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Ph.D. Thesis

The impact of iron-sulfur assembly on the mitochondrial tRNA import in *Trypanosma brucei*

Zdeněk Paris

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ANOTATION

This thesis addresses several aspects of mitochondrial iron sulfur (Fe-S) cluster biogenesis and mitochondrial tRNA import and modifications in *Trypanosoma brucei*. Using RNAi interference it uncovers essential role of Fe-S cluster assembly in tRNA(s) thiolation in both the cytosol and the mitochondrion of *T. brucei.* Further, this thesis describes the role of modifications in tRNA editing and in mitochondrial import of tRNAs. Finally, it provides evidence that in contrast to protein import, mitochondrial membrane potential is dispensable for import of tRNAs into the mitochondrion of *T. brucei*.

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DECLARATION

I hereby declare that I did all the work presented in this thesis by myself or in collaboration with co-authors of the presented papers and only using the cited literature.

České Budějovice, 15 August, 2010 Zdeněk Paris

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V Českých Budějovicích dne 15. srpna 2010 Zdeněk Paris

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CONTENTS

1. Summary

Trypanosoma brucei is a unicellular eukaryote that is the causative agent of human sleeping sickness and nagana in livestock. Due to its capacity for RNA interference (RNAi) and other methods of forward and reverse genetics, which can be exploited for the functional analysis of genes, this protozoan parasite has become the representative model species of the order Kinetoplastida. Thanks to many unique features of its molecular and cellular biology, *T. brucei* is also an extensively studied organism. One of these unusual features is the complete lack of tRNA genes in the mitochondrial genome, which is compensated for by the import of the cytosolic tRNA molecules into the organelle. There is a very limited knowledge about the mechanisms and factors participating in this essential process for the survival of trypanosomes and related leishmanias. This thesis focuses on several aspects of mitochondrial iron sulfur (Fe-S) cluster biogenesis and mitochondrial tRNA import and modifications, as well as possible interactions between these fundamental and interesting processes.

In the first part of this work we were testing whether the thio-modified nucleotide s^2U is a negative determinant of mitochondrial tRNA import in *T. brucei*. We demonstrated that silencing cysteine desulfurase (TbNfs), the key enzyme for tRNA thiolation and Fe-S cluster formation, plays no role in tRNA partitioning. We have also investigated function of the Rieske Fe-S protein in tRNA import. This key subunit of the complex III has been controversially proposed to be an essential subunit of the RNA import complex (RIC) in *Leishmania tropica*. However, we showed that neither the s^2U modification nor the Rieske protein is essential for tRNA import in *T. brucei.* **(Results 3.1 - Paris et al. 2009 *RNA*)**.

In the next study we wanted to understand in more detail the role of cysteine desulfurase in the process of *de novo* synthesis of Fe-S clusters. We examined the level of C to U editing required for the only thiolated mitochondrial $tRNA^{Trp}$, a process necessary for the decoding of mitochondrial tryptophanyl codons in genes encoded in the organellar genome. We proved that thiolation affects tRNA stability and plays role as a negative determinant of mitochondrial tRNA editing in *T. brucei* **(Results 3.2 - Wohlgamuth-Bendum et al. 2009 *JBC*)**.

In order to understand the connection between Fe-S cluster assembly and thio-modifications of tRNAs, we employed appropriate biochemical and molecular

methods to characterize the role of a small protein termed Isd11, an essential binding partner of eukaryotic cysteine desulfurase. We have demonstrated that Isd11 also partakes in both cytosolic and mitochondrial tRNA thiolation, whereas Mtu1 (another protein implicated in tRNA thiolation in other organisms) is required for this process **(Results 3.3 - Paris et al. 2010 *JBC*)**.

In the most recent work in this thesis, we showed that proteins involved in mitochondrial RNA synthesis and processing as well as tRNAs are still imported into the mitochondrion of the *T. b. brucei* subspecies, *T. b. evansi.* This flagellate lacks the requisite organellar genome encoding subunits required for the assembly and function of canonical respiratory complexes. *T. b. evansi* is thus locked in the bloodstream stage with a mitochondrion that does not produce but rather consumes ATP. We also demonstrate for the first time that tRNAs are imported into and thiolated within the mitochondrion of *T.b. brucei* bloodstream form, the disease causing life cycle stage, while tRNAs are not thiolated in the mitochondrion of *T.b. evansi* **(Results 3.4 - Paris et al. *resubmitted for MBP*).**

Finally, the thesis includes preliminary and therefore unpublished data obtained in frame of an ongoing project. Since we showed that 2-thiomodification plays no role in tRNA import in *T. brucei*, we suggest a base methylation could be involved as a negative determinant in this process **(Results 3.5. - Paris et al. *unpublished data*).**

2. Overview

2.1 *Trypanosoma brucei* as a model organism

Trypanosoma brucei and related flagellates are responsible for African sleeping sickness and numerous other serious diseases that afflict the humans living in tropical regions. During their life cycle, trypanosomes undergo complex changes in the course of transmission from the bloodstream of their mammalian host into the insect vector, the tsetse fly (Fig.1). To these dramatically different environments they respond by significant morphological and physiological changes, including adaptation of their energy metabolism (Bringaud et al. 2006). When compared to the procyclic form, the bloodstream form has a simple type of energy metabolism relying on glycolysis as the main metabolic source of ATP. However, the procyclic stage of the insect host is characterized by the presence of a metabolically fully active mitochondrion, equipped with a branched electron transport chain that uses oxygen as a final electron acceptor (Tielens and Hellemond 2009).

A major reason for trypanosomatids being extensively studied is their medical and economical impact. African sleeping sickness (human African trypanosomiasis) is affecting more than 60 million people in sub-Saharan Africa. As no vaccination is available, treatment solely depends on chemotherapeutic drugs. Unfortunately, the widely used drugs such as suramin, pentamidine, melarsoprol and eflornithine, are highly toxic and ineffective (Rodgers et al. 2009, Steverding et al. 2010).

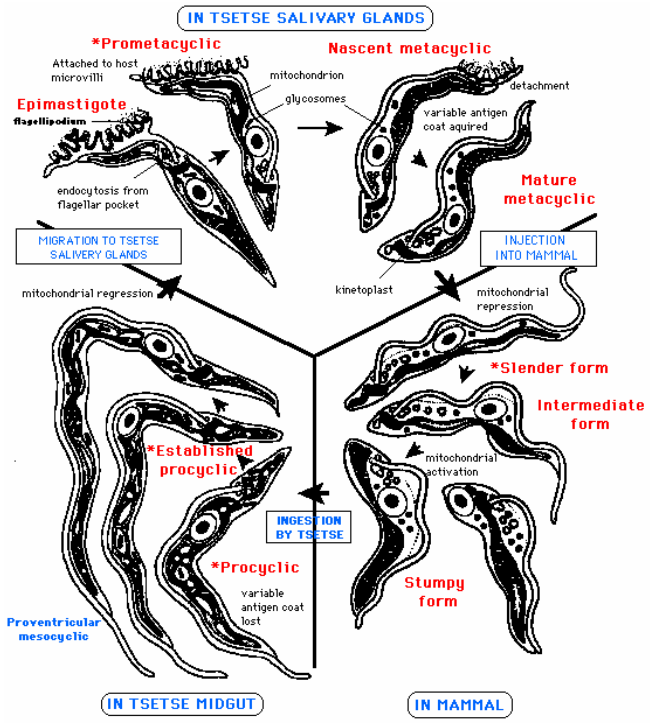

Fig. 1. *Tryoanosoma brucei* schematic representation of developmental cycle in a mammal and in the ts-tse fly vector (adapted from Vickerman 1985).

However, the medical aspect it is not the only reason why these parasites are of interest for

the research community. These unicellular flagellates posses a plethora of biochemical, genetic and morphological features that are unique. They include antigenic variation via programmed DNA rearrangement, glycosomes with localized glycolytic pathway, polycistronic transcription and trans-splicing to generate mature mRNA, mitochondrial RNA editing, lack of tRNA genes in the mitochondrial genome to name just the best known examples (Tan et al. 2002, Fenn and Matthews 2007, Martínez-Calvillo et al. 2010).

Recently, the so-called Tritryp sequencing project has yielded the complete genomes of *T. brucei,* and two other pathogenic trypanosomatids - *Leishmania major* responsible for clinically diverse leishmaniases and *Trypanosoma cruzi* causing Chagas disease (Berriman et al. 2005). This achievement not only allowed comparative genomics, but also provided the groundwork for additional studies focusing on the proteome, transcriptome and other aspects of these related flagellates (Acestor et al. 2009, Panigrahi et al. 2009, Nilson et al. 2010). Although thanks to this and other laudable initiatives we have entered the postgenomic era with these organisms, more than 50 % of their genes have unknown functions of which we know nothing (Berriman et al. 2005).

There are numerous ways how one could assign functions to proteins. The simplest way is via homology search using BLAST and other engines. However, this approach is not very useful in *T. brucei* due to its large evolutionary distance from other model organisms. A powerful technique is tandem affinity purification with subsequent mass-spectrometry analysis. Using this attempt, binding partners of a known protein can be identified. The discovery of RNA interference (RNAi) in *T. brucei* provided a powerful tool to rapidly generate cells in which only the target gene is silenced (Ngo et al. 1998). Unfortunately, neither *L. major* nor *T. cruzi* are amenable to this approach, perhaps due to the loss of the RNAi machinery during evolution (Balaña-Fouce and Reguera 2007). In *T. brucei,* the silencing system is usually based on the ectopic expression of double stranded RNA (dsRNA) from two opposing T7 promoters, stably integrated into a genetic locus, which is regulated by tetracycline induction (Ullu et al. 2004). Alternative and widely used approaches include generation of knock-out cells via homologous recombination, over-expression of variously tagged or mutated versions of genes in questions and other methods.

At the same time forward genetics by which mutant cells are screened for a phenotype of interest followed by identification of genetic lesion(s) that are

responsible for a given phenotype, would enhance our ability to place genes within pathways (Morris et al. 2004). That is why several RNAi-based genomic libraries in procyclic trypanosomes were constructed, in which theoretically each gene in the genome has been silenced in a subset of cells (Morris et al. 2004). To date, this system has been more or less successfully used in different screen (Morris et al. 2002, Drew et al. 2003, Motyka et al. 2006, Monerat et al. 2009). Using this approach, we have identified novel candidates genes the products of which are likely involved in the maintenance of mitochondrial membrane potential (Verner et al. 2010).

2.2 The mitochondrial tRNA import

The mitochondrion is an essential organelle present in various forms in all extant eukaryotes (Dolezal et al. 2006). The typical mitochondrion participates in numerous cellular processes including ATP production via oxidative phosphorylation, synthesis of key metabolites and Fe-S clusters assembly (Kakkar and Singh 2007). In the course of evolution, most bacterial genes from the ancestral alpha-proteobacterial endosymbiont have been either lost or transferred into the nucleus. Consequently, the majority of mitochondrial proteins are encoded in the nucleus and have to be imported into the organelle. Despite the apparently ongoing process of gene transfer to the nucleus, there is a limited number of genes that have been retained in most, if not all mitochondrial genomes (Adams and Palmer 2003). With few exceptions, proteins encoded by these genes are highly hydrophobic components of the respiratory chain or factors of the mitochondrial translation system that produce them (Lithgow and Schneider 2010).

In contrast to proteins, mitochondria generally encode all structural RNAs (rRNA and tRNA) that are needed for organellar translation (Adams and Palmer 2003). However, losses of one or more mitochondrial tRNA genes occurred in a number of unrelated organisms. In such cases the corresponding nucleus-encoded tRNAs have to be imported in order to sustain mitochondrial translation (for recent reviews see Lithgow and Schneider 2010, Alfonzo and Söll 2009, Duchêne et al. 2009, Salinas et al. 2008, Bhattacharyya and Adhya 2004a). To date, tRNA import systems have been experimentally documented in a number of diverse organisms including the ciliate *Tetrahymena* (Suyama 1986; Rusconi and Cech 1996a,b), the apicomplexan *Toxoplasma* (Esseiva et al. 2004a), the kinetoplastid flagellates *Trypanosoma* and *Leishmania* (Simpson et al. 1989; Mottram et al. 1991; Hancock et al. 1992), plants (Marechal-Drouard et al. 1988), the yeast *Sacharomyces* (Martin et al. 1979; Rinehart et al. 2005) and very recently and rather surprisingly, mammals (Rubio et al. 2008). The most extreme situation evolved in two groups of parasitic protozoa, namely the apicomplexans and the trypanosomatids, as both of them completely lost their mitochondrial tRNA genes and therefore must import their whole set from the cytosol (Hanckock and Hajduk 1990, Tan et al. 2002, Esseiva 2004b).

However, in these evolutionary distant protozoan groups cytosol-specific tRNAs are still present (Esseiva et al. 2004b, Geslain et al. 2006). A number of studies published so far have investigated necessary and sufficient factors and/or mechanisms that perform and control tRNA import. Despite laudable efforts, these mechanisms remain poorly understood and much more detailed biochemical and genetic data is needed to shed light on this interesting and enigmatic process.

3.5.1 Determinants for tRNA import

A number of different tRNA import determinants and/or antideterminants were identified by *in vitro* and *in vivo* studies in different organisms (Tab. 1). Most efforts focused on comparing the sequences of imported tRNAs versus non-imported (for recent review see Duchêne et al. 2009). Taken together, the data obtained so far suggest that tRNA import signals are present on mature tRNAs. There are two known exceptions to this rule, namely the 5´ flanking sequence of a precursor $tRNA^{Leu}$ and dicistronic precursor containing the $tRNA^{Ser}$ and $tRNA^{Leu}$, which were shown to be important for its targeting into the *T. brucei* mitochondrion (LeBlanc et al. 1999, Sherrer et. al. 2003). However, *in vivo* experiments with different over-expressed tRNA precursors generally favor a scenario in which import occurs independent of genomic context, with only mature tRNA serving as an import substrate (Tan et al. 2002).

Table 1. Import determinants or antideterminants in matures tRNAs (modifdified form Duchêne et al. 2009)

Organisms	RNA species	Import determinants	Import antideterminants	References
Protozoans				
T. brucei	All except $tRNA^{Meti}$ and $tRNA^{Sec}$	T-stem pair 51–63		Bouzaidi-Tiali et al. (2007)
	$tRNA^{Meti}$		U51-A63 base-pair	
	$tRNA^{Sec}$		U8-U66 base-pair	
Leishmania spp.	$tRNA^{Tyr}$	D-arm		Mahapatra et al. (1998)
	$tRNA^{Trp}$	Anticodon		Kapushoc et al. (2000)
	$tRNA^{Thr}$	Variable loop		Chen et al. (1994)
	$tRNA^{Glu}$		Thio-modification of U34	Kaneko et al. (2003)
T. pyriformis	$tRNA^{Gln}$(UUG)	UUG anticodon		Rusconi and Cech (1996)
Yeast				
S. cerevisiae	tRNALys(CUU)	Anticodon Acceptor stem		Entelis et al. (1998)
Plants				
N. tabaccum	$tRNA^{Val}$	Anticodon		Delage et al. (2003b)
		D- and T-loop		Laforest et al. (2005)
	$tRNA^{Gly}$	Anticodon D-loop		Salinas et al. (2005)

From these findings it is apparent that no universal signal exists and that organellar tRNA import evolved independently multiple times in the course of evolution. Moreover, in the studied systems it remains to be established what is responsible for different localization of imported tRNAs. It has been described that in

some organisms the quantity of imported tRNAs represent only a small fraction (1-7,5 %) of their total cellular amount, which is also the case in *T. brucei* (Schneider and Maréchal-Drouard 2000, Tan et al. 2002). Suprisingly, the relative tRNA steady state levels of individual tRNAs in the related trypanosomatid flagellate *Leishmania tarentolae* were different. The two-dimensional quantitative Northern analysis of the intracellular distribution using specific probes revealed three major groups of tRNA: mainly cytosolic, mainly mitochondrial and tRNAs shared between both compartments (Kapushoc et al. 2002). For some organisms it has been proposed that gene dosage is an important regulatory component of the intracellular tRNA concentration and that this factor may be responsible for the selectivity of tRNA import (Kanaya et al. 2001).

Comparison of cytosolic and mitochondrial codon usage with the subcelullar distribution of tRNAs revealed that the steady state levels of a particular tRNA species are linked not only to the frequency of the cognate codon in the mitochondria but also to its frequency in the cytosol, and are supposed to be fine tuned to allow optimal mitochondrial translation (Vinogradova et al. 2009). The pattern of steady state levels with predominantly cytosolic or mitochondrial localization of a given tRNA resembles the one observed in *L. tarentolae* (Kapushock et al. 2002). However, in *T. brucei* and in *L. tarentolae* no correlation was detected between the extent of mitochondrial localization of different tRNAs and their codon usage (Suyama et al. 1998, Tan et al. 2002).

The three studies mentioned above have a similar drawback, which could possibly negatively influence the interpretation of presented data, as they compared steady state levels in the cytosol and mitochondria using quantitative Northern analysis. There are several ways how tRNAs can escape detection by Northern blot analysis, such as the existence of permutated or split tRNA genes, or genes with multiple introns (Randau and Söll 2008). Morover, tRNA editing events (i.e. C to U editing in trypanosomes) (Simpson et al. 2000) and the presence of nucleotide modification(s), which prevent hybridization to the oligonucleotide probe could also explain elusiveness of certain tRNAs. This problem can be solved by using multiple independent probes annealing to different regions of a particular tRNA molecule. Nevertheless, we do not consider tRNA modifications only as possible artifact of the methods used, as they are likely actively involved in the processes responsible for distinct distribution of tRNAs in different cellular compartments.

Transfer RNAs are widely known for their crucial role in protein synthesis. A typical tRNA does not represent a nude structure as it carries a high number of modified nucleotides (Fig. 2). On average, each tRNA molecule contains 12 modifications, with 75 diverse tRNA modifications being identified so far (Grosjean 2009). In fact, it has been claimed that the tRNA modifications carry more genetic information than tRNA genes themselves (Björk 1995).

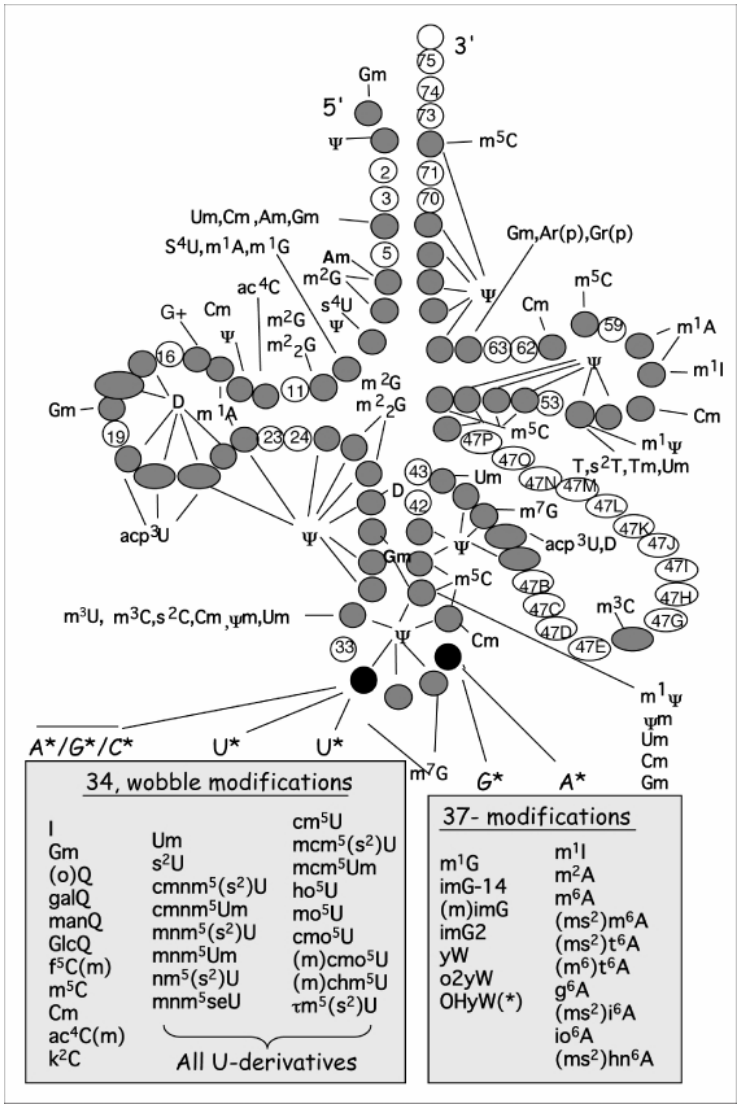

Fig. 2 Schematic representation of tRNA cloverleaf with indicated positions where a given modified nucleoside has been found (taken from Grosjean 2009).

The distribution and roles of tRNA modifications are wide and are based on different structural and physical properties conferred to the tRNA molecules. Nevertheless, there are modification hot spots (see Fig. 2), in particular position 32, the wobble position 34 and the modified purine 37 in the anticodon loop, which have critical function in preventing the loop from translational frameshifting and in accurate codon selection (Gustilo et al. 2008). Additionally, structural modifications located in the core of the tRNA molecule influence its stability and half-life (Motorin and Helm 2010).

As already mentioned above, tRNA modifications can be used by the cell as determinants or antideterminants for tRNA import, since they (potentially) provide a fine tuning mechanism for marking and/or unmarking of tRNAs subjected to organellar import in comparison to their relatively inflexible sequence context (Grosjean 2005). The role of tRNA modifications in relation to tRNA import was investigated in several systems. In plants, three nucleus-encoded $tRNA^{Leu}$ have a methylated guanosine at position 18 if present in the mitochondrion whereas this position is not methylated in their cytosolic counterparts (Maréchal-Drouard et al. 1988,). A similar observation was made in *T. brucei* using enzymatic sequencing of $tRNA^{Lys}$, $tRNA^{Leu}$ and $tRNA^{Tyr}$, each of which bears mitochondrion-specific methylated cytidine residue at the penultimate (32) position 5´ of the anticodon (Schneider et al. 1994). It has been hypothesized that this modification may act as a signal for transport of the tRNAs across the mitochondrial membrane (Schneider et al. 1994). Interestingly, this modification present in the mature mitochondrial $tRNA^{Tyr}$ was not found in the mutant form of this tRNA defective in splicing. However, this mutant $tRNA^{Tyr}$ was properly imported into the mitochondrion suggesting that the modified cytidine residue is indispesable for import and most likely is a general feature of imported tRNAs (Schneider et al. 1994).

In another case, it has been proposed that compartment specific thio modifications (2-thiouridine; s^2U) in the anticodon loop of the $tRNA^{Glu}$ and $tRNA^{Gln}$ may represent negative determinants for tRNA import in *L. tarentolae* (Kaneko et al. 2003). Cytosol-specific localization of this modification was used as evidence supporting this scenario. Moreover, it has been shown that natively purified thio-modified $tRNA^{Glu}$ is imported less efficiently into the mitochondrion than its *in vitro* transcribed counterpart lacking such a thio-modification (Kaneko et al. 2003).

Recently, the same type of retention mechanism was analyzed in *T. brucei* (Paris et al. 2009). This functional study was inspired by the observation that thiolation in yeast depends on components of the Fe-S cluster biosynthesis pathway (ISC) (Nakai et al. 2007, Nakai et al. 2004, Lill and Mühlenhoff 2008). However, the ablation of cysteine desulfurase (Nfs), which is a major player in the ISC assembly (Lill and Mühlenhoff 2008, Lill 2009), did not alter the levels of (non) thio-modified trypanosomal $tRNA^{Glu}$ and $tRNA^{Gln}$. Additionally, the same extent of *in vitro* import was achieved with native thio modified $tRNA^{Glu}$ and its chemically dethiolated counterpart (Paris et al. 2009). This result is in full agreement with the recent publication from the Schneider lab (Bruske et al. 2009) showing that not only Nfs but also Nbp35 and Cfd1 from the cytosolic Fe-S cluster assembly (CIA) machinery are involved in cytosolic tRNA thiolation. Moreover, the latter two proteins were shown be dispensable for thiolation of mitochondrial tRNAs (Bruske et al. 2009). These authors explain the observed mitochondrial enrichment of the non-thiolated $tRNA^{Glu}$ by post-import removal of the modification rather than selective import of non-thiolated tRNAs, a statement supported by experiment with overexpressed tagged $tRNA^{Glu}$ (Bruske et al 2009). So far no protein responsible for this predicted dethiolation activity was identified.

Except for trypanosomatids, the only other group of organisms that imports the whole set of mitochondrial tRNAs are apicomplexans. In *Toxoplasma gondii,* their omnipresent initiator $tRNA^{Met}$ is the only cytosol-specific tRNA, whereas all other nucleus-encoded tRNAs are present both in the cytosol and the mitochondrion (Esseiva et al. 2004a). Same cytosolic retention was observed in *T. brucei*, and a specific localization determinant was implicated with this phenomenon (Bouzaidi-Tiali et al. 2007). Indeed, the same motif is present in *T. gondii* initiator $tRNA^{Met}$ further supporting this notion. Moreover, this tRNA remained in the cytosol when expressed in *T. brucei* (Esseiva et al. 2004a). However, in contrast to trypanosomatids no thio-modifications were detected in the $tRNA^{Gln}$ of *T. gondii* suggesting that in contrast to *L. tarentolae*, thiolation is not involved in regulation of tRNA import in this protist (Esseiva et al. 2004a).

3.5.1 Role of Fe-S cluster assembly in thio-modifications of cytosolic and mitochondrial tRNAs

The precise codon – anticodon selection that occurs during translation requires a post-transcriptional modification at the first anticodon position 34 (so-called wobble position) of the tRNA (Suzuki 2005). The uridines at wobble position 34 in $tRNA^{Lys}$, $tRNA^{Glu}$ and $tRNA^{Gln}$ from many species contains sulfur at the 2-position (s^2U) and are further modified to 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) and 5-methoxycarbonylmethyl-2- thiouridine (mcm^5s^2U) in *Escherichia coli,* yeast and humans. The s^2 group of s^2U_{34} stabilizes anticodon structure, improves ribosome binding ability to the tRNA and helps to maintain a proper reading frame (Agris et al. 2007).

In *E. coli,* two conserved proteins, IscS and MnmA, have been previously reported to be responsible for 2-thiouridine formation of mnm^5s^2U (Kambampati and Lauhon 2003). IscS functions as a general sulfur donor by delivering it to proteins in various metabolic pathways including ISC biosynthesis, where it transfer sulfur atom from L-cystein to a nascent cluster (Ayala-Castro et al. 2008). Moreover, IscS has an important role in providing sulfur for five different thio-modifications (Nilson et al. 2002). MnmA is a thiouridylase that catalyzes 2-thiolation of mnm^5s^2U by recognizing substrate tRNA. *In vitro* 2-thiolation was partially reconstituted with purified IscS and MnmA but low yield and specificity indicated that more factors are involved in this process (Kambampati and Lauhon 2003).

Recent studies revealed a complex sulfur relay system composed of multiple sulfur mediators that select and facilitate sulfur flow to 2-thiouridine (Ikeuchi et al. 2006). Using ribonucleome analysis, a method based on reverse genetic approach combined with mass spectrometry genes TusA,B,C,D and E encoding sulfur mediators essential for 2-thiolation of mnm^5s^2U in *E. coli* were identified and biochemicaly characterized (Ikeuchi et al. 2006). The efficient 2-thiouridine formation was reconstituted *in vitro* with recombinant proteins TusA, complex TusBCD and TusE and the previously identified IscS and MnmA. By directly interacting with IscS and stimulating its desulfurase activity, TusA accepts the persufide group from IscS and transfers the sulfur to TusD in the TusBCD complex. TusE interacts with the TusBCD complex, accepting the sulfur from TusD, Moreover, TusE also interacts with the MnmA-tRNA complex (Ikeuchi et al. 2006). Subsequently, the sulfur is transferred by the nucleophilic attack to the activated C2 position of the wobble

uridine, forming thiocarbonyl group and releasing AMP (Numata et al. 2006). These processes seem to work independently of any Fe-S cluster containing protein (Leipuviene et al. 2004). On the other hand, some thio-modifications of adenosine and cytidine depend on the Fe-S proteins MiaB and TtcA, respectively. In these cases, the sulfur atoms assembled into the Fe-S cluster of MiaB or TtcA have been proposed to be used for the 2-thiomodification (Jäger et al. 2004, Hernández et al. 2007). Nevertheless, the ISC-dependent as well as ISC-independent pathways require IscS as a sulfur donor (Leipuviene et al. 2004).

The connection between 2-thiouridine formation and the Fe-S cluster assembly was demonstrated also in the yeast *S. cerevisiae,* where thiolation occurs in both cytosol and mitochondrion. Three components of the CIA pathway (Cfd1, Nbp35 and Cia1) and two scaffold proteins (Isu1 and Isu2) from the mitochondrial ISC assembly pathway are required for 2-thiolation of cytoplasmic but not mitochondrial tRNAs (Nakai et al. 2007). Moreover Nfs, a homolog of the bacterial IscS was shown to be indispensable for the biogenesis of 2-thiouridine in both cytoplasmic and mitochondrial tRNAs (Nakai et al. 2004). This indicates that cytoplasmic 2-thiouridine formation is dependent on an Fe-S cluster-containing protein, while mitochondrial tRNA thiolation is independent on ISC (Nakai et al. 2007).

Several other Fe-S cluster carrying proteins are likely involved in synthesis of tRNA modifications. A member of the yeast elongator complex, Elp3 was implicated with the early steps of modification of the wobble uridine (Huang et al. 2005). However, the Elp3 mutant still retained the 2-thiomodification although it was lacking the 5-methoxycarbonyl group (Huang et al. 2005). It was also demonstrated that s^2U and mcm^5 in the cytosolic tRNA influence each others efficiency (Nakai et al 2008), which indicates that this complex process requires additional yet unidentified factors. Finally a role of the CIA pathway itself in tRNA thiolation cannot be excluded since Nbp35 and Nar1 are known to contain Fe-S clusters (Balk et al. 2004, Hausmann et al. 2005).

Thiolation of tRNAs in both cellular compartments of the yeast cell directly depends on mitochondrial Nfs (Nakai et al 2004, Umeda et al. 2005, Nakai et al. 2007) but apparently does not require the involvement of Fe/S-containing enzymes. This suggests a role for this desulfurase in sulfur-relay, independent of its function in the Fe/S cluster assembly.

The factors that relay the sulfur from Nfs to tRNAs differ in the two compartments. In mitochondria, Mtu1, a homolog of the bacterial MnmA, acts as the tRNA-specific 2-thiouridylase (Umeda et al. 2005). However, in contrast to the bacterial system, the TusABCDE complex was not found in the mitochondria. Since in bacteria this complex transfers sulfur from IscS to MnmA (Ikeuchi et al. 2006), some other mitochondrial protein(s) may have taken over this function in eukaryotes. One possible candidate is the recently identified Isd11, a small mitochondrial protein that represents an evolutionary invention of the eukaryotic cells (Richards and van der Giezen 2006). Isd11 forms a tight complex with mitochondrial Nfs, via which it participates in the mitochondrial Fe-S cluster biogenesis (Adam et al. 2006, Wiedemann et al. 2006). Recently, our group proved that Isd11 partakes in both cytoplasmic and mitochondrial tRNA thiolation in *T. brucei* (Paris et al. 2010). This finding supports the notion that Nfs-Isd11 complex works as a functional equivalent of the bacterial TusABCDE sulfur relay system.

Recently, significant progress has been achieved in the identification and characterization of proteins mediating transfer of the activated sulfur from the cysteine residue to the 2-thiogroup of mcm^5s^2u in the cytoplasmic tRNAs. Several groups have independently characterized novel function for Urm1, the earliest ubiquitin-like protein in the pathway leading to the thio-modification of the cytosolic tRNAs. Specifically, along with Urm1 several other genes (Uba4, Ncs2, Ncs6 and Tum1) were identified in a genome-wide screens in *S. cerevisiae* to be responsible for 2-thiouridine formation (Huang et al. 2008, Nakai et al. 2008, Noma et al. 2009, Liedel et al. 2009). Urm1 belongs to the UBL (ubiquitin-like modifiers) family, members of which are involved in various biological processes, mostly regulating activity and function of numerous target proteins by conjugation with them via a so-called urmylation. This labeling is derived from Urm1, likely the most ancient member of the UBL family, which emerged as a sulfur carrier in the process of tRNA modification (Perdioli et al. 2008).

Despite the identification of novel sulfur-flow components, it remains unclear where in the cell 2-thiouridine formation take place. Nfs is mainly located in the mitochondrion but trace amounts of this protein in the nucleus are essential for cell survival (Land and Rouault 1998, Naamati et al. 2009). Moreover, it was proposed that nuclear Nfs has an essential role in a pathway other than the Fe-S protein biogenesis and 2-thiomodification (Nakai et al. 2007). Based on the protein targeting

motif and *in vitro* experiments, a plausible model for Urm1-mediated 2-thiouridylation has been proposed, which is detailed below (Noma et al. 2009). The first member of the eukaryotic sulfur flow, Tum1, is predicted to occur in both the cytosol and the mitochondria. Thus, Tum1 could interact with Nfs and accept the persulfide group within the organelle. Upon its export to the cytosol, Tum1 was proposed to release the activated sulfur from by means of Uba4. This way Tum1 may function as a shuttle between both cellular compartments. The rest of the machinery is predicted to work in the cytoplasm, commencing with thiocarboxylation of Urm1 by Uba4, and followed by 2-thiouridine formation of tRNAs mediated by Ncc2p/Nc6p (Noma et al. 2009).

3.5.1 tRNA Import factors and mechanisms

Since tRNAs are relatively large hydrophilic and negatively charged macromolecules, they most likely require an active import machinery against the proton electrochemical gradient on the mitochondrial inner membrane. the only common feature of tRNA import machineries examined so far is the use of ATP as the energy source for tRNA translocation, while other import factors and mechanisms widely differ among studied organisms (Fig. 3).

In the yeast *S. cerevisiae* one of the nucleus-encoded isoacceptor $tRNA^{Lys}_{CUU}$ is imported into the mitochondrion by a mechanism similar to the protein translocation machinery (Tarassov et al. 1995a). This import pathway is dependent on the electrochemical potential on the mitochondrial inner membrane with some additional factors involved. Mitochondrial targeting requires the aminoacylated form of $tRNA^{Lys}_{CUU}$, for which the cytosolic lysyl-tRNA synthetase is responsible (Tarassov et al. 1995b). Next the charged tRNA is targeted by the glycolytic enzyme enolase to the surface of the mitochondrion. Enolase is subsequently released, allowing the tRNA to form a complex with the precursor of mitochondrial lysyl-tRNA synthetase (Tarassov et al. 1995b), which its N-terminal domain was reported to be essential and sufficient for import (Kamenski et al. 2010). Finally, the complex is translocated into the organellar matrix through the TOM and TIM complexes of the protein import channel (Entelis et al. 2006). The efficiency of this import pathway was shown to be regulated by the ubiquitin proteasome system (Brandina et al. 2007). Moreover, import of $tRNA^{Lys}_{CUU}$ was shown to be essential for mitochondrial protein synthesis under temperature stress condition, when the mitochondria-encoded $tRNA^{Lys}_{UUU}$ becomes undermodified at the wobble position of the anticodon and thus can no

longer recognize the AAG codon for lysine in mitochondrial transcripts (Kamenski et al. 2007).

In contrast to $tRNA^{Lys}_{CUU}$, the two cytosolic isoacceptors for $tRNA^{Gln}$ were shown to be imported by a mechanism completely different from the protein translocation pathway. Surprisingly, the organellar import of these cytosolic $tRNA^{Gln}$ was reconstituted *in vitro* without the addition of any cytosolic factors and and in the absence of aminoacylation (Reinhart et al. 2005).

The first evidence of tRNA import in mammals was obtained from the mitochondria of several marsupial species, in which the mitochondria-encoded lysyl-tRNA gene deteriorated into a pseudogene. An *in vitro* aminoacylation assay showed that the absence of this tRNA is indeed compensated by import of the nuclear-encoded $tRNA^{Lys}$ into the marsupial mitochondria (Dörner at al. 2001).

Until recently, it has been believed that human mitochondria do not import tRNAs under normal physiological conditions. However, contrary to the expectations the nucleus-encoded $tRNA^{Gln}$ was shown to be imported into the human and rat mitochondria (Rubio et al. 2008). *In vitro* translocation of this tRNA does require neither addition of protein factors, nor mitochondrial membrane potential, yet the mechanism remains to be further characterized (Rubio et al. 2008).

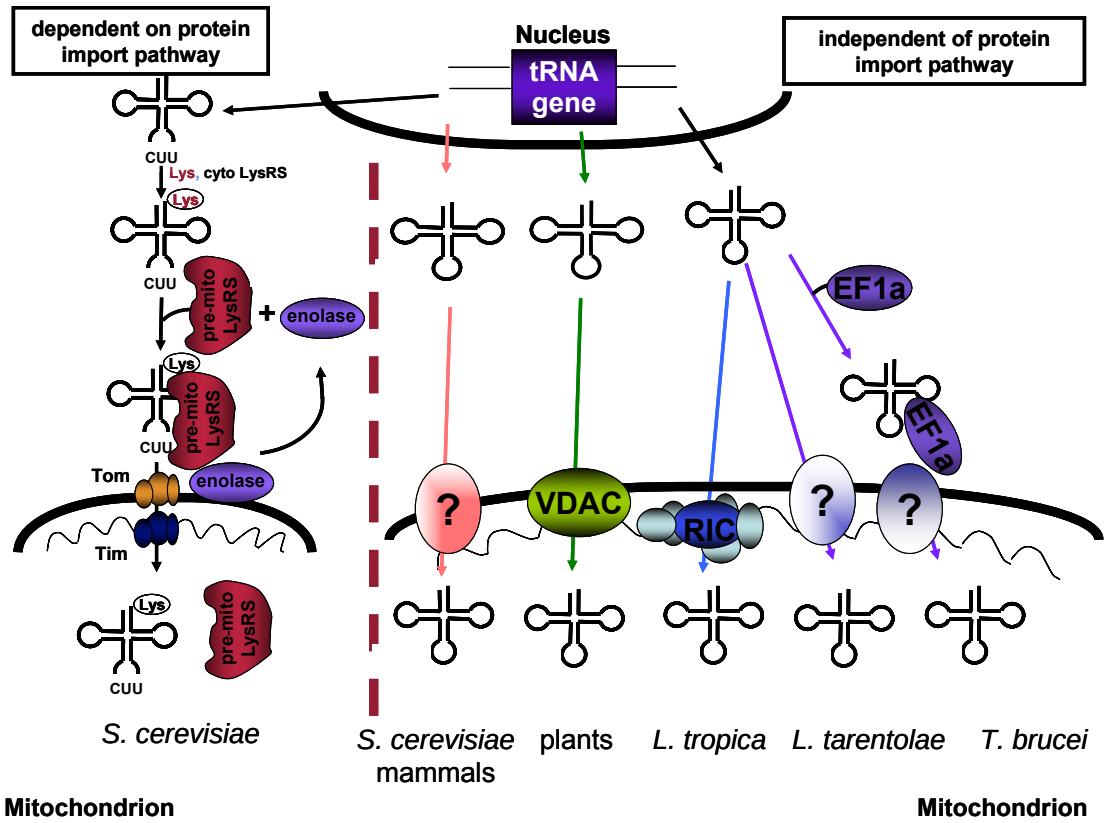

Fig. 3 Different mechanisms of mitochondrial tRNA import (modified from Alfonzo and Söll 2009)

In plants, the voltage dependent anion channel (VDAC) known as an evolutionary conserved metabolite transporter, was shown to be crucial for the translocation of tRNAs through the outer mitochondrial membrane (Salinas et al. 2006) Plant mitochondrial tRNA import was proven to be independent on additional cytosolic factors, while mitochondrial membrane potential was needed (Delage et. al. 2003). This indicates that the tRNA import pathway is similar to protein translocation a proposal backed by the fact that two key components of the translocase of the outer mitochondrial membrane (TOM22 and TOM40) are important for tRNA binding at the surface of the organelle (Salinas et al. 2006). However, an *in vitro* competition assay with the mitochondrial pre-sequence of the ATP synthase $F_1\beta$ subunit previously described as inhibitor of the protein import, indicated that proteins and tRNAs in plants are imported via two distinct albeit perhaps partially overlapping mechanisms (Salinas et al. 2006).

In trypanosomatids such as *T. brucei* and *Leishmania* spp. contradictory data regarding the requirement of mitochondrial membrane potential and additional cytosolic factors for tRNA import have been published. Indeed, no cytosolic factors were needed in the reconstituted tRNA import assays *in vitro* (Mahapatra et al. 1994, Yermovsky-Kammerer and Hajduk 1999, Nabholz et al. 1999, Rubio et al. 2000), while in *T. brucei* the interaction between the cytosolic elongation factor (eEF1) and some tRNAs was shown *in vivo* to mediates the specificity of tRNA import via the eEF1 interaction with single T-stem nucleotide pair at position 51:63 in *T. brucei* (Bouzaidi-Tiali et al. 2007). This situation resembles the protein-dependent import pathway in yeast, where the $tRNA^{Lys}$ is co-imported with the pre-lysyl tRNA synthetase (Tarasov et al. 1995). However, this does not seem to be the case in *T. brucei,* since higher amount of tRNA in the *in vitro* assay did not compete with protein import (Nabholz et al. 1999) and since the eEF1 remains in the cytosol (Bouzaidi-Tiali et al. 2007). Taken together, this data indicate that proteins and tRNAs are imported by different mechanisms, while *in vitro* experiments in some studies suggested that tRNA import requires electrochemical gradient across the mitochondrial inner membrane (Mukherjee et al. 1999, Yermovsky-Kammerer and Hajduk 1999, Bhattacharyya et al. 2004b), although such a requirement was questioned by others (Nabholz et al. 1999, Rubio et al. 2000).

These differences may reflect the variability among different trypanosomatid species used in experiments, or may even by due to the applied mitochondrial

isolation procedures. The indispensability of the mitochondrial membrane potential, the main prerequisite for protein import was proven *in vivo* by the inhibition of import of over-expressed tRNA upon incubation with CCCP, a mitochondrial uncoupler, which destroys membrane potential (Bouzaidi-Tiali et al. 2007). We can only speculate how artificial the used system was. There is a possibility that tetracycline-induced over-expression resulted in an excessively high production of tRNA, which cannot be properly folded and then imported into the organelle. Contradictory results were obtained by our group, since despite decreased membrane potential in cells with silenced Nfs (Smíd et al. 2006), the steady state levels of imported tRNAs remained unaltered both *in vitro* and *in vivo* (Paris et al. 2009).

The plant homolog of VDAC was suggested to be an essential metabolite transporter in *T. brucei*, although, is not required for mitochondrial tRNA import (Pusnik et al. 2009). Moreover, in plants, several subunits of the TOM complex are important binding of tRNAs to the outer membrane, but such a scenario is unlikely in trypanosomes due to the apparent absence of most TOM subunits (Schneider et al. 2008, Pusnik et al. 2009).

The only tRNA import mechanism identified so far in trypanosomatid flagelates is a large multisubunit complex (~ 640 kDa) called RNA import complex RIC that has been isolated from the inner mitochondrial membrane of *Leishmania tropica* and shown to promote ATP-dependent tRNA import upon its incorporation into liposomes (Bhattacharyya et al. 2003). A working model of tRNA import based on this and oter studies postulates that binding of tRNAs to the RIC triggers a series of reactions that lead to tRNA translocation, activation of ATPase, ATP hydrolysis in the mitochondrial matrix, export of protons and finally the proton-dependent tRNA translocation via an RNA channel (Bhattacharyya and Adhya 2004b):539-46, Goswami et al. 2006, Mirande 2007) (Fig. 4). Recently, a detailed characterization of individual subunits of the RIC complex was presented (Mukherjee et al. 2007, Mirande 2007). Using affinity purification of RIC and subsequent mass spectrometry analysis, 11 putative RIC subunits were detected, eight of which are nucleus-encoded (Tab. 2). The most surprising feature of RIC is that five components are shared with the respiratory complexes, and therefore appear to be bi-functional (Mukherjee et al. 2007). Systematic RNAi knock-downs of individual RIC components revealed that six of them are essential *in vivo* (Mukkherjee et al. 2007).

However, in recent reviews several controversial issues were raised concerning the RIC in *L. tropica* such as the use of RNAi anti-sense strategy for conditional knock-downs or reconstitution of functional RIC from subunits isolated from denaturating SDS gels (Salinas et al. 2008, Lithgow and Schneider 2010). Moreover, the editor-in-chief of PNAS recently announced an “Editorial Expression of Concern” regarding another article by the Adhya group - Goswami et al. 2006. The validity of the results was questioned by some readers, not only because of an apparent duplication of certain figures in this article but also to other issues (Schekman 2010). The fact that to date RIC has been found only in *L. tropica* by the Adhya laboratory and that we and others have observed surprising differences between the relatively closely related *T. brucei* and *L. tropica* only contributes to the ambiguous situation in the field.

It was shown previously in *T. brucei* that the Rieske protein, which is a subunit of the respiratory complex III, is required for both the maintenance of membrane potential and respiration (Horváth et al. 2005). However, in contrast to its function in *L. tropica* (Mukherjee et al. 2007), the Rieske protein is not necessary for tRNA import machinery in *T. brucei* (Paris et al. 2009). In addition, our recent detection of tRNA import in the bloodstream stage of *T. brucei* (Paris et al. resubmitted) argues strongly against any involvement of respiratory complexes in this process, as most of them are absent in this life cycle stage (Bringaud et al. 2006). Consequently, the involvement in tRNA import of other essential RIC subunits, allegedly shared with respiratory complexes, is highly unlikely in *T. brucei* (Paris et al. resubmitted).

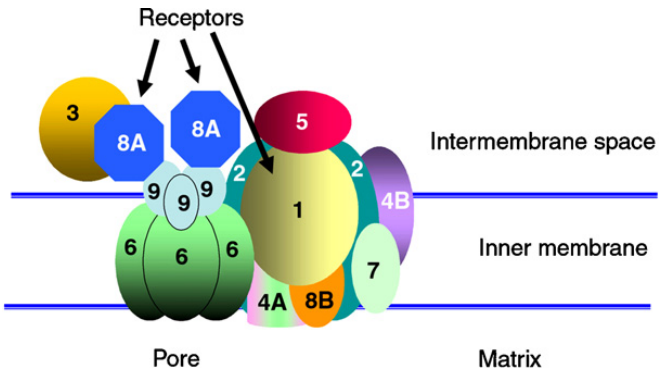

Fig. 4 RNA import complex (RIC) as a model for the tRNA import through the inner mitochondrial membrane in *Leishmania. tropica* (taken from Adhya 2007)

Table 2 Identities of RNA import complex subunits. (taken from Mukherjee et al. 2007)

Subunit no.	Size (kDa)	Mole ratio*	Gene identity[++]	Gene copy no.	Sequence/ structural similarity with known proteins[§]	Complex with which shared	Role in import
1	62	1	*LmjF05.0510*	2	F1-ATP synthase subunit α	V	Essential; type I tRNA receptor; tRNA-dependent ATPase; type II tRNA activator
2	56	2	***MURF1***	1	None	None	Not essential[#]
3	47	1	*LmjF21.0340*	1"	Core proteins (complex III)	None[¶]	Not essential
4A	43	1	*LmjF07.1110*	1	None	None	Essential
4B	43	0,5	***MURF2***	1	None	None	Not essential[#]
5	40	1	*LmjF12.0670*	1	None	IV	Not essential
6	35	3	*LmjF35.1540*	1	Complex III iron-sulphur protein	III	Essential
7	28	1	***MURF2***	1	None	None	Not essential[#]
8A	21	2	*LmjF35.0100*	1	Complex III subunit 6b	III	Essential; type II tRNA receptor; negative regulator of type I tRNA
8B	2	1	*LmjF04.0630*	1	None	None	Essential
9	19	3	*LmjF21.1710*	1	Complex IV subunit VI	IV	Essential

*Stoichiometry determination described in the supplementary information online. [++]Nuclear-encoded genes (italics) designated as in the Leisgmania major database; mitochondrion-encoded genes (bold italics) as in the kinteoplastid U-insertion/deletion editing database. [§]BLAST and SWISS MODEL. "Multigene family of M16 metalloproteases. [¶]Other homologues in complex III. [#]By in vitro import assay. MURF, maxicircle unidentified reading frame.

3.5.1 The scope of the tRNA import

The interest in the mitochondrial tRNA import is steadily increasing during the last decade. This phenomenon is being extensively studied not only for its essentiality but also thanks to some practical implications.

Causative agents of several serious parasitic diseases, such as human African trypanosomiasis, leishmaniases and/or malaria completely lack genes encoding tRNAs in their mitochondrial genomes (Feagin 2000), their organellar translation exclusively depends on tRNA import from the cytosol, which distinguishes the parasitic cells from their hosts and thus represents a promising drug target (Monzote and Gille 2010)

Mutations in mitochondrial DNA are associated with a range of human diseases. Most of these mutations are located within mitochondrial tRNA genes or in genes encoding proteins responsible for tRNA maturation, modification, folding or amino-acylation, their association with translational factors and various functions on the ribosome (Kirino and Suzuki 2005). For example, a pathogenic point mutation at position 8344 in the mitochondrial $tRNA^{Lys}$ gene is found in most patients with myoclonous epilepsy associated with ragged-red fibers (MERRF) (Shoffner et al. 1990). Recent studies showed that the MERRF phenotype, which is caused by the decreased mitochondrial translation and impaired respiration can be rescued by several approaches. In a high profile study was reported that the RIC complex identified in *L. tropica* can enter cultured human cells via calveolin-dependent pathway, inducing import of cytosolic human tRNAs, which cured mitochondrial defects associated with MERRF syndrome (Mahata et al. 2006). However, it seems that human cells have a natural ability to import tRNAs, only prerequisite is the presence of sufficient amount of ATP (Rubio et al. 2008). Another approach resulted in partial rescue of mitochondrial function after the import of mutated $tRNA^{Lys}$, which was stably over-expressed in patient-derived fibroblast bearing the MERRF mutation (Kolesnikova et al. 2004). In the most recent study, a set of small RNA molecules significantly improving efficiency of import into yeast and human mitochondria was characterized, opening the possibility of creating a new mitochondrial vector system able to target therapeutic oligonucleotides into deficient human mitochondria (Kolesnikova et al. 2010).

Another interesting aspect of mitochondrial tRNA import is the intimate connection between tRNA and apoptosis. The best known type of apoptosis is defined by the release of cytochrome *c* from the mitochondria, which triggers a series of reactions leading to cell death (Riedl and Salvesen 2007). Recent data reported binding of cytochrome *c* by various tRNAs, consequently preventing its association with caspase activator Apaf-1, tRNAs therefore posses the capacity to directly inhibit apoptosis (Mei et al 2010). Import of cytosolic tRNAs may thus represent another way by which cells tightly regulate apoptosis. This speculation is supported by the experimental enhancement of apoptosis by tRNA hydrolysis in living cells, whereas microinjection of tRNA had an inhibitory effect (Mei et al. 2010).

3. Results

3.1 Thiolation and the Rieske protein (an essential component of the *Leishmania* RIC complex) play negligible roles in *Trypanosoma brucei* tRNA import

Paris Z, Rubio MAT, Lukeš J, Alfonzo JD.

RNA (2009) 15: 1398-1406

Due to a complete lack of the tRNA genes in the mitochondrial genome of *Trypanosoma brucei*, all tRNAs needed for mitochondrial translation have to be imported into the organelle from the cytosol. A previous study showed that the modified nucleotide s^2U could act as a negative determinant for mitochondrial tRNA import in another kinetoplastid, *Leishmania tarentolae*. We have investigated whether the same type of cytosolic control for tRNA retention exists in *T. brucei*. Based on Northern analysis with subcellular RNA fractions and in vitro import assays, we demonstrate that silencing of the cysteine desulfurase, TbNfs (TbIscS), the key enzyme in tRNA thiolation (s^2U) and Fe-S cluster formation in vivo, has no effect on tRNA partitioning. This observation is especially surprising in light of a recent report suggesting that in *L. tropica* the Rieske Fe-S protein is an essential component of the RNA import complex (RIC). In line with the above observation, we also show that down-regulation of the Rieske protein by RNA interference, similar to the TbNfs knockdowns, has no effect on import. The data presented here supports the view that in T. brucei: (1) s^2U is not a negative determinant for tRNA import; (2) the Rieske protein is not an essential component of the import machinery, and (3) since the Rieske protein is essential for respiration and maintenance of inner mitochondrial membrane potential, neither process plays a critical role in tRNA import. We therefore suggest that the *T. brucei* import machinery differs substantially from what has been described in *Leishmania*.

Thiolace tRNA ani protein Rieske nemají vliv na import tRNA do mitochodrie *Trypanosoma brucei.*

Všechny mitochondriální tRNA geny jsou kódovány v jádře *Trypanosoma brucei*, a proto musí být jednotlivé tRNA importovány z cytosolu. V této práci jsme ukázali, že vypnutí cystein desulfurázy (TbNfs), klíčového enzymu thiolace tRNA (s^2U) a tvorby Fe-S center, nemá žádný vliv na tRNA import. Výsledek je zvláště překvapující s ohledem na nedávné studie naznačující, že Fe-S protein Rieske je nezbytnou součástí komplexu importu tRNA u *Leishmania tropica*. Dále jsme prokázali, že vypnutí genu kódující protein Rieske pomocí RNA interference, podobně jako vypnutí genu pro TbNfs, nemá žádný vliv na tRNA import. Z našich dat vyplývá, že s^2U není negativním prvkem ovlivňujícím tRNA import, protein Rieske není zásadní složkou importu a jelikož je Rieske nezbytný pro dýchání a udržování mitochondriálního membránového potenciálu, nehraje žádná z těchto funkcí rozhodující roli v importu tRNA do mitochondrie *T. brucei*.

3.2 Thiolation controls cytoplasmic tRNA stability and acts as a negative determinant for tRNA editing in mitochondria

Wolgamuth-Benedum JM, Rubio MAT, **Paris Z**, Long S, Poliak P, Lukeš J, Alfonzo JD.

J Biol Chem (2009) 284: 23947-23953

Kinetoplastids encode a single nuclear tryptophanyl tRNA that contains a CCA anticodon able to decode the UGG codons used in cytoplasmic protein synthesis but cannot decode the mitochondrial UGA codons. Following mitochondrial import, this problem is circumvented in *Trypanosoma brucei* by specifically editing the $tRNA^{Trp}$ anticodon to UCA, which can now decode the predominant mitochondrial UGA tryptophan codons. This tRNA also undergoes an unusual thiolation at position 33 of the anticodon loop, the only known modification at U33 in any tRNA. In other organisms, tRNA thiolation is mediated by the cysteine desulfurase, Nfs1 (IscS). However, *T. brucei* encodes two Nfs homologues, one cytoplasmic and the other mitochondrial. We show by a combination of RNA interference and Northern and Western analyses that the mitochondria-targeted TbNfs, and not TbNfs-like protein, is essential for thiolation of both cytosolic and mitochondrial tRNAs. Given the exclusive mitochondrial localization of TbNfs, how it mediates thiolation in the cytoplasm remains unclear. Furthermore, thiolation specifically affects thiolated tRNA stability in the cytoplasm but more surprisingly acts as a negative determinant for the essential C to U editing in *T. brucei*. This provides a first line of evidence for mitochondrial C to U editing regulation in this system.

Thiolace cytosolických tRNA má vliv na jejich stabilitu a zároveň slouží jako negativní determinant pro editovani tRNA v mitochondrii.

Kinetoplastida kódují jedinou jadernou tryptophanyl tRNA, která nese CCA antikodon, schopen dekódovat UGG kodony používané v cytoplazmatické syntéze bílkovin. Avšak nemůže dekódovat UGA kodony v mitochondrii. U *Trypanosoma brucei* je tento problém vyřešen následným mitochondriálním importem a editací tRNA (Trp) antikodonu na UCA, který lze nyní dekódovat převládajícím mitochondriálním kodonem UGA pro tryptofan. Tato tRNA také podstupuje neobvyklou thiolaci na pozici 33. U jiných organismů je tRNA thiolace zprostředkována cystein desulfurázou, Nfs. Prokázali jsme, že TbNfs, je zásadní pro thiolaci jak cytosolických, tak mitochondriálních tRNA. Kromě toho, že thiolace tRNA ovlivňuje její stabilitu v cytoplasmě, se dále navíc chová jako negativní determinant pro editaci C-U v *T. brucei*. Tato práce poskytuje první linii důkazů pro regulaci editingu C-U v mitochondrii u tohoto systému.

3.3 The Fe/S cluster assembly protein Isd11 is essential for tRNA thiolation in *Trypanosoma brucei*

Paris Z, Changmai P, Rubio MA, Zíková A, Stuart KD, Alfonzo JD, Lukeš J.

J Biol Chem (2010) 285: 22394-22402

Fe/S clusters are part of the active site of many enzymes and are essential for cell viability. In eukaryotes the cysteine desulfurase Nfs (IscS) donates the sulfur during Fe/S cluster assembly and was thought sufficient for this reaction. Moreover, Nfs is indispensable for tRNA thiolation, a modification generally required for tRNA function and protein synthesis. Recently, Isd11 was discovered as an integral part of the Nfs activity at an early step of Fe/S cluster assembly. Here we show, using a combination of genetic, molecular, and biochemical approaches, that Isd11, in line with its strong association with Nfs, is localized in the mitochondrion of *T. brucei*. In addition to its involvement in Fe/S assembly, Isd11 also partakes in both cytoplasmic and mitochondrial tRNA thiolation, whereas Mtu1, another protein proposed to collaborate with Nfs in tRNA thiolation, is required for this process solely within the mitochondrion. Taken together these data place Isd11 at the center of these sulfur transactions and raises the possibility of a connection between Fe/S metabolism and protein synthesis, helping integrate two seemingly unrelated pathways.

Protein Isd11 podílející se na tvorbě Fe/S center je nezbytný pro thiolaci tRNA u *Trypanosoma brucei*

Fe/S centra jsou nedílnou součástí aktivních míst mnoha proteinů a jsou nezbytná pro životaschopnost buňky. Původně bylo předpokládáno, že cystein desulfuráza Nfs (IscS) je sama o sobě dostačující při poskytování síry v reakci pro tvorbu Fe/S center. Nedávno byl identifikován protein Isd11 , který se společně s Nfs účastní výše popsané reakce. Pomocí kombinace metod molekulární biologie a biochemie jsme prokázali, že Isd11 je v komplexu s Nfs, který je lokalizovaný v mitochondrii *T. brucei*. Kromě role Isd11 při tvorbě Fe/S center jsme prokázali, že tento protein se účastní rovněž thiolace cytosolických a mitochondriálních tRNA, zatímco protein Mtu1 se účastní pouze thiolace tRNA v mitochondrii. Naše data naznačují jasnou spojitost mezi Fe/S metabolismem a syntézou bílkovin, což napomáhá propojit dva zdánlivě nesouvisející procesy.

3.4 Futile import of tRNAs and proteins into the mitochondrion of *Trypanosoma brucei evansi*

(resubmitted to Molecular and Biochemical Parasitology)

(This part comprising of 15 pages contains still unpublished data, which is present in the original thesis deposited at the Faculty of Science)

3.5 Unpublished data

(This part comprising of 9 pages contains still unpublished data, which is present in the original thesis deposited at the Faculty of Science)

4. Conclusions

This Ph.D. thesis has illuminated several aspects of mitochondrial Fe-S cluster biogenesis and tRNA import and modifications, as well as possible interactions between these fundamental processes. It is composed of three published papers, one resubmitted paper and unpublished results.

The main conclusions are as follows:

1. The Fe-S cluster assembly protein Isd11 partakes in both cytosolic and mitochondrial tRNA thiolation, whereas Mtu1 (another protein implicated in tRNA thiolation in other organisms) is required for this process only within the organelle.
2. Since thiolation of tRNAs is not involved in partitioning of tRNA in *T. brucei,* we proposed a model where tRNA methylation plays a critical role as a negative determinant for tRNA import.
3. Thiolation affects tRNA stability and plays a role as a negative determinant of mitochondrial tRNA editing in *T. brucei* .
4. Membrane potential is dispensable for tRNA translocation into the mitochondrion.
5. tRNAs were for the first time shown to be imported into the bloodstream stage of *T. brucei.*

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6. Curriculum vitae

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