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Mitochondrial translation is essential in bloodstream forms of *Trypanosoma brucei*

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Summary

The parasitic protozoa *Trypanosoma brucei* has a complex life cycle. Oxidative phosphorylation is highly active in the procyclic form but absent from bloodstream cells. The mitochondrial genome encodes several gene products that are required for oxidative phosphorylation, but it completely lacks tRNA genes. For mitochondrial translation to occur, the import of cytosolic tRNAs is therefore essential for procyclic *T. brucei*. Whether the same is true for the bloodstream form has not been studied so far. Here we show that the steady-state levels of mitochondrial tRNAs are essentially the same in both life stages. Editing of the imported $tRNA^{Trp}$ also occurs in both forms as well as in mitochondria of *Trypanosoma evansi*, which lacks a genome and a translation system. These results show that mitochondrial tRNA import is a constitutive process that must be mediated by proteins that are expressed in both forms of the life cycle and that are not encoded in the mitochondrial genome. Moreover, bloodstream cells lacking either mitochondria-specific translation elongation factor Tu or mitochondrial tryptophanyl-tRNA synthetase are not viable indicating that mitochondrial translation is also essential in this stage. Both of these proteins show trypanosomatid-specific features and may therefore be excellent novel drug targets.

Introduction

All eukaryotes contain mitochondria whose main function in most species is the production of ATP by oxidative phosphorylation (OXPHOS). Mitochondria contain an own genome reflecting their bacterial ancestry. However, this genome contains only a very small number of protein-coding genes (3–44) that either encode proteins involved in OXPHOS or factors that are required to produce them (Gray *et al*., 1999; Barbrook *et al*., 2010). Thus more than 95% of mitochondrial proteins are nuclear encoded, synthesized in the cytosol and imported into mitochondria. But even though much of the mitochondrial proteome is directly or indirectly devoted to OXPHOS this is not the essential function of the organelle in all organisms. A few eukaryotes have degenerated mitochondria that lack DNA. These mitochondria are not capable of OXPHOS but are nevertheless essential for cellular function. This can be explained by the fact that mitochondria house the pathway for the formation of Fe/S clusters which are inorganic cofactors of many mitochondrial and of some essential cytosolic proteins (Lill and Mühlenhoff, 2008). Thus, while the mitochondrial genome is dispensable under some conditions, the mitochondrion itself is essential.

The parasitic protozoon *Trypanosoma brucei* contains a single mitochondrion that has proven to be a great model to study mitochondrial biology (Schneider, 2001; Lukes *et al*., 2005). It contains two genetic elements, the maxi- and the minicircles, which are highly topologically interlocked, building the so-called kinetoplast DNA (kDNA) network. The maxicircles are homologous to the mitochondrial genome of other species and encode 18 proteins. These proteins are required either for OXPHOS or for mitochondrial translation or have an as yet unknown function (Feagin, 2000). Expression of mitochondrial proteins in *T. brucei* shows some unusual features. Many mitochondrial genes represent cryptogenes, meaning that their primary transcripts have to be modified by multiple uridine insertions and/or deletions in order to become functional mRNAs. This process of RNA editing is mediated by small transcripts termed guide RNAs that for the most part are encoded on the minicircle DNA. The central steps of RNA editing are catalysed by multiprotein complexes that co-ordinate the large number of chemical reactions required to produce the mature mRNAs (Stuart *et al*., 2005). Moreover, unique trypanosome-specific features do also extend to mitochondrial translation (discussed below).

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During its life cycle, *T. brucei* alternates between an insect and a mammalian host. The environmental conditions in the two hosts are radically different and necessitate significant structural and physiological adaptations, many of which concern the energy metabolism. Insect-stage or procyclic *T. brucei* has a highly active mitochondrion and generates ATP by OXPHOS as well as by mitochondrial and cytosolic substrate level phosphorylation. In contrast, the long slender bloodstream form present in the mammalian host contains much reduced mitochondria that are of smaller volumes and are essentially devoid of cristae (Fenn and Matthews, 2007). The respiratory complexes as well as most of the citric acid cycle enzymes are lacking and ATP is generated exclusively through glycolysis (Durieux *et al*., 1991; Tielens and VanHellemond, 1998).

Does the absence of OXPHOS in bloodstream *T. brucei* mean that they do not require mitochondrial translation? The fact that dyskinetoplastic and akinetoplastic bloodstream-stage trypanosomes, which partially or completely lack mitochondrial DNA, exist naturally and can also be generated in the lab seems to suggest so (Schnaufer *et al*., 2002). However, it has also been shown that replication of mitochondrial DNA (Timms *et al*., 2002), RNA editing (Schnaufer *et al*., 2001; Hashimi *et al*., 2009) and the mitochondrial ATPase (Schnaufer *et al*., 2005) – one subunit of which is encoded on the mitochondrial genome (Hashimi *et al*., 2010) – are essential in both life cycle stages. These observations suggest that mitochondrial translation is essential also in the bloodstream stage and raises the question of how these seemingly contradictory results can be explained.

The mitochondria of both life stages require a membrane potential to import proteins across the inner membrane as well as for transport of metabolites. In the insect-stage forms, this potential is generated by electron flow through the respiratory chain that is coupled to the export of protons. The proton gradient is then used by the ATP synthase to form intramitochondrial ATP. Bloodstream forms of *T. brucei* do not contain respiratory complexes and create the membrane potential by the ATP synthase working in reverse. Thus, consumption of mitochondrial ATP by the ATP synthase induces an active transport of protons into the intermembrane space, and by doing so generates a membrane potential (Schnaufer *et al*., 2005).

Recently a hypothesis has been proposed that could explain why dyskinetoplastic and akinetoplastic strains of *T. brucei* can exist. It is clear that without the mitochondrially encoded A6-subunit the mitochondrial ATP synthase cannot pump protons. It may however still be able to hydrolyse ATP. Akinetoplastic and dyskinetoplastic *T. brucei* strains appear to have a nuclear encoded γ-subunit of the ATP synthase that carries specific mutations which boost the ATPase activity and cause a reduction of the ATP levels in the matrix. As a consequence, cytosolic ATP is imported via the ADP/ATP carrier in quantities that generate a membrane potential. However, whether mutations in the γ-subunit are the only adaptation required for overcoming the loss of kDNA is still an open question (Schnaufer *et al*., 2005; Jensen *et al*., 2008; Lai *et al*., 2008).

Mitochondrial translation in trypanosomatids shows many unique features (Schneider, 2001). One special characteristic is that their mitochondrial genome is devoid of tRNA genes. Translation therefore depends entirely on nucleus-encoded tRNAs that are imported from the cytosol. As a consequence trypanosomatid mitochondria have a diverged translation initiation mechanism (Tan *et al*., 2002a; Charrière *et al*., 2005) and many mitochondrial translation factors such as elongation factor Tu (EF-Tu) and some aminoacyl-tRNA synthetases (aaRSs) (Charrière *et al*., 2006; 2009; Español *et al*., 2009) show interesting trypanosome-specific adaptations. Moreover, mitochondrial ribosomes of *T. brucei* are highly diverged and contain rRNAs that are among the smallest known (Sharma *et al*., 2009; Lithgow and Schneider, 2010).

Trypanosoma brucei is the causative agent of the human sleeping sickness that occurs in sub-Saharan Africa. Forty-five million people are at risk of contracting sleeping sickness and 300 000 die each year. Thus, new drug targets that allow the development of efficient and safe treatments of sleeping sickness are desperately needed (Lüscher *et al*., 2007). The unique mitochondrial translation system in trypanosomes might be an optimal novel drug target. However, direct evidence whether mitochondrial translation *per se* is essential for the bloodstream form of *T. brucei* has so far been missing. Here we provide an analysis of mitochondrial tRNA import for the bloodstream from of *T. brucei*. Moreover, we show that EF-Tu and tryptophanyl-tRNA synthetase (TrpRS), two mitochondria-specific translation factors, are essential for bloodstream forms of *T. brucei*. In summary these results validate mitochondrial protein synthesis as a potential novel drug target.

Results

Mitochondria of bloodstream T. brucei *import tRNAs*

The energy metabolism of procyclic *T. brucei* relies to a large extent on OXPHOS and therefore requires the efficient translation of essentially all mitochondrially encoded proteins. Moreover, as the mitochondrial genome of *T. brucei* is devoid of tRNA genes mitochondrial translation exclusively depends on tRNAs that are imported from the cytosol (Hancock *et al*., 1992; Schneider *et al*., 1994a;

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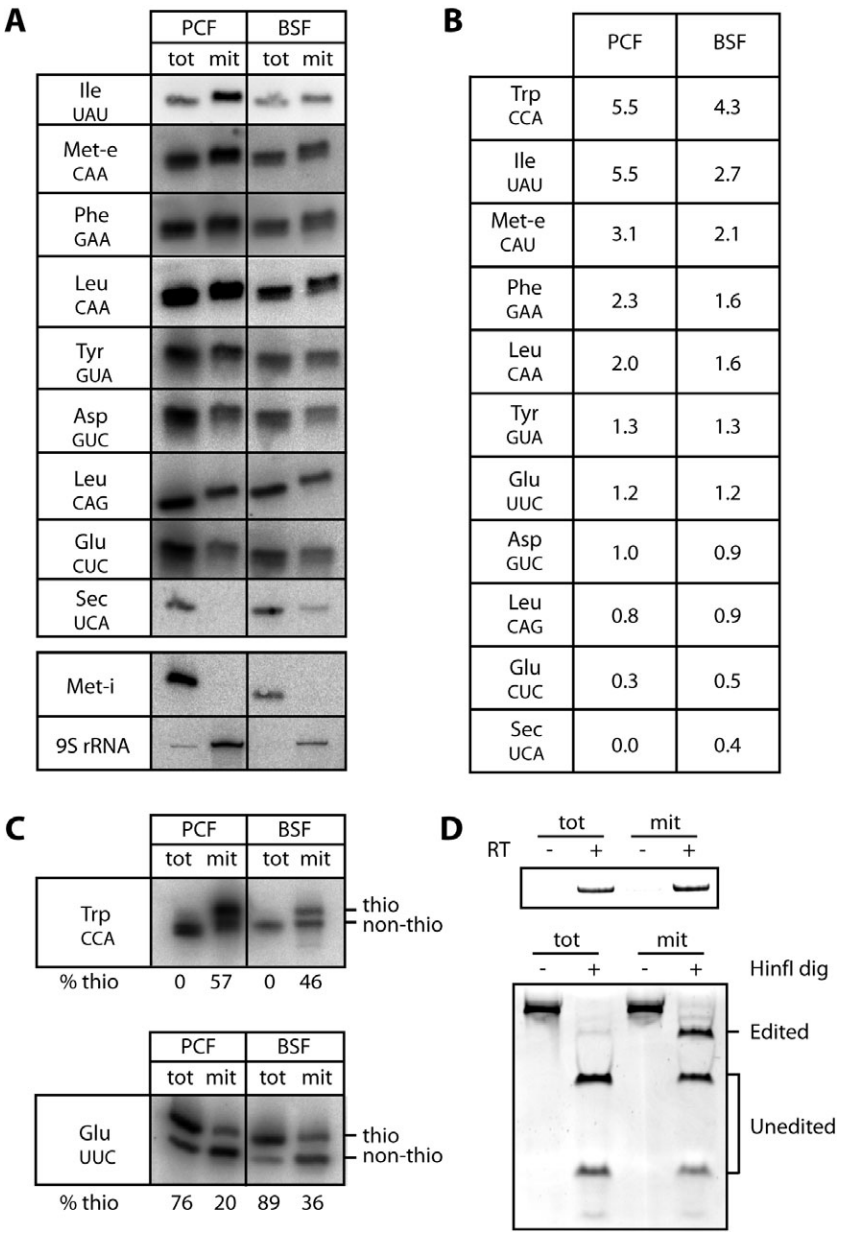

Fig. 1. The steady-state levels of mitochondrial tRNAs in procyclic and bloodstream forms of *T. brucei* are very similar.
A. RNA isolated from total cells (tot) and from digitonin-extracted mitochondrial fractions (mit) of procyclic *T. brucei* 427 (PCF) and of bloodstream *T. brucei* NYSM cells (BSF) were analysed by Northern blots using specific oligonucleotides. The amino acids specified by the tRNAs that are detected in the different panels and their corresponding anticodons are indicated. Met-e, elongator $tRNA^{Met}$; Met-i, initiator $tRNA^{Met}$; 9S rRNA, mitochondrial 9S rRNA.
B. Table showing the percentage of the total cellular content of the indicated elongator tRNAs that are present in the mitochondrial fraction of procyclic and bloodstream-form cells.
C. As in (A) but RNA fractions were resolved on an APM-containing polyacrylamide gel in order to separate thiomodified (thio) from non-thiomodified tRNAs (non-thio). Analysis was performed with oligonucleotides that specifically detect the $tRNA^{Trp}$ or $tRNA^{Glu}$. The extent of thiomodification is indicated at the bottom of each panel.
D. The extent of $tRNA^{Trp}$ editing in bloodstream form of *T. brucei* was analysed by an RT-PCR-based assay as described before (Charrière *et al.*, 2006). The top panel shows that the cytosolic and mitochondrial RNA fractions used as templates for RT are free of DNA. The extent of RNA editing was analysed by using a HinfI restriction digest (lower panel). RNA editing destroys a HinfI site that is only present in the cDNA derived from the unedited $tRNA^{Trp}$. cDNA amplified from unedited tRNATrp contains two HinfI sites and, thus, will be digested into three fragments (46, 22 and 21 nt; unedited). The cDNA derived from edited $tRNA^{Trp}$ contains the synthetic HinfI site only and will be digested into two fragments only (68 and 21 nt; edited).

Schneider, 2001). Mitochondria in the bloodstream stage of the parasite, however, are much reduced, they lack cytochromes and are not capable of OXPHOS (Tielens and VanHellemond, 1998). They only synthesize an unknown but very small number of proteins (Feagin, 2000). Whether and to which extent they import tRNA from the cytosol has so far not been investigated. We therefore decided to compare the steady-state levels of nuclear-encoded tRNAs in the mitochondrial fractions of procyclic and bloodstream stage of *T. brucei*. To that end, total RNA and RNA isolated from digitonin-extracted mitochondrial pellets were analysed by Northern blots (Tan *et al.*, 2002b). The quality of the cell fractionation was evaluated using probes detecting the mitochondrial 9S rRNA and the initiator $tRNA^{Met}$ which serve as mitochondrial and cytosolic markers respectively. In procyclic cells it has previously been shown that the initiator $tRNA^{Met}$ is not compatible with mitochondrial translation initiation and therefore is not imported into mitochondria (Tan *et al.*, 2002a). The lower panel of Fig. 1A shows that, as expected, the mitochondrial 9S rRNA is highly enriched in the mitochondrial fractions whereas the initiator $tRNA^{Met}$ is exclusively detected in total RNA fractions, which essentially consist of cytosolic RNAs. Moreover, Fig. 1 shows that 10 of 11 tested elongator tRNAs are present in the mitochondrial fraction of both procyclic and bloodstreams

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cells. We detect a very small amount of the $tRNA^{Sec}$ in the mitochondrial fraction of the bloodstream-stage cells even though this tRNA is cytosol-specific in procyclic *T. brucei* (Geslain *et al*., 2006). The absence of mitochondria-encoded selenoproteins further suggests that this might represent cytosolic contamination although a low level of import of the $tRNA^{Sec}$ cannot be excluded. The percentage of a given tRNA that was recovered in the mitochondria was calculated and is listed in descending order in Fig. 1B. The percentage of mitochondrial localization was different for different tRNA species but surprisingly very similar between the two life cycle stages.

Compartment-specific tRNA modifications in bloodstream forms of T. brucei

It has been shown in procyclic *T. brucei* that mitochondrial tRNAs are often modified in a compartment-specific manner (Schneider *et al*., 1994b; Crain *et al*., 2002), despite the fact that they are derived from the same nuclear gene as their cytoplasmic counterparts. The $tRNA^{Glu}$ is thiolated at the wobble nucleotide preferentially in the cytosol (Kaneko *et al*., 2003; Bruske *et al*., 2009; Paris *et al*., 2009), whereas for the $tRNA^{Trp}$ the thiolation of the penultimate nucleotide before the anticodon is specific for the mitochondrion (Crain *et al*., 2002; Charrière *et al*., 2006). In order to analyse the extent of thiolation the RNA fractions were resolved on acrylamide gels containing [(*N*-acryloylamino)phenyl] mercuric chloride (APM) which causes retardation in the migration of tRNAs that contain a thio carbonyl group (Igloi and Kössel, 1987). Northern analysis of total and mitochondrial RNA fractions shows that both tRNAs are also thiolated in the bloodstream forms (Fig. 1C). Besides thiolation, the imported $tRNA^{Trp}$ of procyclic *T. brucei* also undergoes C to U RNA editing at the wobble position in order to recognize the UGA codon that in mitochondria has been reassigned to tryptophane (Alfonzo *et al*., 1999; Charrière *et al*., 2006). The occurrence and the extent of RNA editing can be analysed by HinfI restriction digests of RT-PCR-amplified $tRNA^{Trp}$ since molecules derived from the edited $tRNAs^{Trp}$ lack the recognition site for this restriction enzyme. Figure 1D shows that approximately 50% of imported $tRNA^{Trp}$ becomes edited in the bloodstream forms, which is very similar to what is observed in procyclics (Charrière *et al*., 2006). The imported $tRNA^{Trp}$ appears to be subject to the same type of modifications in bloodstream forms that have already been described for procyclic *T. brucei*. The fact that the thiomodification and the C to U editing of $tRNA^{Trp}$ is exclusively detected in the mitochondria illustrates that digitonin fractionation is a highly suitable method to obtain pure mitochondrial RNA fractions from bloodstream forms of *T. brucei*.

$tRNA^{Trp}$ is imported and edited in the absence of mitochondrial translation

Trypanosoma evansi is closely related to *T. brucei* and causes disease in livestock in Africa, South America and large parts of Asia (Lun *et al*., 1992). *T. evansi* strains are either dyskinetoplastic or akinetoplastic, meaning that they lack large parts of or even the entire mitochondrial genome and due to this are locked in the bloodstream stage. *T. evansi* cells are mechanically transmitted by a large number of different arthropod vectors which explains their widespread geographical distribution. It has been observed that dyskinetoplastic *T. evansi* strains when kept in culture spontaneously convert to an akinetoplastic form by losing their residual mitochondrial genome (Fig. 2A) (Brun *et al*., 1998) (R. Brun, pers. comm.). Thus, *T. evansi* does not require mitochondrial protein synthesis for growth. As discussed in the introduction for the bloodstream form of *T. brucei* this is most likely due to compensatory changes in the nuclear-encoded γ-subunit of the mitochondrial ATPase (Schnaufer *et al*., 2005; Lai *et al*., 2008). Here we have used *T. evansi* as a model to investigate the interdependence of tRNA import and mitochondrial translation. In the immunoblot shown in Fig. 2B whole-cell extracts of procyclic and bloodstream forms of *T. brucei* and *T. evansi* were analysed for the presence of the voltage-dependent anion channel (VDAC), a mitochondrial outer membrane protein (Pusnik *et al*., 2009), and for the matrix localized Hsp70. The result shows that there is much less VDAC present in the outer membrane of bloodstream than in that of procyclic mitochondria. In *T. evansi* VDAC is hardly detectable anymore. This contrasts with the mitochondrial heat shock protein 70 (mHsp70) where similar levels are observed, and it illustrates the large qualitative differences between the mitochondria in the three types of trypanosomes. The very low amount of VDAC in mitochondria of *T. evansi* may reflect the fact that in the absence of replication, transcription and translation much fewer metabolites need to be transported across the outer membrane.

Using the same digitonin-based extraction that was used for bloodstream *T. brucei* we prepared mitochondrial fractions of *T. evansi*. The Northern analysis in Fig. 2C shows that the $tRNA^{Trp}$ is imported into mitochondria whereas the $tRNA^{Met\text{-}i}$ and the $tRNA^{Sec}$ appear to remain in the cytosol. Moreover, in approximately 10% of the imported $tRNA^{Trp}$ the wobble nucleotide becomes edited from C to U (Fig. 2D). Analysis on an APM-containing polyacrylamide gel furthermore shows that in *T. evansi* mitochondria no thiolated $tRNA^{Trp}$ can be detected (Fig. 2C, bottom panel).

Thus, as in bloodstream *T. brucei*, the $tRNA^{Trp}$ of *T. evansi* is imported into mitochondria and becomes partially edited after import. These results show that the

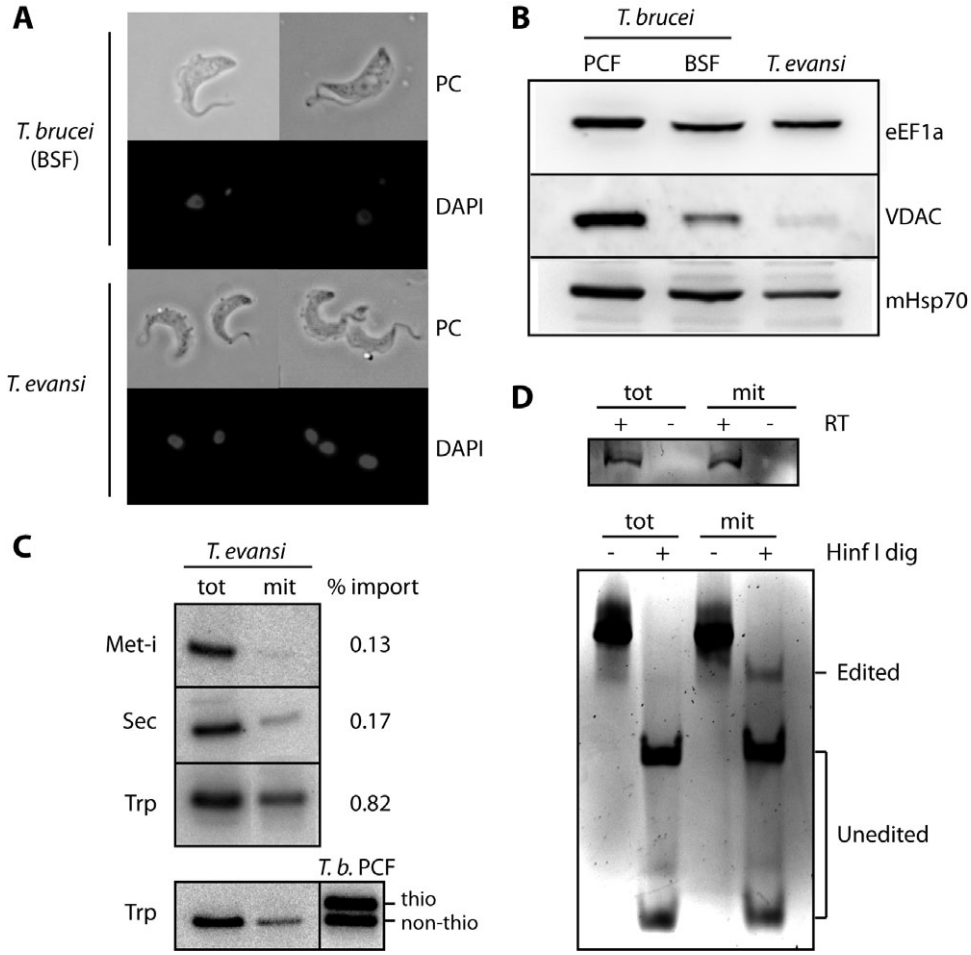

Fig. 2. tRNA^{Trp} is imported into mitochondria of *Trypanosoma evansi* and edited.
A. Images of phase-contrast (PC) and DAPI-stained *T. brucei* bloodstream form and *T. evansi* showing the absence of the kDNA from the latter.
B. Total extracts of 10^6 cells each of *T. brucei* procyclic (PCF) and bloodstream forms (BSF) as well as of *T. evansi* were analysed by Western blot to compare the levels of the voltage-dependent anion channel (VDAC) and mitochondrial Hsp70 (mHsp70). Eukaryotic elongation factor 1a (eEF1a) serves as a loading control.
C. Top panel, RNA isolated from total cells (tot) and from digitonin-extracted mitochondrial fractions (mit) of *T. evansi* cells were analysed by Northern blots using specific oligonucleotides detecting the initiator tRNA^{Met} (Met-i), the tRNA^{Sec} (Sec) and the tRNA^{Trp} (Trp). The percentage of the total cellular content of the indicated tRNAs that are present in the mitochondrial fractions are depicted on the right. Bottom, the same RNA samples as above were separated on an APM-containing polyacrylamide gel. No thiomodified tRNA^{Trp} could be detected. The lane on the left shows a mitochondrial RNA sample from procyclic *T. brucei* that was run on the same gel as a positive control.
D. The extent of tRNA^{Trp} editing in *T. evansi* was analysed by an RT-PCR-based assay as described in Fig. 1D.

processes of tRNA import and mitochondrial translation are not tightly coupled.

EF-Tu is required for survival of bloodstream T. brucei

In *T. brucei*, all mitochondrial tRNAs are imported from the cytosol and are therefore of eukaryotic evolutionary origin. However, as in all other mitochondria the mitochondrial translation system is of the bacterial type. Thus, the processes of translation initiation and elongation are highly similar to the ones in bacteria. One of the best characterized bacterial translation factors is EF-Tu. It is a small GTPase that in the presence of GTP binds the aminoacylated elongator tRNAs. The ternary complex consisting of EF-Tu, GTP and tRNA is then directed to the ribosome where after GTP hydrolysis the amino acid is incorporated into the nascent polypeptide (Krab and Parmeggiani, 2002). Trypanosomal EF-Tu (Tb-EF-Tu) is, except for a trypanosomatid-specific 30-amino-acid insertion close to the carboxy-terminus, more than 50% identical to bacterial and other mitochondrial EF-Tus. In order to show whether mitochondrial translation is essential in bloodstream forms of *T. brucei* we produced a cell line allowing inducible RNAi-

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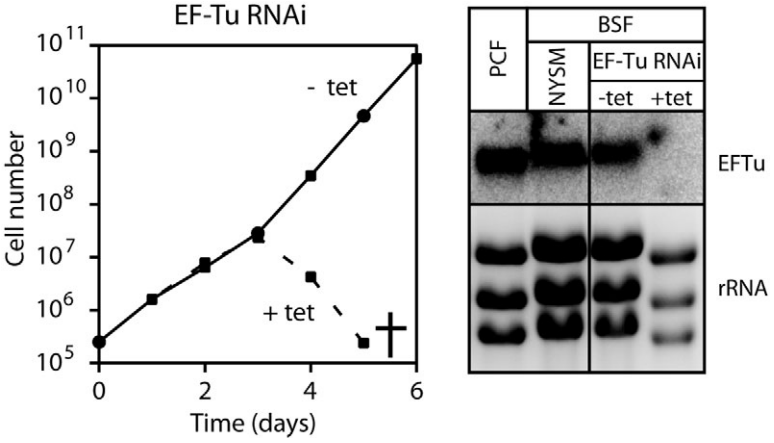

Fig. 3. Tb-EF-Tu is essential for growth of bloodstream form *T. brucei*. Representative growth curve of an uninduced and induced (–tet, +tet) clonal Tb-EF-Tu RNAi cell line of bloodstream forms of *T. brucei* NYSM. The Northern blot verifying mRNA ablation is shown on the top panel on the right. For a comparison total RNA of procyclic *T. brucei* 427 (PCF) and of the bloodstream form RNAi parent cell line *T. brucei* NYSM cells was also analysed. The lower panel shows the ethidium bromide stained rRNAs and serves as a loading control.

mediated ablation of Tb-EF-Tu. Using this cell line we could show that ablation of Tb-EF-Tu mRNA in bloodstream *T. brucei* not only results in a growth arrest but actually kills the cell (Fig. 3).

Translation elongation is presently the only known function of EF-Tu suggesting that mitochondrial protein synthesis is essential for growth and survival of the bloodstream form of *T. brucei*.

Tb-TrpRS2 is required for survival of bloodstream T. brucei

Based on the results with Tb-EF-Tu we predict that all factors required for mitochondrial translation should be essential in bloodstream forms of *T. brucei*. Cases of particular interest regarding a possible chemotherapeutic intervention are mitochondria-specific aaRSs. These are indispensable enzymes for translation and catalyse the addition of specific amino acids to their cognate tRNAs. In line with the fact that all of its mitochondrial tRNAs are imported from the cytosol the large majority of aaRSs of *T. brucei* are encoded by single genes suggesting the use of the same enzymes in the cytosol and the mitochondrion (Rinehart *et al.*, 2004; Geslain *et al.*, 2006). However *T. brucei* contains distinct cytosolic and mitochondrial tryptophanyl-, aspartyl- and lysyl-tRNA synthetases (TrpRS, AspRS and LysRS) (Charrière *et al.*, 2006; 2009; Español *et al.*, 2009). The mitochondrial TrpRS (Tb-TrpRS2) is of the eukaryotic type and has been studied in detail in procyclic *T. brucei*: it is required to aminoacylate the imported thiolated $tRNA^{Trp}$ that due to the mitochondrial recoding of UAG to tryptophan undergoes C to U editing of the wobble nucleotide (Charrière *et al.*, 2006). In order to investigate whether Tb-TrpRS2 is essential in bloodstream forms of *T. brucei*, we produced a cell line allowing an inducible RNAi-mediated ablation of Tb-TrpRS2. However, induction of RNAi did not affect growth (data not shown). Since RNAi cannot be relied on to deplete protein levels completely, negative data can be misleading. We therefore constructed a cell line in which both alleles of the Tb-TrpRS2 were knocked out by homologous recombination (Fig. 4A). One allele was replaced by the blasticidine resistance gene resulting in the Tb-TrpRS2+/– single knock-out cell line. Several attempts of knocking out the second allele in this cell line were unsuccessful, suggesting that Tb-TrpRS2 is essential. For this reason the cells were transfected with a tetracycline-inducible ectopic copy of the Tb-TrpRS2 gene. The resulting cell line, Tb-TrpRS2+/–::Tb-TrpRS2 (Fig. 4B), was then used in the presence of tetracycline to replace the second allele of Tb-TrpRS2 by the puromycin resistance gene yielding the double knock-out cell line Tb-TrpRS2–/–::Tb-TrpRS2. Insertion of the two resistance genes and the absence of the wild-type Tb-TrpRS2 genes was verified by Southern blotting (Fig. 4C). Figure 4D shows that removal of tetracycline which prevents expression of the ectopic copy of Tb-TrpRS2 results in a growth arrest after 2 days and that few days later the cells start dying. Northern blots of long acid gels that allow to separate charged from uncharged tRNAs showed that the observed growth arrest coincides with the loss of the aminoacylated form of mitochondrial $tRNA^{Trp}$ (Fig. 4E). The accumulation of non-aminoacylated $tRNA^{Trp}$ is a specific effect of the lack of Tb-TrpRS2 since the levels of aminoacylated cytosolic $tRNA^{Trp}$ and $tRNA^{Ile}$ are not affected.

Removal of tetracycline led to a highly reproducible growth arrest followed by an 10-fold reduction of cell numbers within the following 3 days. Surprisingly, at even later time points the culture starts to grow again and reaches generation times comparable to the ones of cells grown in the presence of tretracycline (Fig. 4D). RT-PCR analysis using primers that can detect either all Tb-TrpRS2 mRNAs or selectively the ones that are derived from the endogenous Tb-TrpRS2 gene (Fig. 5A) shows that in recovering cells the ectopic copy of

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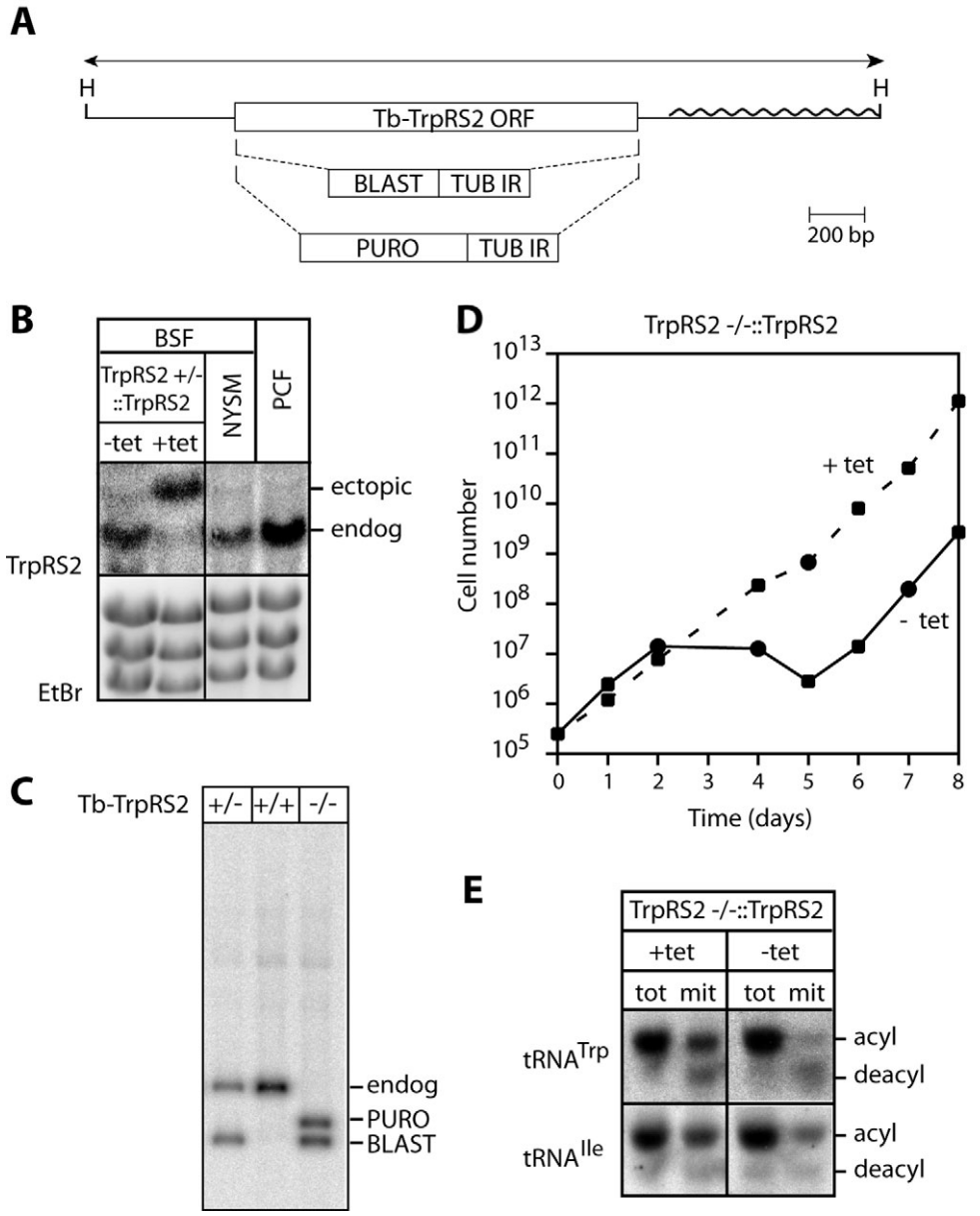

Fig. 4. Tb-TrpRS2 is essential for growth of bloodstream forms of *T. brucei* and responsible for mitochondrial tryptophanyl-tRNA^{Trp} formation.
A. Generation of Tb-TrpRS2 double knock-out cell line. Schematic to scale drawing of the wild-type Tb-TrpRS2 locus and the situation after homologous recombination leading to replacement of the two alleles by the blasticidine (BLAST) and the puromycin (PURO) resistance genes. HindIII restriction sites that were used for Southern blot analysis are indicated (H). The region where the probe used for the Sothern analysis hybridizes is shown as a wiggly line.
B. Northern blot analysis of the Tb-TrpRS2 single knock-out cell line allowing tetracycline-inducible ectopic expression of Tb-TrpRS2 (TrpRS+/–::TrpRS2). Cells were analysed in the presence (+tet) and 2 days after removal of tetracycline (–tet). For a comparison total RNA of procyclic *T. brucei* 427 (PCF) and of the bloodstream-form RNAi parent cell line *T. brucei* NYSM cells was also analysed. The positions of the mRNAs derived form the endogenous and ectopic copy of the Tb-TrpRS2 genes are indicated.
C. Southern blot analysis of the Tb-TrpRS2 knock-out cell lines. HindIII-digested genomic DNA of the parent cell line *T. brucei* NYSM (+/+) and the resulting cell lines after replacement of the first (+/–) and the second (–/–) Tb-TrpRS2 allele were hybridized with the probe indicated in (A). The hybridizing fragments for the endogenous Tb-TrpRS2 gene as well as the fragments obtained after insertion of the two resistance markers are indicated on the right.
D. Growth curve in the presence and absence of tetracycline (+tet and –tet) of the Tb-TrpRS2–/–::Tb-TrpRS2 cell line.
E. Northern blot analysis of RNA isolated from total cells (tot) and from digitonin-extracted mitochondrial fractions (mit) of the Tb-TrpRS2–/–::TrpRS2 cell line. Samples were taken in the presence (+tet) or 3 days after removal of tetracycline (–tet). The RNA fractions were resolved on long acid urea gels, which allow separation of aminoacylated (acyl) from deacylated tRNAs (deacyl). The blots were probed with specific oligonucleotide hybridization for the *T. brucei* tRNA^{Trp} (top panel) and for tRNA^{Ile} as a control that is not affected by the knock-out.

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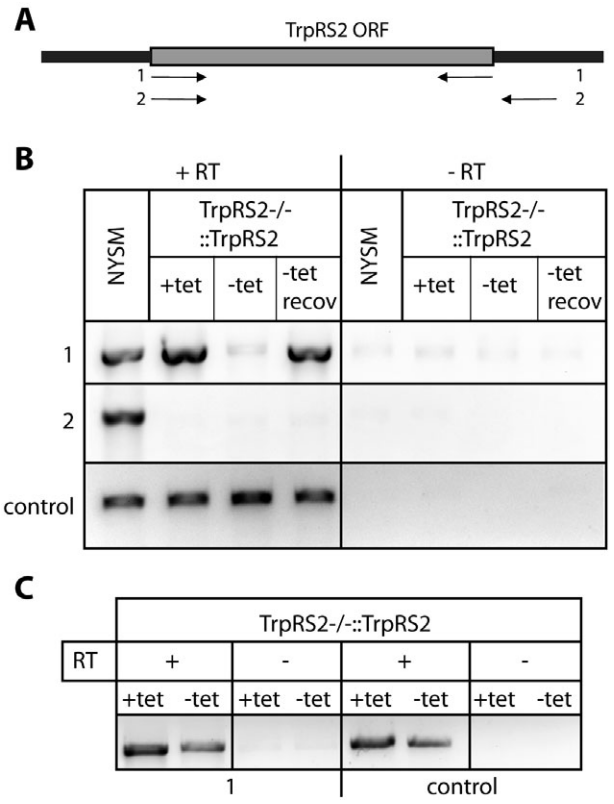

Fig. 5. Analysis of Tb-TrpRS2–/–::TrpRS2 cells that grow in the absence of tetracycline.
A. Primers used in the RT-PCR analysis of Tb-TrpRS2 mRNA. Primer pair 1 amplifies cDNA derived from both the endogenous and the ectopic copy of Tb-TrpRS2. Primer pair 2 selectively amplifies cDNA derived from the endogenous Tb-TrpRS2 locus.
B. RT-PCR analysis of the NYSM parental strain and the Tb-TrpRS2–/–::TrpRS cell line grown in culture. For the latter cells were grown in the presence of tetracycline (+tet) as well as for 3 (–tet) and more than 6 days (–tet, recov) after tetracycline removal. Cells at last time point were collected since they were able to grow even in the absence of tetracycline. RT-PCR analysis was performed using primer pair 1 (top panel), primer pair 2 (middle) panel and a primer pair amplifying the Tb-EF-Tu mRNA that serves as a loading control (bottom panel).
C. As in (B) but cells were grown *in vivo* in a mouse that was initially resistant to *T. brucei* infection but later on relapsed. RT-PCR analysis was performed using primer pair 1 (left two panels) or the primer pair amplifying the Tb-EF-Tu mRNA as loading control (right two panels).
All reactions in (B) and (C) were performed in the presence (+RT) and absence (–RT) of RT to control for contaminating DNA.

Tb-TrpRS2 is expressed even in the absence of tetracycline (Fig. 5B). Thus, after prolonged incubation a population of cells is selected where the expression of the ectopic Tb-TrpRS2 gene is no longer under control of the tetracycline repressor anymore.

In summary, these results are entirely consistent with the results for Tb-EF-Tu and establish that Tb-TrpRS2 and therefore mitochondrial translation is essential for the growth and survival of bloodstream form of *T. brucei*.

Tb-TrpRS2 is essential for establishing an infection in vivo

We also wanted to explore the role Tb-TrpRS2 plays during *in vivo* infections. Five mice were infected with the Tb-TrpRS2–/–::Tb-TrpRS2 knock-out strain that had been cultured in the absence of tetracycline for 2 days to shut down the expression of the ectopic copy of Tb-TrpRS2. Figure 4D shows that for this time period the presence or absence of tetracycline does not affect growth. As controls, five animals received doxycycline in their drinking water for 2 days before infection and throughout the experiment. These were infected with the Tb-TrpRS2–/–::Tb-TrpRS2 knock-out strain that had been cultured continuously in the presence of tetracycline to allow full expression of the ectopic Tb-TrpRS2. All five control animals presented high parasitaemia 3–4 days after infection and had to be sacrificed. In contrast, three out of five animals infected with the Tb-TrpRS2-ablated knock-out strain remained trypanosome-free over the entire 4-day period. Two mice of this group however did develop high parasitaemia suggesting that in these cases the downregulation of the ectopically expressed Tb-TrpRS2 was too inefficient to prevent virulence. Moreover, the three initially resistant animals relapsed and developed parasitaemia at later time points. RT-PCR analysis of the trypanosomes recovered from these animals showed that the relapse can be readily explained by tetracycline-independent expression of the ectopic copy of Tb-TrpRS2 (Fig. 5C), in good agreement with our cell culture results (see above).

These results suggest that Tb-TrpRS2 is not only required for cell growth in culture but also for establishing an infection in mice and therefore indicate an essential role of mitochondrial translation for virulence in *T. brucei*.

Discussion

Studies of mitochondrial translation in *T. brucei* are technically very challenging which is why we know relatively little about the process (Horvath *et al.*, 2000a,b; 2002). Essentially all work that has been done on the subject was limited to procyclic forms. A survey of these studies shows that there are a number of trypanosome-specific features of mitochondrial translation that are relevant for mitochondrial biology in general as well as for the identification of novel potential drug targets. The most important one of these in the context of this work is the complete dependence of mitochondrial translation on imported eukaryotic-type tRNAs (Hancock *et al.*, 1992; Schneider *et al.*, 1994a).

Here we provide an analysis of mitochondrial translation in the bloodstream form of *T. brucei*. We first focused on mitochondrial tRNA import and could show that the

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specificity and the extent of tRNA import in bloodstream forms is very similar to what is observed in procyclic forms. This is surprising since the relative volume of the mitochondrion per cell is much smaller in bloodstream forms (Böhringer and Hecker, 1975) and suggests that the intramitochondrial concentration of tRNAs is either the same in both life cycle stages or even higher in the bloodstream form. Connected with tRNA import we also analysed thiolation and C to U editing of the imported $tRNA^{Trp}$ and again could show that they are qualitatively and quantitatively essentially identical in both life cycle stages. The fact that mitochondrial tRNA import is not regulated between the procyclic and the bloodstream forms suggests that the as yet elusive components of the mitochondrial tRNA import machinery are constitutively expressed. Thus, assuming the machinery for tRNA import is the same in both life cycle stages we would expect that all proteins that are specific for the procyclic stage, such as components of the respiratory complexes, cannot be involved in mitochondrial tRNA import.

This is interesting for the following reason: it has been claimed that the tRNA import machinery of the mitochondrial inner membrane of *Leishmania tropica* consists of a protein complex (termed RIC) composed of nine proteins six of which are essential for tRNA import (Bhattacharyya and Adhya, 2004). Three of the essential subunits of RIC are bona fide components of the respiratory chain and therefore are proposed to have a dual function in OXPHOS and mitochondrial tRNA import (Mukherjee *et al*., 2007). The orthologues of these three proteins are not detectable in bloodstream forms *T. brucei* and thus cannot be required for tRNA import. This finding is not limited to *T. brucei* since we show that also akinetoplastic *T. evansi* which is not able to perform OXPHOS and even lacks a mitochondrial genome imports tRNAs into its mitochondrion. These results furthermore demonstrate that mitochondrial tRNA import does not rely on any mitochondrial-encoded proteins. In summary our results show that the inner membrane tRNA import machinery in *T. brucei* cannot be the same as the one proposed for *L. tropica* which is surprising considering how close the two species are related.

The fact that tRNA import and the mitochondria-specific $tRNA^{Trp}$ editing are also detected in akinetoplastic *T. evansi* shows that the presence of correctly processed tRNAs does not necessarily indicate a functional mitochondrial translation system. In line with this observation it has been reported that the RNA-editing complexes are still present in *T. evansi* despite the absence of editable RNA substrates (Lai *et al*., 2008). The same is the case for the DNA replication machinery. This is surprising since producing the numerous components that mediate transcription, RNA editing and translation – processes that are redundant in the absence of mitochondrial DNA – is costly for the cells. We would therefore expect them to disappear or at least to degenerate over time. The fact that this did only partially happen in *T. evansi* suggests that the loss of the kDNA in evolutionary terms may have happened fairly recently.

However, while still operational both import and editing of the $tRNA^{Trp}$ are much less efficient in *T. evansi* than in the bloodstream form of *T. brucei* and thiolation of the tRNA is not even detectable anymore. It has recently been shown that editing of the $tRNA^{Trp}$ acts as a negative determinant for thiolation (Bruske *et al*., 2009; Wohlgamuth-Benedum *et al*., 2009). The concomitant decrease of $tRNA^{Trp}$ editing and thiolation that we observe in *T. evansi* indicates that the two processes become downregulated independently of each other. This shows that in the absence of selection for functional translation, the import, editing and thiolation of the $tRNA^{Trp}$ start to deteriorate.

Besides its value as a model system for cell biology, *T. brucei* is also a clinically important pathogen that causes sleeping sickness in Africa. As no vaccination is available, treatment is solely dependent on chemotherapeutic drugs. However, the few approved drugs are either only effective against the first stage of the disease or have a high toxicity. This is exemplified by melarsoprol which is used to treat the second stage of sleeping sickness and which directly causes death in 5–10% of all patients. Moreover, resistance is emerging and limits the efficacy of the drugs. Thus, it is clear that novel drugs are urgently needed (Lüscher *et al*., 2007).

The pragmatic definition of a drug target is a process or a structure that is essential for the parasite and that does not occur in the host. Here we show that the two translation factors, Tb-EF-Tu and Tb-TrpRS2, are essential for survival of bloodstream forms of *T. brucei* which strongly suggests that mitochondrial translation is essential. This raises the question of how similar mitochondrial translation is in humans as compared with *T. brucei.*

Humans have a complete set of mitochondrial tRNA genes but nevertheless may import a few cytosolic tRNAs (Rubio *et al*., 2008). Trypanosomes on the other hand lack organellar tRNA genes and therefore have to import the entire set of mitochondrial tRNAs from the cytosol whereas the mitochondrial translation system is of bacterial descent. It is therefore not surprising that many imported tRNAs require adaptations in mitochondrial translation factors in order to be functional. Thus, many of these factors are highly diverged when compared with their human orthologues. Examples include methionyl-tRNA formyl-transferase (Tan *et al*., 2002a), as well as a number of mitochondrial aaRSs (Charrière *et al*., 2006; 2009; Español *et al*., 2009). For our *in vivo* analysis we chose mitochondrial Tb-EF-Tu and Tb-TrpRS2. Tb-EF-Tu is conserved but shows a trypanosomatid-specific insertion of unknown function

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close to the carboxy-terminus. Mitochondrial Tb-TrpRS2, on the other hand, is a eukaryotic-type mitochondrial TrpRS that is specific for trypanosomatids (data not shown) and that as an enzyme is of great interest as a potential novel drug target.

Why should *T. brucei* have a unique mitochondrial TrpRS? In mitochondria of trypanosomatids, the stop codon UGA has been reassigned to tryptophan. The organellar $tRNA^{Trp}$ therefore has to decode UGA in addition to the normal tryptophan codon UGG. It is not obvious how this can be achieved by an imported cytosolic $tRNA^{Trp}$ that does not recognize the UGA stop codon. Trypanosomatids solve this problem by a mitochondria-specific RNA-editing event that converts the CCA anticodon of the imported $tRNA^{Trp}$ to UCA (Alfonzo *et al*., 1999; Charrière *et al*., 2006; Bruske *et al*., 2009; Wohlgamuth-Benedum *et al*., 2009). However, the CCA anticodon is an identity determinant for the eukaryotic TrpRS. Thus, unlike most other imported tRNAs of trypanosomes, the edited $tRNA^{Trp}$ in mitochondria cannot be charged by an aaRS that is dually targeted to the cytosol and the mitochondrion. Instead, trypanosomatids evolved a highly divergent eukaryotic-type TrpRS that is specific for mitochondria and that, unlike its cytosolic counterpart, can aminoacylate both edited and unedited $tRNA^{Trp}$ (Charrière *et al*., 2006).

We have shown that Tb-TrpRS2 is essential for both procyclic and bloodstream forms of *T. brucei*. The protein is specific for trypanosomatids and distinct from its cytosolic counterpart. It should be possible to exploit these differences for the development of drugs that selectively inhibit the mitochondrial Tb-TrpRS2 of *T. brucei* without interfering with the human enzyme in the cytosol. The human mitochondrial TrpRS does not need to be considered here since it is of the bacterial type. On the other hand Tb-TrpRS2 is still sufficiently similar to its cytosolic orthologues that it should be possible to model its structure by using the solved structures of human or yeast TrpRS as templates (Shen *et al*., 2006; Yang *et al*., 2006). These structures could then provide a platform for rational drug design. Finally, recombinant active Tb-TrpRS2 has been produced (Charrière *et al*., 2006). Thus its activity can easily be assayed by methods that are amenable to high-throughput screening.

Tb-TrpRS2 of *T. brucei* is not the only enzyme linked to mitochondrial translation that might be an excellent drug target. There are two other aaRSs – the AspRS (Charrière *et al*., 2009) and the LysRS (Español *et al*., 2009) – where we know that trypanosomatid-specific mitochondrial variants exist. Both of these enzymes have been characterized recently and although it was not tested whether they are essential for survival of bloodstream forms of *T. brucei*, in analogy to our results with TrpRS2 it is likely they are.

The idea of using aaRSs as drug targets is not new. Many natural antimicrobial agents synthesized by bacteria and fungi have been characterized that inhibit specific aaRSs (Pohlmann and Brotz-Oesterhelt, 2004; Kim *et al*., 2003). Numerous screening programmes aimed at identifying aaRS inhibitors have been performed and promising lead compounds have been identified. A few agents have moved into clinical development and the antibiotic mupirocin that targets the bacterial isoleucyl-tRNA synthetase is in clinical use (Thomas *et al*., 2010). Most efforts of using aaRSs as drug targets have targeted bacterial infections. However, it has been suggested that cytosolic aaRS could be used as drug targets against eukaryotic parasites including *Plasmodium* spp. and *T. brucei* (Farrera-Sinfreu *et al*., 2008; Bour *et al*., 2009).

The fact that Tb-TrpRS2 of *T. brucei* is essential for bloodstream form and the fact that it defines a unique trypanosomatid-specific family of TrpRSs suggest that it might even be a better drug target than cytosolic aaRSs. Since the situation is very similar for trypanosomal AspRS and LysRS the same might be true for these enzymes.

Experimental procedures

Culture of cells

Bloodstream *T. brucei* NYSM line (Wirtz *et al*., 1999) and akinetoplastid *Trypanosoma evansi* STIB806 (Lun *et al*., 1992) were cultivated in HMI-9 medium (Hesse *et al*., 1995) containing 10% fetal calf serum and 1 $\mu g\ ml^{-1}$ G418, in the case of the NYSM strain. Transfections were done as described previously (Burkard *et al*., 2007). Selection of the transfectants was done with 0.1 $\mu g\ ml^{-1}$ puromycin, 0.5 $\mu g\ ml^{-1}$ phleomycin or 5 $\mu g\ ml^{-1}$ blasticidine. Expression was induced with 1 $\mu g\ ml^{-1}$ tetracycline. Procyclic *T. brucei* 427 was grown as described (Bochud-Allemann and Schneider, 2002).

Constructs and cell lines

RNAi of Tb-EF-Tu was done by using pLew-100-based stem loop constructs containing the blasticidin resistance gene (Wirtz *et al*., 1999; Bochud-Allemann and Schneider, 2002). As insert, we used a 536 bp fragment (nucleotides 7–543) of the Tb-EF-Tu ORF (Tb927.10.13360, Uniprot ID Q388RI). The knock-out cell line for the first allele, Tb-TrpRS2+/–, was generated by transfecting NYSM cells with a DNA fragment containing a blasticidin resistance cassette followed by the α-β tubulin intergenic region, flanked by approximately 100 bp each of 5′ and 3′ flanking regions of Tb-TrpRS2. After selection, cells were tested for correct genomic integration of the construct by PCR. A positive clone was selected and transfected with a pLew-100-based construct, allowing tetracycline-inducible expression of Tb-TrpRS2, that carries the phleomycin resistance gene. The resulting cell line, Tb-TrpRS2+/–::Tb-TrpRS2, allowing inducible ectopic expression of Tb-TrpRS2, was further transfected (in the

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presence of 1 μg ml^{-1} tetracycline) with a DNA fragment for the second allele knock-out which is essentially identical to the first one except that it contains a puromycin resistance cassette. Clones of the final double knock-out cell line, Tb-TrpRS2–/–::Tb-TrpRS2, were tested for the absence of endogenous copies of Tb-TrpR2 by Southern blot analysis.

Cell fractionation by digitonin extraction

Crude mitochondrial fractions of bloodstream *T. brucei* NYSM and procyclic *T. brucei* 427 were prepared by digitonin extraction as described (Tan *et al*., 2002b). Washed *T. brucei* cells (2×10^8 cells) were resuspended in 0.5 ml of SoTE (0.6 M sorbitol/20 mM Tris-HCl, pH 8/ 2 mM EDTA). After the addition of 0.5 ml of SoTE containing 0.05% (w/v) of digitonin, the samples were mixed by pipetting and incubated on ice for 5 min. The suspensions were centrifuged and the resulting pellet, corresponding to the crude mitochondrial fraction, was resuspended in 0.5 ml of SoTE and treated with RNase A (10 μg ml^{-1}) for 5 min on ice. After centrifugation, RNA was extracted from the pellet using the acid guanidinium method (Chomczyinski and Sacchi, 1987). Finally total RNA (from 0.3×10^7 cell equivalents) and crude mitochondrial RNA (from 1.33×10^8 cell equivalents) were separated on 10% polyacrylamide/8 M urea gels and analysed by Northern blots as described (Tan *et al*., 2002b). The oligonucleotide used to detect tRNA^{Sec} and the two tRNAs^{Glu} are described in (Geslain *et al*., 2006) and (Bruske *et al*., 2009) respectively. All other oligonucleotides are described in (Tan *et al*., 2002b).

Mouse infections

Animals (young adult females, outbred NMRI) were purchase from Charles River Laboratories and were given feed and water *ad libitum*. Two days before infection and throughout the experiment, one group of animals received 0.5 mg ml^{-1} doxycycline (Sigma D9891) in deionized drinking water. The doxycycline was replaced daily. Water uptake was monitored daily and was not different between animals receiving water only and those receiving water with doxycyline (*c*. 4.5 ml per mouse per 24 h). Animals were infected intraperitoneally with 5×10^5 cultured trypanosomes. Parasitaemia was monitored daily by collecting 2 μl of tail blood into 48 μl of 0.85% NH_4Cl, 10 mM Tris-HCl, pH 7.5 on ice, followed by counting in a Neubauer chamber. For transferring of trypanosomes from blood into cultures, the tail tips were thoroughly cleaned with 70% ethanol, and a 2 μl drop of blood was collected with a sterile pipette tip and transferred into 1 ml of HMI-9 medium containing antibiotics. Cells were grown until the culture was fully adapted to the medium. All animal experimentation was performed under a permit of and according to the rules and regulations of the government committee on animal experimentation.

Miscellaneous

Editing of tRNA^{Trp} in Figs 1D and 2D was analysed using an RT-PCR-based assay as described (Charrière *et al*., 2006). Aminoacylation levels of the tRNA^{Trp} in total RNA (isolated from 0.5×10^7 cell equivalents) and in digitonin-extracted crude mitochondrial RNA fraction (isolated from 9.5×10^7 cell equivalents) was analysed on long acidic gels and subsequent Northern analysis (Varshney *et al*., 1991; Charrière *et al*., 2006) (Fig. 4E). For microscopic analysis cells were washed in phosphate-buffered saline (PBS), fixed in 1% formaldehyde and after subsequent washes with PBS mounted on glass slides with Vectashield containing 1.5 μg ml^{-1} DAPI.

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