp}$ which in the cytosol exclusively recognizes the CCA codon can decode both the CCA and the UGA codons when present in mitochondria. Trypanosomatids solve this problem by a mitochondria-specific RNA editing event that converts the CCA anticodon of the imported $tRNA^{Trp}$ to UCA, which allows decoding of both UGG and UGA triplets [14–16]. Thus, the use of the imported $tRNA^{Trp}$ in mitochondrial translation requires an enzyme that catalyzes the C to U editing reaction. This trypanosomatid-specific enzyme could possibly make an excellent drug target; unfortunately, it has not been identified yet.

The imported $tRNA^{Trp}$ is not only edited at the wobble nucleotide (C34 to U34), but the adjacent nucleotide U33 also becomes thiomodified [15]. Recently, it was shown that a reduction in the level of the thiomodification results in an increase of editing of the $tRNA^{Trp}$ [17,18]. However, RNAi-mediated ablation of the mRNA encoding for one of the enzymes responsible for the thiomodification and the corresponding increase in $tRNA^{Trp}$ editing did not affect growth of procyclic *T. brucei* [18,19] Whether the thiomodification serves to regulate the extent of tRNA editing *in vivo* is therefore unclear at present.

Mitochondrial tryptophanyl-tRNA synthetase

In all eukaryotic organisms the mitochondrial aaRSs are encoded in the nucleus and imported into mitochondria [20]. Considering that all mitochondrial tRNAs of trypanosomatids are imported from the cytosol, the same aaRSs could in principle be used in both compartments. This is typically the case; for most aaRSs only one gene is found, and for some aaRSs dual localization has been confirmed [21]. The finding that two genes coding for cytosolic and mitochondrial versions of tryptophanyl-, aspartyl- and lysyl-tRNA synthetases (TrpRS, AspRS and LysRS) exist has therefore been puzzling.

In the case of the TrpRS, it was found that editing of the anticodon or thiolation of U33 of the imported $tRNA^{Trp}$ changes an identity determinant (the ability of the TrpRS to identify the cognate tRNA, $tRNA^{Trp}$) for cytosolic TrpRS. *T. brucei* therefore needs two distinct TrpRSs: a cytosolic one (TbTrpRS1) that charges the $tRNA^{Trp}$ with the anticodon CCA and a mitochondrial one (TbTrpRS2) that can aminoacylate the $tRNA^{Trp}$ after it has been edited and/or thiolated [16].

Unlike the human mitochondrial enzyme, TbTrpRS2 is of eukaryotic origin. However, it is markedly divergent from its cytosolic counterpart TbTrpRS1, as well as from the cytosolic TrpRS of humans. Interestingly, it was recently shown that TbTrpRS2 is essential for proliferation of the bloodstream stage of *T. brucei in vitro* and for establishment of an infection in mice, making it another

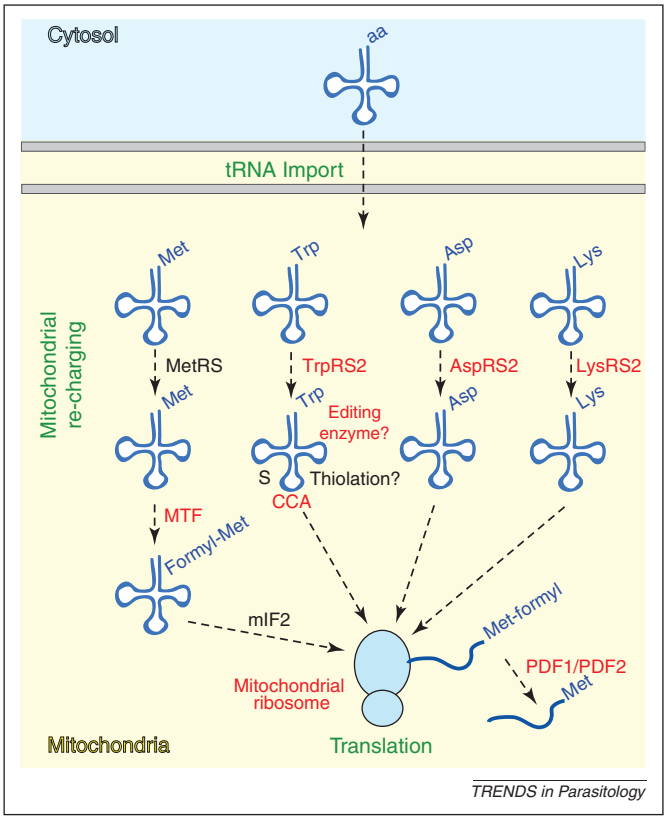

Figure 2. Unique features of mitochondrial translation in *T. brucei*. The pathways and processes that show trypanosome-specific features are indicated in green with upper case letters. Enzymes and processes that participate in the different pathways that are conserved within most or all eukaryotes are depicted in black. Enzymes and structures that: **(i)** are specific for trypanosomes or that have unique features and **(ii)** that are expected to be essential and therefore are potential drug targets, are shown in red. The enzyme responsible for C to U editing of the imported $tRNA^{Trp}$ is unknown. The imported $tRNA^{Trp}$ is thiolated at U33, the nt 5′ of the anticodon. The thiolation reaction is catalyzed by enzymes that (at least in part) are also required for the biogenesis of mitochondrial and cytosolic iron sulfur clusters [18,19]. It is unknown whether thiolation is essential, but it has been shown to prevent $tRNA^{Trp}$ editing. tRNAs are most probably imported in their aminoacylated state but can be recharged inside mitochondria [37]. Abbreviations: MetRS, methionyl-tRNA synthetase; TrpRS2, eukaryotic-type mitochondrial tryptophanyl-tRNA synthetase; AspRS2, eukaryotic-type mitochondrial aspartyl-tRNA synthetase; LysRS2, mitochondrial lysyl-tRNA synthetase; mIF2, mitochondrial initiation factor 2; MTF, methionyl-tRNA formyltransferase; PDF1 and 2, peptide deformylase 1 and 2.

potential target for drug development [22]. It should be emphasized in this context that aaRSs have been successfully used as drug targets in bacteria and that cytosolic aaRSs have previously been proposed as drug targets in several parasitic protozoa (for a review see [23] and references therein; also consult Box 2).

Mitochondrial aspartyl- and lysyl-tRNA synthetases

The two other trypanosomal aaRSs encoded by two different genes, AspRS2 and LysRS2, have also been studied but are less well understood. For the two AspRSs the situation is analogous to the TrpRSs. Whereas both enzymes can charge the cytosolic $tRNA^{Asp}$, the mitochondrial $tRNA^{Asp}$ can only be charged by the mitochondrial enzyme [24]. This different specificity is also probably due to an as yet uncharacterized compartment-specific modification(s) of the $tRNA^{Asp}$.

The situation is different for the LysRSs. The mitochondrial LysRS contains an N terminal extension that functions as a targeting signal and a C terminal one that blocks its function [25]. After import the N terminus of the mitochondrial LysRS is cleaved, and this in turn triggers processing of the C terminal extension resulting in an active protein. The substrate specificities of the cytosolic and mitochondrial LysRSs and why the activity of the mitochondrial enzyme has to be blocked when in transit to the mitochondria are currently unknown.

Box 2. Aminoacyl-tRNA synthetases as drug targets

aaRSs catalyze the attachment of amino acids to their cognate tRNAs. Most organisms have at least 20 different aaRSs, one for each amino acid. aaRSs are universal and essential for cell viability, yet some of them differ markedly in structure from one organism to the other [23,55,56] and for this reason have long been proposed as targets for new antibiotics to treat bacterial infections. Many natural products have been described with the ability to inhibit different bacterial aaRSs. However, only one aaRS inhibitor, mupirocin, has been developed into a commercially available drug. Mupirocin was originally isolated from *Pseudomonas fluorescens* [57]. It inhibits isoleucyl-tRNA synthetases (IleRS) from Gram-positive bacterial pathogens including *Staphylococcus aureus*, *Streptococcus pyogenes* and *Staphylococcus epidermidis* [55]. More recently, aaRSs have also been suggested as drug targets in eukaryotic pathogens. Substances have been found that inhibit the activity of recombinant cytosolic LysRS of trypanosomes [58,59], and more recently inhibitors of the cytosolic MetRS of *T. brucei* were characterized that suppress the onset of trypanosomiasis in the mouse model [60]. aaRSs are attractive drug targets owing to the above-mentioned structural divergence [23,55,56] but also for several practical reasons that need to be taken into account for rational drug design: (i) many have been recombinantly expressed in a soluble and active state; (ii) their activity can be measured by methods amenable to high-throughput screening; (iii) the mechanism of catalysis of aaRSs has been extensively studied and is well understood; and (iv) the structures of many aaRSs are solved at high resolution, providing a platform on which more divergent aaRSs can be modeled. With this information in hand, the rational design of aaRSs inhibitors is within reach.

Mitochondrial ribosomes of trypanosomatids

Mitochondrial ribosomes in trypanosomatids are unique. Their small subunit (SSU) and the large subunit (LSU) rRNAs are extremely short, consisting of only 610 nt and 1150 nt (9 and 12S of *T. brucei*, respectively) [26–28]. Whereas the core regions of the peptidyl transferase center are conserved, other domains of the rRNAs are completely absent [29–31]. The mitochondrial ribosome of *T. brucei* consists of 133 proteins (77 in the LSU and 56 in the SSU) [32], which include orthologs of all mitochondrial ribosomal proteins that were previously identified in *Leishmania tarentolae* [30]. Mitochondrial ribosomes are known to contain more proteins than their bacterial counterparts. However, 133 is an extraordinarily high number compared to, for example, the 77 proteins present in the mammalian mitochondrial ribosome [33], indicating that some of the proteins might not be components of the stable ribosome but part of a super-complex that also performs other functions [32]. Only approximately 30% of the ribosome-associated proteins in *T. brucei* have orthologs in either bacteria or in other eukaryotes. The remaining 70% are unique to the trypanosomatids [32]. It has been suggested that the large number of ribosomal proteins in mitochondria of trypanosomatids might be needed to compensate for the markedly short rRNAs [32]. This view is supported by the recently solved Cryo-EM structure of the *L. tarentolae*

mitochondrial ribosome, which shows that despite the small rRNAs, the overall size and morphology of the particle is very similar to the bacterial ribosome [31].

In summary, even though presently no drug is known that specifically inhibits mitochondrial ribosomes of trypanosomatids, their unique composition makes them an excellent potential drug target.

Mitochondrial translation as drug target

Perhaps the unique features of mitochondrial translation in *T. brucei* and other trypanosomatids discussed above could offer as yet unexploited drug targets (Figure 2); interestingly, many of these features are directly or indirectly linked to the fact that trypanosomatid mitochondria need to import the entire set of tRNAs from the cytosol (reviewed in [6]).

Although the tRNA import machinery in *T. brucei* is very poorly characterized, a series of publications on the tRNA import system of *Leishmania tropica* are deemed highly controversial as has been discussed before [34]. Owing to concerns about the validity of these results [35,36], it is premature to discuss the value of the mitochondrial tRNA import system as a drug target. However, some of the adaptations of the mitochondrial translation system that allow it to use imported, eukaryotic-type tRNAs have been characterized in sufficient detail to evaluate them as serious drug targets (Figure 2 and Box 2, [37]). One of the key questions in this context is whether or not mitochondrial translation is in fact essential in the disease-causing forms of trypanosomatids.

In *T. brucei* this question has recently been the focus of much interest. Bloodstream forms of *T. brucei* do not perform oxidative phosphorylation but produce their energy exclusively by glycolysis (reviewed in [2]). Most of the mitochondrially encoded genes in trypanosomes code for components required for oxidative phosphorylation (Box 1) and therefore might not be expressed in the bloodstream form. Moreover, *T. brucei* strains exist that are lacking mitochondrial DNA; these so-called dyskinetoplastic strains are locked in the bloodstream stage (see [38] and references therein). These observations suggested that mitochondrial translation is dispensable in the bloodstream form. In addition, a recent publication reports the failure to detect *de novo* synthesized proteins in bloodstream form mitochondria [39]. Nevertheless, several studies also showed that kinetoplast DNA replication [40], mitochondrial transcription [41] and RNA editing [42] are essential throughout the life cycle of *T. brucei*. Moreover, these results were confirmed by more direct investigations of the mitochondrial translation system. It is now known that survival of the bloodstream form of *T. brucei* depends on the mitochondrial ATP synthase, one subunit of which is mitochondrially encoded and translated. In this life stage, the ATP synthase functions in reverse, consuming mitochondrial ATP to pump protons to the intermembrane space. In this way it generates the membrane potential that is needed to maintain transport of metabolites and proteins across the mitochondrial inner membrane [43,44]. But how can dyskinetoplastic strains survive? To explain this conundrum it has been hypothesized that dyskinetoplastic forms of *T. brucei* contain compensatory mutations in one nuclear-encoded subunit of the mitochondrial ATP synthase that allow it to hydrolyze ATP even in the absence of the mitochondrial encoded subunit [38,44,45].

Data on mitochondrial translation in *Leishmania* spp. or *Trypanosoma cruzi* are scarce. In *L. tarentolae*, it has been shown that mitochondrial translation takes place in the promastigote stage that occurs in the sand fly [46,47]. The respiratory chain is present in both the amastigote and the promastigote life stages of *Leishmania* spp. [2]. Several studies suggest that the respiratory chain could be a target for chemotherapy [48,49]. The mitochondrial metabolism of *Leishmania* spp. therefore bears a resemblance to procyclic *T. brucei* [2]. The same appears to be the case for the trypomastigote and epimastigote forms of *T. cruzi* [2,50,51].

Concluding remarks

Since the advent of antibiotics, protein synthesis has been a classic target for antibacterial compounds. Analogously, many of the unique features of mitochondrial protein synthesis discussed above will provide novel drug targets against all major trypanosomatid pathogens, if further investigated in the future. It is now clear that mitochondrial translation is essential for bloodstream forms of *T. brucei*. However, experiments regarding the inhibition or ablation of specific translation factors and the resulting consequences on parasite survival in the bloodstream stage are missing. The same is the case for *T. cruzi* and *Leishmania* spp. This is unfortunate as a pilot study has shown that the mitochondrial translation elongation factor Tu and TrpRS2 are indeed essential for survival of bloodstream form of *T. brucei*, suggesting that further studies in this direction might be very rewarding [22]. We hope this article stimulates discussion regarding the mitochondrial translation system in pathogenic eukaryotes as a potential drug target, and the sparks of this discussion ignite further research efforts beyond the trypanosomatid field.

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