Mitochondrial translation factors of *Trypanosoma brucei*: elongation factor-Tu has a unique subdomain that is essential for its function

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Summary

Mitochondrial translation in the parasitic protozoan *Trypanosoma brucei* relies on imported eukaryotic-type tRNAs as well as on bacterial-type ribosomes that have the shortest known rRNAs. Here we have identified the mitochondrial translation elongation factors EF-Tu, EF-Ts, EF-G1 and release factor RF1 of trypanosomatids and show that their ablation impairs growth and oxidative phosphorylation. *In vivo* labelling experiments and a SILAC-based analysis of the global proteomic changes induced by EF-Tu RNAi directly link EF-Tu to mitochondrial translation. Moreover, EF-Tu RNAi reveals downregulation of many nuclear encoded subunits of cytochrome oxidase as well as of components of the bc1-complex, whereas most cytosolic ribosomal proteins were upregulated. Interestingly, *T. brucei* EF-Tu has a 30-amino-acid-long, highly charged subdomain, which is unique to trypanosomatids. A combination of RNAi and comple-

mentation experiments shows that this subdomain is essential for EF-Tu function, but that it can be replaced by a similar sequence found in eukaryotic EF-1a, the cytosolic counterpart of EF-Tu. A recent cryo-electron microscopy study revealed that trypanosomatid mitochondrial ribosomes have a unique intersubunit space that likely harbours the EF-Tu binding site. These findings suggest that the trypanosomatid-specific EF-Tu subdomain serves as an adaption for binding to these unusual mitochondrial ribosomes.

Introduction

Mitochondria derive from an α-proteobacterial endosymbiont that during evolution either has transferred most of its genes to the nucleus or lost them for good. Most of the mitochondrial proteome, consisting of 1000 or more proteins, is therefore produced in the cytosol and subsequently imported into the organelle. However, a typical mitochondrial genome still encodes a small set of proteins (8 in yeast and 13 in humans) that is essential for oxidative phosphorylation (OXPHOS), the main function of mitochondria. To produce these proteins, mitochondria need their own translation system consisting of ribosomes, a full set of tRNAs and soluble translation factors. In line with their evolutionary descent the translation system of mitochondria is of the bacterial type (Gray, 2012). However, mitochondria and bacteria diverged approximately 2 billion years ago, resulting in many unique features of the mitochondrial translation system. Mitochondrial ribosomes are anchored to the membrane, have much shorter rRNAs and a larger number of ribosomal proteins. Mitochondrial mRNAs lack Shine–Dalgarno sequences and often are translated by a variant genetic code. Translation uses a reduced set of tRNAs that generally are shorter than their bacterial or cytosolic counterparts (Agrawal and Sharma, 2012; Gray, 2012).

The soluble mitochondrial translation factors, such as elongation factor Tu (EF-Tu), elongation factor Ts (EF-Ts), elongation factor G (EF-G) and release factor 1 (RF1) are orthologues of the corresponding factors in bacteria (Spremulli *et al.*, 2004). The small GTPase EF-Tu forms a complex with GTP and aminoacylated elongator tRNAs,

bringing the latter to the A site of the ribosome. There the GTPase activity of EF-Tu is stimulated, causing a conformational change that eventually leads to the release of the aminoacylated tRNA. The GTP exchange factor EF-Ts binds to the GDP-form of EF-Tu and induces its recycling by exchanging GDP for GTP. The conserved bacterial GTPase EF-G has two functions. It catalyses the translocation step after peptide bond formation and is involved in ribosome recycling. Interestingly, mitochondria have two orthologues of EF-G: EF-G1 and EF-G2, which are required for ribosomal translocation and recycling respectively. Finally, translation is terminated by binding of RF1 to the stop codons UAA and UAG, which releases the completed polypeptide (Spremulli *et al.*, 2004; Rorbach *et al.*, 2007).

Most of what we know about mitochondrial translation stems from work in yeast and mammals, which are quite closely related. To understand the conserved features of mitochondrial translation and the evolutionary forces that shaped it, it is important to study the process in a more diverse group of eukaryotes. The parasitic protozoan *Trypanosoma brucei* and its relatives are excellent systems to do so, since they appear to have diverged from other eukaryotes very early in evolution (Dacks *et al.*, 2008). This is reflected in a number of unusual features of its organellar translation system. The ribosomes of trypanosomal mitochondria for example have very unique structural features including the shortest rRNAs found in nature and a very high protein content (Sharma *et al.*, 2009; Agrawal and Sharma, 2012). Unlike yeast and mammals, the mitochondria of trypanosomatids lack tRNA genes and therefore need to import all tRNAs from the cytosol. Imported tRNAs represent a small fraction of the tRNA population that is used for cytosolic translation. Thus, in trypanosomatids the bacterial-type translation system of the mitochondrion must function exclusively with imported tRNAs, all of which are of the eukaryotic type (Alfonzo and Söll, 2009; Schneider, 2011). This creates problems for mitochondrial translation initiation, which requires a bacterial-type initiator tRNA^{Met}. Another conflict arises from the fact that in mitochondria the universal UGA stop codon has been reassigned to tryptophan and therefore cannot be decoded by the tRNA^{Trp}(CCA) that is imported from the cytosol. In order to cope with these challenges, trypanosomatids evolved adaptations in their mitochondrial translation system. They have a tRNA^{Met} formyl-transferase that recognizes the imported elongator tRNA^{Met} and allows it to function in organellar translation initiation (Tan *et al.*, 2002a). In the case of the tRNA^{Trp}, trypanosomatids acquired an RNA editing enzyme that changes the anticodon of the imported tRNA^{Trp} from CCA to UCA (Alfonzo *et al.*, 1999) and a specific tryptophanyl-tRNA synthetase that is able to charge the edited tRNA^{Trp} (Charrière *et al.*, 2006).

Here we present an *in vivo* analysis of the conserved mitochondrial translation factors EF-Tu, EF-Ts, EF-G1 and RF1 in *T. brucei*. Individual ablation of each of these factors inhibits OXPHOS and consequently normal growth of the procyclic form of the parasite. *In vivo* labelling experiments demonstrate the requirement of EF-Tu for mitochondrial protein synthesis and a quantitative proteomic analysis of the EF-Tu RNAi cell line documents the central role mitochondrially encoded subunits play for the stability and/or assembly of respiratory complexes. Finally, a bioinformatic analysis identified a trypanosomatid-specific motif of EF-Tu that is critical for EF-Tu function but dispensable for its interaction with EF-Ts.

Results

EF-Tu, EF-Ts, EF-G1 and RF1 are essential for normal growth of T. brucei

In silico analysis using the BLAST algorithm readily identifies the trypanosomal orthologues of the mitochondrial translation factors EF-Tu (Tb927.10.13360), EF-Ts (Tb927.3.3630), EF-G1 (Tb927.10.5010) and RF1 (Tb927.3.1070) (Figs S1–S4) (Berriman *et al.*, 2005). In order to determine their localization, we prepared transgenic cell lines allowing the expression of variants of the four proteins carrying either the haemagglutinin (HA) or the cMYC tag at their C-termini. Subsequent digitonin extractions were used to prepare crude mitochondrial and cytosolic fractions (Charrière *et al.*, 2006), which were analysed by immunoblotting. The results showed that, as expected, all four tagged proteins co-purify with mitochondrial heat shock protein 70 (mHSP70) that was used as a mitochondrial marker (Fig. 1). To study their importance for growth, we established stable transgenic cell lines allowing inducible RNAi-mediated ablation of each of the four proteins in procyclic *T. brucei*. For each cell line, the efficiency of RNAi was verified by Northern blot analysis. Figure 2 shows that ablation of all four translation factors resulted in an impairment of normal growth. Whereas for EF-Tu, EF-G1 and RF1 we observe a growth arrest, the ablation of EF-Ts caused a less severe slow growth phenotype. In summary these results show that in procyclic *T. brucei* all four mitochondrial translation factors are essential for normal growth.

Ablation of mitochondrial translation factors impairs OXPHOS

At early time points after induction ablation of mitochondrial translation factors should interfere with mitochondrial protein synthesis without affecting cytosolic translation. All known mitochondrially encoded proteins of *T. brucei* either function directly in OXPHOS or are components of
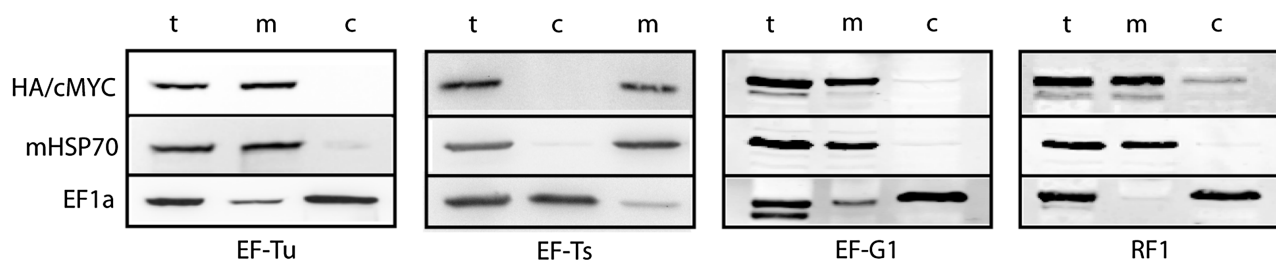
Fig. 1. Localization of epitope-tagged EF-Tu, EF-Ts, EF-G1 and RF1. A total of 0.3×10^7 cell equivalents each of total cellular (t), cytosolic (c) and crude mitochondrial extracts (m) of cell lines expressing C-terminally HA-tagged versions of EF-Tu, EF-G1 and RF1 or cMYC-tagged EF-Ts were analysed by immunoblots using anti-HA- or anti-cMYC-antibodies (top panels). Comparison with molecular mass markers showed that the sizes of the tagged proteins were consistent with the prediction. mHSP70 served as a mitochondrial marker (middle panels) and eukaryotic translation elongation factor 1a (EF1a) was used as a cytosolic marker (bottom panels). Only the relevant regions of the blots are shown.

the mitochondrial ribosomes that produce them (Feagin, 2000). Thus, inhibition of mitochondrial translation will primarily affect OXPHOS but not substrate level phosphorylation (SUBPHOS), which relies exclusively on nuclear-encoded proteins. Measuring OXPHOS activity can therefore be used as a proxy for the functionality of the mitochondrial translation system. The mitochondrion of procyclic *T. brucei* produces ATP by OXPHOS as well as by SUBPHOS catalysed by the citric acid cycle enzyme succinyl-CoA synthetase (Bochud-Allemann and Schneider, 2002). We have established an assay that allows quantification of both modes of ATP production. Extraction of whole cells with low concentrations of digitonin was used to obtain crude mitochondrial fractions that are incubated either with α-ketoglutarate, the substrate for SUBPHOS, or with succinate, a substrate for OXPHOS. Atractyloside, which prevents mitochondrial import of ADP, inhibits both types of mitochondrial ATP production, whereas antimycin an inhibitor of complex III of the respiratory chain selectively blocks OXPHOS (Schneider *et al.*, 2007). Figure 3 shows that ablation of each of the four mitochondrial translation factors of *T. brucei* abolishes OXPHOS without significantly impairing mitochondrial SUBPHOS. These results suggest that the growth phenotypes that are observed in the four RNAi cell lines (Fig. 2) are caused by the inhibition of mitochondrial protein synthesis.

*Ablation of EF-Tu abolishes mitochondrial
protein synthesis*

EF-Tu was studied in more detail. To that end we produced a cell line, termed EF-Tu-3′UTR RNAi, in which in contrast to the cell line shown in Fig. 1A the RNAi was targeting the 3′UTR of the EF-Tu mRNA. Induction of RNAi in this cell line causes essentially the same growth phenotype (Fig. 7A) than was observed in the original RNAi cell line, in which the coding region of EF-Tu was targeted (Fig. 2).

The advantage of this new RNAi cell line is that it allows complementation experiments (see below). Uninduced as well as induced EF-Tu-3′UTR RNAi cell lines were subjected to [^{35}S]-methionine labelling of *de novo* synthesized mitochondrially encoded proteins. The labelled proteins were subsequently resolved on a 9%/14% two-dimensional denaturing acrylamide gel (Horváth *et al.*, 2002; Hashimi *et al.*, 2013). The results in Fig. 4 show that in uninduced cells spots corresponding to mitochondrially encoded cytochrome b (CYTB) and cytochrome oxidase subunit 1 (COX1), as well as a number of still unidentified mitochondrial gene products were detected. Induction of EF-Tu RNAi for 5.5 days, the time of the onset of the growth arrest, abolishes this labelling, indicating that EF-Tu as expected is essential for mitochondrial protein synthesis.

*Quantitative proteomic analysis of the EF-Tu RNAi
cell line*

In order to get a more detailed, unbiased and global picture on the effects of ablation of EF-Tu in trypanosomes, we devised a powerful proteomics approach which comprises RNAi combined with stable isotope labelling by amino acids in cell culture (SILAC) and high-resolution mass spectrometry (MS). Uninduced and induced EF-Tu RNAi cells were grown in media containing heavy or light isotopes of lysine and arginine respectively. Equal cell numbers of both populations were then mixed and mitochondria-enriched fractions were prepared by digitonin extraction. Quantitative MS-based analysis allowed to accurately determine the abundance ratio of proteins in uninduced and induced RNAi cells. The experiment was performed in triplicate and statistical analysis allowed us to identify, on a global scale, which proteins exhibit significant changes in abundance in the induced EF-Tu RNAi cell line (Table S1). All in all 1848 proteins were quantified in at least two replicates (data not shown). After 4 days of induction, at the time point the growth arrest becomes
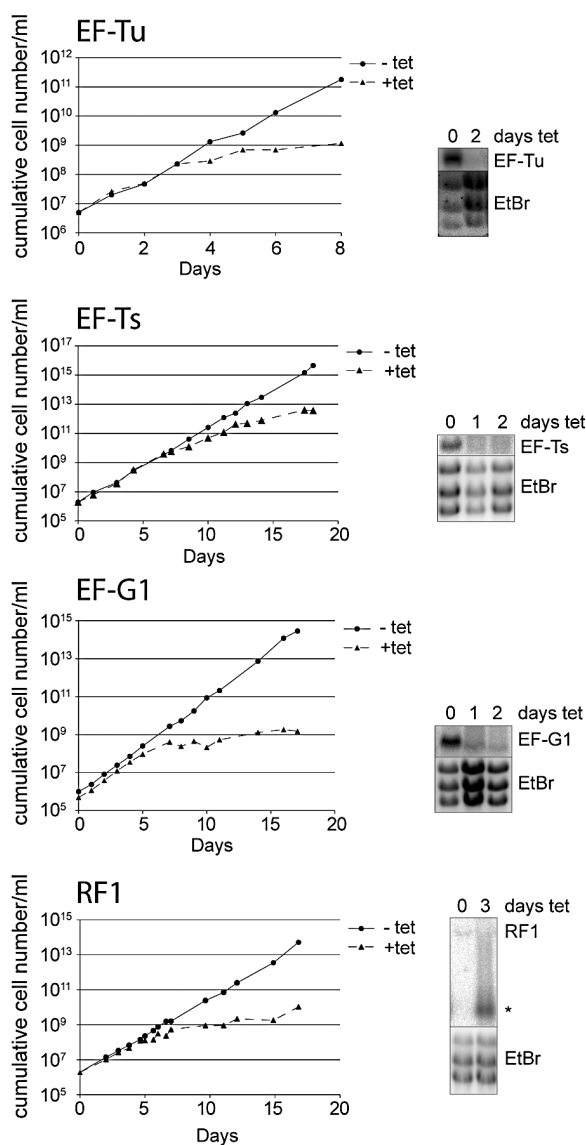
Fig. 2. EF-Tu, EF-Ts, EF-G1 and RF1 are required for normal growth of procyclic *T. brucei*. Growth curves of uninduced (− tet) and induced (+ tet) representative clonal RNAi cell lines directed against the translation factors indicated at the top are shown. The panels on the right depict Northern blots of the corresponding mRNAs isolated at the indicated time points after induction of RNAi. The ethidium bromide (EtBr) stained rRNAs in the lower panels serve as loading controls.

apparent, 21 proteins were more than twofold downregulated (Fig. 5A). As expected the most highly downregulated protein was EF-Tu itself (20-fold), the direct target of the RNAi. Moreover, mitochondrially encoded COX2 was also among the top hits (4.3-fold downregulated). The mitochondrial gene product COX3 was threefold downregulated but only detected in a single replicate (data not shown). A total of 55% of the remaining downregulated proteins are predicted subunits or assembly factors of COX or the bc1-complex (Fig. 5A), which contain three and one

mitochondrially encoded subunits respectively. The results were confirmed by blue-native gel analysis which show a decline of the levels of COX4 and cytochrome C1, a subunit of the bc1-complex, in the corresponding respiratory complexes (Fig. 5B). In summary, the proteome-wide quantitative EF-Tu RNAi analysis and the [³⁵S]-methionine *in vivo* labelling experiments allow to directly link the expression of EF-Tu to mitochondrial protein synthesis.

Interestingly, we also report 61 proteins that are more than twofold upregulated upon EF-Tu RNAi (Fig. 5C). Approximately 60% of these are cytosolic ribosomal proteins, suggesting that ablation of mitochondrial translation causes a global upregulation of cytosolic protein synthesis. The remaining more than twofold upregulated proteins include two glucose transporters, alternative oxidase and fumarate hydratase (Table S1). The latter two may indicate that in the absence of a functional respiratory chain the cell tries to balance the redox state by other means.

EF-Tu of T. brucei *contains a trypanosomatid-specific motif*

Unlike in other eukaryotes, mitochondrial translation in *T. brucei* and its relatives has to function with imported cytosolic tRNAs and with highly derived ribosomes having ultra-short rRNAs (Niemann *et al.*, 2011). Of all the translation factors mentioned above, EF-Tu is therefore of special interest since it must directly interact with both imported, eukaryotic-type tRNAs and the uniquely structured ribosome. EF-Tu of trypanosomatids is highly similar to EF-Tu of other species (Fig. S1). However, a multiple sequence alignment of EF-Tu orthologues from bacteria and mitochondria of various organisms reveals a trypanosomatid-specific motif of approximately 30 amino acids in length, located 100 amino acid residues from the C-terminus (aa 370–400) (Fig. 6A; Fig. S1). This subdomain is conserved within trypanosomatids and consists of almost 50% charged amino acids, with nine basic and eight acidic residues in *T. brucei*. EF-Tu has a three domain structure consisting of domain 1 (position 1–199 in *Escherichia coli* EF-Tu), which includes the GTP binding site, and domains 2 (aa: 209–296 in *E. coli* EF-Tu) and 3 (aa: 300–393 in *E. coli* EF-Tu) that consist of β-strands only (Krab and Parmeggiani, 1998, Krab & Parmeggiani, 2002). A high-resolution structure of *Thermus thermophilus* EF-Tu in complex with aminoacyl-tRNA and GDP bound to the 70S ribosome has been solved (Schmeing *et al.*, 2009). Using the alignment shown in Fig. 6A it is therefore possible to map the trypanosomatid-specific subdomain onto the structure of EF-Tu. This analysis shows that the subdomain localizes to domain 3, where it most likely contributes to a loop that connects two betafolds (data not shown).
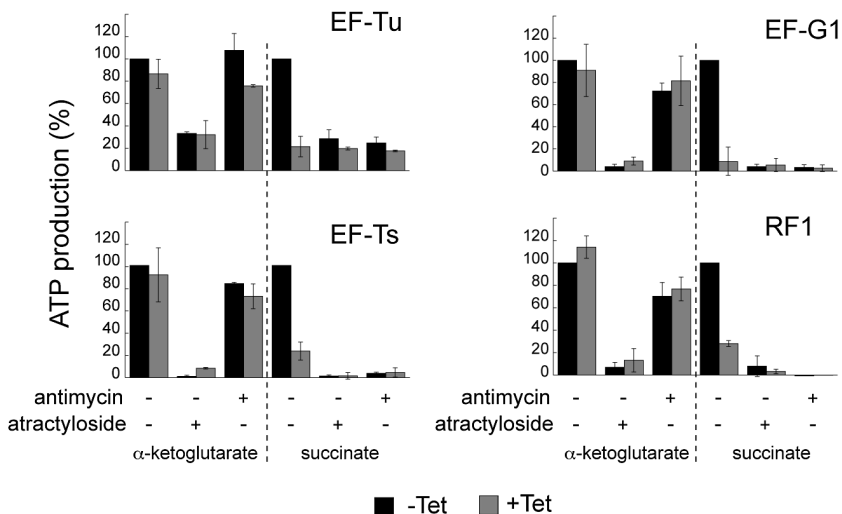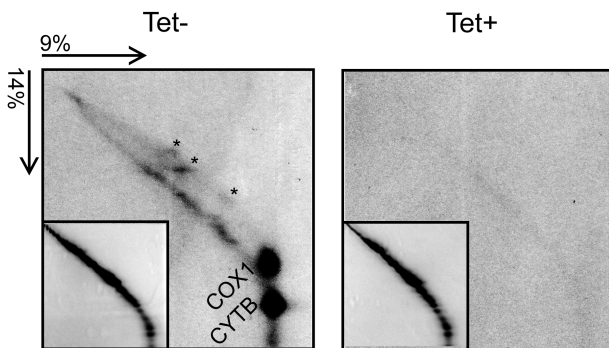
Fig. 3. EF-Tu, EF-Ts, EF-G1 and RF1 are required for OXPHOS but not for mitochondrial SUBPHOS. *In organello* mitochondrial ATP production triggered by α-ketoglutarate and succinate of the indicated uninduced (− Tet) and induced (+ Tet) RNAi cell lines was determined using luciferase-mediated luminescence. The substrates tested and the additions of antimycin and atractyloside are indicated at the bottom. ATP production in mitochondria isolated from uninduced cells tested without antimycin or atractyloside is set to 100%. The bars represent means expressed as percentages. Standard errors of at least three independent biological replicates are indicated. Induction times were: 5 days for EF-Tu; 8 days for EF-Ts; 5 days for EF-G1 and 7 days for RF1.

Eukaryotic translation elongation factor 1a (EF1a) is the orthologue of EF-Tu in the eukaryotic cytosol. Interestingly, a multiple sequence alignment of trypanosomal EF-Tu with cytosolic EF1a orthologues from a number of eukaryotes allows the identification of a conserved sequence segment in EF1a that shows similarity to the trypanosomatid-specific EF-Tu motif (Fig. 6B). With 18 amino acids in length it is shorter than its EF-Tu counterpart, but it is found 60 amino acids from the C-terminus of EF1a in domain 3, which is the same relative position as is seen for the trypanosomatid-specific motif in EF-Tu. Interestingly, the sequence segments in EF-Tu and EF1a consist of almost 50% of charged amino acids and have a high degree of similarity in their central regions.
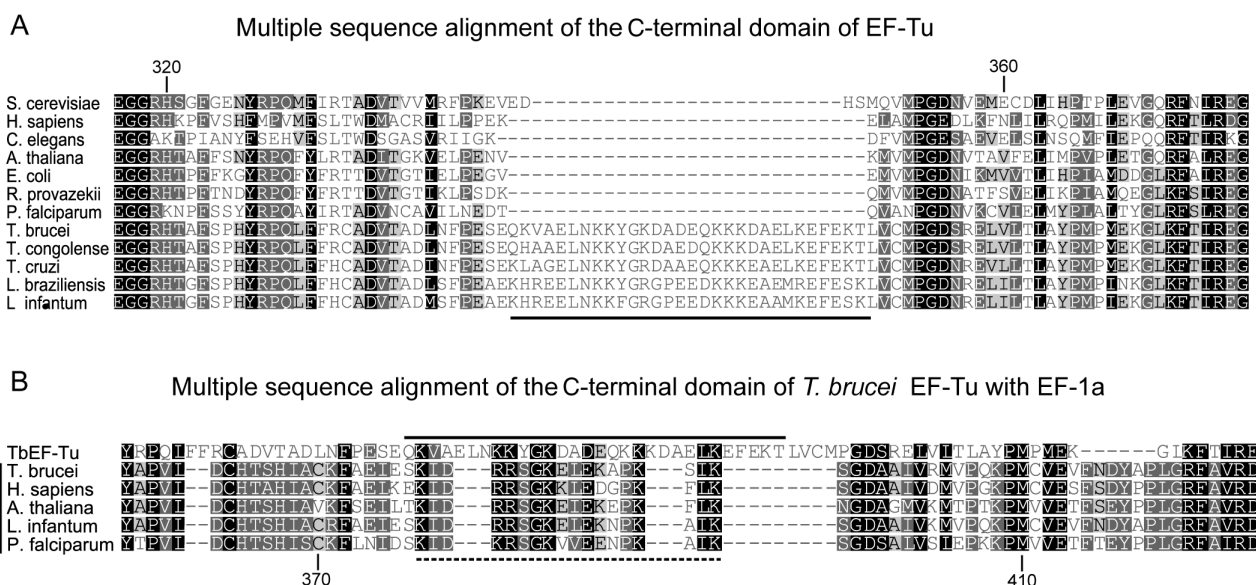

Fig. 5. Global proteomic changes in EF-Tu RNAi cells analysed by SILAC and blue-native gel electrophoresis of respiratory complexes. Equal numbers of uninduced and induced (4 days) EF-Tu RNAi cells, grown in the presence of light or heavy isotopes of lysine and arginine, were mixed and fractionated using digitonin prior to mass spectrometric analysis.

A. Pie chart depicting the 21 proteins that are downregulated at least twofold upon ablation of EF-Tu. Seven proteins are putative components or assembly factors of COX. Four proteins are putative subunits or assembly factors of the bc1-complex. Seven proteins are annotated as hypothetical proteins with unknown function in TriTrypDB (http://tritrypdb.org/tritrypdb/) and three proteins show homology to proteins of known function. The latter include EF-Tu.

B. Blue-native gel immunoblot analysis of COX (COX4) and the bc1-complex (Cyt C1) in uninduced (− Tet) and induced (+ Tet; 4 days) EF-Tu RNAi cells. An immunoblot of an SDS-gel containing the same samples was decorated with voltage-dependent anion channel (VDAC) antiserum to serve as a loading control. Molecular weight markers (kDa) are indicated.

C. Pie chart depicting the 62 proteins that are upregulated at least twofold following induction of EF-Tu RNAi. Thirty-six proteins are putative components of the cytosolic ribosome. Five proteins are annotated as hypothetical proteins with unknown function in TriTrypDB and 21 other proteins show homology to proteins of known function. See Table S1 for the complete data set.

Fig. 4. RNAi silencing of EF-Tu results in inhibition of mitochondrial translation. *De novo* synthesized mitochondrial proteins from parallel uninduced (Tet−) and induced (Tet+, 5.5 days) EF-Tu-3′UTR RNAi cell lines were labelled with S[35]-methionine and subsequently resolved by two-dimensional SDS-PAGE electrophoresis. Acrylamide concentrations of each gel dimension are indicated in the upper left-hand corner. Identified spots corresponding to CYTB and COX1 are indicated along with unidentified polypeptides (*). Insets show same gels with Coomassie-stained cytoplasmic proteins as loading control.

A
Multiple sequence alignment of the C-terminal domain of EF-Tu

B
Multiple sequence alignment of the C-terminal domain of *T. brucei* EF-Tu with EF-1a

Fig. 6. Multiple sequence alignment reveals a trypanosomatid-specific motif in EF-Tu.
A. Multiple sequence alignment of a domain 3 subregion of the indicated mitochondrial and bacterial EF-Tu orthologues. The five trypanosomatid proteins are listed at the bottom. The trypanosomatid-specific motif is underlined. Numbers at the top indicate the amino acid positions in *E. coli* EF-Tu.
B. Multiple sequence alignment of a domain 3 subregion of *T. brucei* EF-Tu with its eukaryotic orthologue EF1a. The trypanosomatid-specific subdomain of EF-Tu is overlined and the segment of EF1a that was used to replace EF-Tu subdomain is indicated by the dashed line. Numbers at the bottom indicate the amino acid positions in the human EF1a.

The trypanosomatid-specific motif of EF-Tu is essential for function

The presence of the trypanosomatid-specific subdomain in EF-Tu raises the questions whether it is required for EF-Tu function and whether it is connected to the similar sequence segment that is found in EF1a. These questions can be addressed using the EF-Tu-3′UTR RNAi cell line, since it allows complementation experiments with wild-type and mutant versions of EF-Tu provided that they are expressed in a different genomic context. The experiment in Fig. 7B shows that ectopic expression of wild-type EF-Tu (EF-Tu-WT) fully restores growth of the EF-Tu-3′UTR RNAi cell line and therefore serves as a positive control. Ectopic expression of an EF-Tu variant lacking the trypanosomatid-specific subdomain (EF-Tu-ΔELKE), on the other hand, was not able to complement the growth arrest (Fig. 7C). However, if a chimeric EF-Tu was expressed, in which the trypanosomatid-specific motif was replaced by the corresponding sequence segment of EF1a (EF-Tu-1a), growth was restored to wild-type levels (Fig. 7D). *In vivo* labelling experiments and blue native gels furthermore showed that the complemented cell line was able to synthesize mitochondrial protein and that it had an intact COX and bc1-complex (Fig. S5).

Northern blots were performed to confirm that in all tested cell lines addition of tetracycline results in (i) ablation of the endogenous EF-Tu mRNA and (ii) induction of

the expression of the ectopic EF-Tu gene copy. Ectopically expressed EF-Tu and its two variants all contained a C-terminal HA-tag, which allows us to confirm that the proteins are efficiently targeted to mitochondria (Fig. 8A). In summary these experiments demonstrate that the trypanosomatid-specific subdomain is essential for EF-Tu function and that it can be replaced by the corresponding sequence segment in EF1a.

One explanation for these results would be that removal of the trypanosomatid-specific motif simply results in the misfolding and potential collapse of the EF-Tu tertiary structure. The resolved structure of the *E. coli* EF-Tu/EF-Ts complex shows that both domain 1 and domain 3 of EF-Tu are in contact with EF-Ts (Kawashima *et al.*, 1996). Thus, misfolding of these EF-Tu domains would impair interaction with EF-Ts and inhibit cell growth.

In order to test whether the EF-Tu variants can still interact with EF-Ts, we used cell lines that simultaneously expresses the HA-tagged EF-Tu variants and cMYC-tagged EF-Ts.

Immunoprecipitation experiments using anti-HA antiserum showed that cMYC tagged EF-Ts efficiently interacts with wild-type EF-Tu as well as with the two EF-Tu variants (Fig. 8B). Interestingly, interaction of EF-Ts with the EF-Tu variant that lack the trypanosomatid-specific domain was even slightly more efficient than with the chimeric EF-Tu, in which the trypanosomatid-specific subdomain was replaced by the corresponding sequence
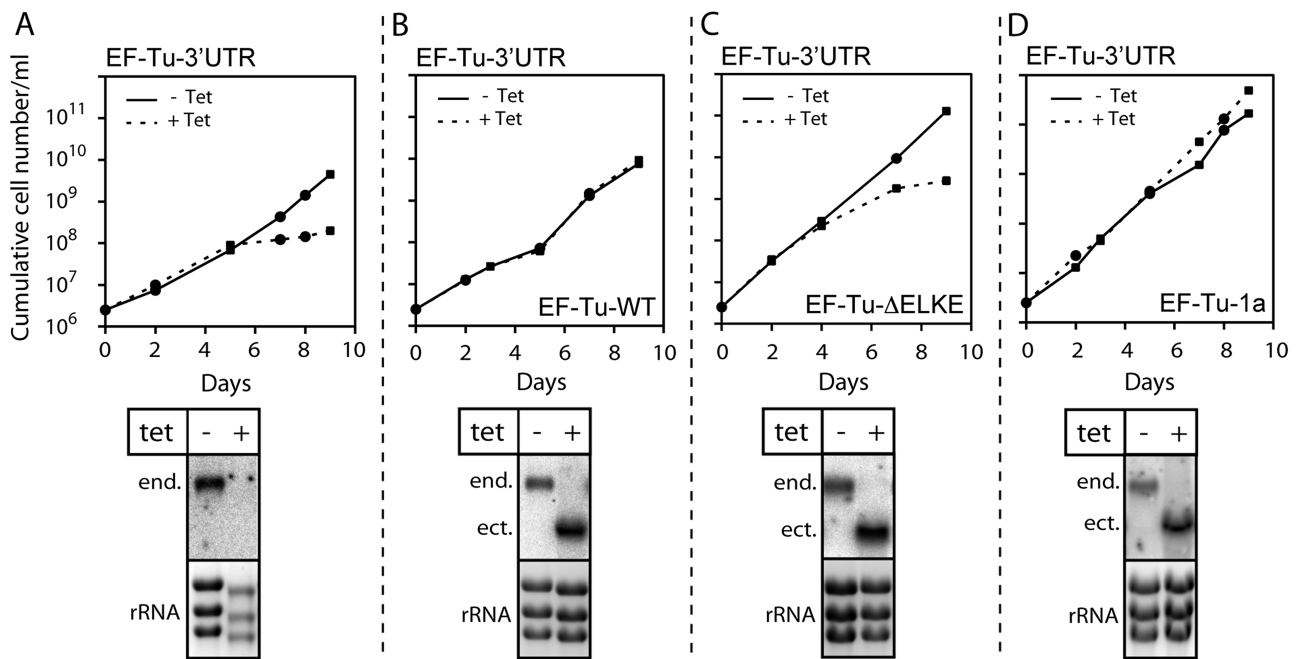
Fig. 7. Complementation experiments using an EF-Tu RNAi cell line targeting the 3′UTR.
A. Top panel, growth curve of uninduced (− Tet) and induced (+ Tet) EF-Tu-3′UTR RNAi cell line. Bottom panel, Northern blots probed for the coding region of the EF-Tu mRNA (end.). The EtBr-stained rRNAs in the lower panels serve as loading controls.
B. EF-Tu-3′UTR RNAi cell line ectopically expressing C-terminally HA-tagged versions of wild-type EF-Tu (EF-Tu-WT) under Tet control. The addition of Tet induces simultaneous downregulation of the endogenous EF-Tu mRNA (end.) and upregulation of the ectopic copy (ect.) of the EF-Tu gene respectively.
C. As in (B) but the complementation was performed using a C-terminally HA-tagged version of EF-Tu lacking the trypanosomatid-specific motif (EF-Tu-ΔELKE).
D. As in (B) but the complementation was done using a HA-tagged version of EF-Tu in which the trypanosomatid-specific subdomain was replaced by the corresponding region of EF1a (EF-Tu-1a).

segment of EF1a, even though only the latter could complement growth. These results strongly suggest that removal of the trypanosomatid-specific subdomain does not result in misfolding of domain 3 of EF-Tu but interferes with its function in a more subtle way.

Discussion

In contrast to mitochondrial RNA editing, which has been a major focus of research in trypanosomes during the last two decades, the study of mitochondrial translation has been largely neglected. This is somewhat ironic since the only function of RNA editing is to convert transcripts of mitochondrially encoded cryptogenes into functional mRNAs that can be translated. Here, we provide the first analysis of four *T. brucei* mitochondrial translation factors, with a special emphasis on EF-Tu. All four proteins are essential for normal growth of procyclic trypanosomes. Whereas EF-Tu has previously been shown to be essential for the bloodstream from Cristodero *et al.* (2010), the other three proteins have not been studied in this life cycle stage yet. However, since it is well established that mitochondrial translation is essential in bloodstream forms of

the parasite, we would expect EF-G1, EF-Ts and RF1 to be essential in this pathogenic stage as well. The requirement of EF-Ts for normal growth is interesting in the light of the fact that the genome of *Saccharomyces cerevisiae* lacks an EF-Ts orthologue, indicating that its EF-Tu has the capability for self-recycling (Chiron *et al.*, 2005). In contrast, mitochondrial translation in *Schizosaccharomyces pombe* and humans depends on EF-Ts and therefore, in this respect, groups together with the *T. brucei* system.

In order to get a more precise picture of the proteomic changes that are caused by the EF-Tu RNAi, we developed a SILAC-based method that in principle is applicable to any inducible RNAi cell line of trypanosomes. It allowed to quantify the changes in the abundance levels of 1721 proteins that were induced by EF-Tu RNAi. The results show a strong downregulation of mitochondrially encoded COX2 and COX3. The remaining 16 predicted mitochondrially encoded proteins were not detected. This is not surprising since trypanosomal proteins encoded by mitochondrial genes are notoriously difficult to detect due to their small size and extreme hydrophobicity (Horváth *et al.*, 2000; 2002). In fact the only MS-based study that identified mitochondrially encoded proteins before also
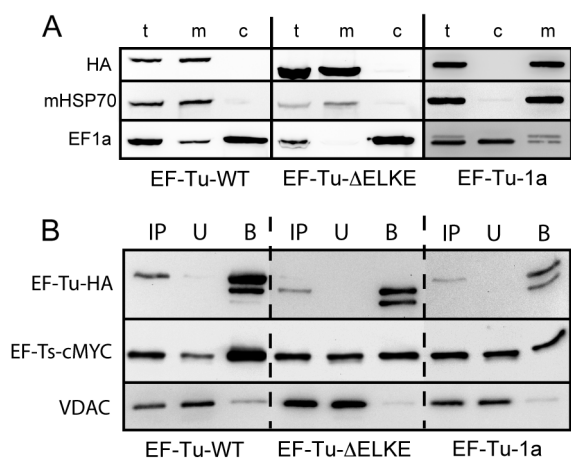
Fig. 8. All versions of EF-Tu localize to mitochondria and interact with EF-Ts.
A. A total of 0.3×10^7 cell equivalents each of total cellular (t), cytosolic (c) and crude mitochondrial extracts (m) of cell lines expressing C-terminally HA-tagged wild-type EF-Tu (EF-Tu-WT) or mutated EF-Tu versions (EF-Tu-ΔELKE, EF-Tu-1a) were analysed by immunoblots as in Fig. 1.
B. Digitonin extracts of cell lines expressing EF-Ts carrying a C-terminal cMYC-tag and the indicated HA-tagged EF-Tu variants were immunoprecipitated using anti-HA antibodies and analysed by immunoblotting. Ten per cent each of total extract (IP) and unbound fraction (U) as well as 95% of the bound fraction (B) were analysed by immunoblot. The panels were probed with anti-HA, anti-cMYC and anti-VDAC antibodies, as indicated.

only detected COX2 and COX3, even though in this case isolated respiratory complexes were analysed (Acestor *et al.*, 2011). Thus, the strong reduction of COX2 and COX3 levels upon RNAi strongly suggest that EF-Tu is directly required for their synthesis. In addition, we also observed a decrease in abundance of many nuclear-encoded subunits of both COX and the bc1-complex (Fig. 5A), which is consistent with the results from immunoblots of blue-native gels in which the two complexes essentially cannot be detected anymore after induction of RNAi. In summary, this suggests that the mitochondrially encoded subunits play a central role in the assembly and/or stability of these two respiratory complexes and that in their absence most of their nuclear-encoded subunits are degraded. Global proteomic analysis of the changes caused by the EF-Tu RNAi is also an excellent tool to study potential compensatory mechanism with which the cell tries to counteract the loss of mitochondrial protein synthesis. Indeed we observed a co-ordinated increase in expression of essentially all cytosolic ribosomal proteins detected in the EF-Tu RNAi proteome (Fig. 5C, Table S1). This suggests that upregulation of cytosolic protein synthesis is one of the main responses induced by the absence of mitochondrial translation and/or the subsequent loss of OXPHOS.

EF-Tu of *T. brucei* contains a short, highly charged insertion of approximately 30 amino acids close to the

C-terminus that is essential for its function. EF-Tu functions in bringing charged elongator tRNAs to the ribosomes. It must therefore interact with both, tRNAs and ribosomes. We have purified recombinant trypanosomal wild-type EF-Tu from *E. coli*. However, despite extensive efforts we were not able to show that it binds tRNAs of any source. Moreover, the purification of native mitochondrial ribosomes of trypanosomatids in amounts that would be required for EF-Tu binding assays, is also very challenging (Zíková *et al.*, 2008; Sharma *et al.*, 2009). Thus, direct biochemical interrogation of tRNA/EF-Tu or EF-Tu/ribosome interactions is at the moment not possible. However, a comparative analysis of the trypanosomatid mitochondrial translation system with that of other eukaryotes suggests that the trypanosomatid-specific EF-Tu motif may be linked to features of mitochondrial translation that are unique to these flagellates and therefore sheds light on its possible function.

An interesting deviation of mitochondrial translation in trypanosomatids in comparison with other systems is that it functions exclusively with imported tRNAs that are of the eukaryotic-type (Salinas *et al.*, 2008; Alfonzo and Söll, 2009; Schneider, 2011). This means that trypanosomal EF-Tu, in contrast to its homologues in other species, has to bind eukaryotic-type tRNAs. It is therefore tempting to speculate that the trypanosomatid-specific EF-Tu motif represents an adaptation of the bacterial-type mitochondrial translation system to the eukaryotic-type tRNAs. This notion is supported by the fact that it can be replaced by the corresponding sequence of eukaryotic EF1a. However, whereas bacterial-type and eukaryotic-type initiator tRNAs are very different (RajBhandary, 1994) this is not the case for the elongator tRNAs. Some have identity elements that differ between the two domains and therefore must be charged by the matching eukaryotic-type or bacterial-type aminoacyl-tRNA synthetases. Once charged, however, the elongator tRNAs of eukaryotes and bacteria are functionally largely interchangeable. Furthermore, the loss of mitochondrial tRNA genes is not restricted to trypanosomatids but also occurred in another group of parasitic protozoa, the apicomplexans (Crausaz-Esseiva *et al.*, 2004; Pino *et al.*, 2010). These organisms are therefore faced with the same problem than trypanosomatids, namely to use of eukaryotic-type tRNAs in mitochondrial translation. However, apicomplexans have a standard EF-Tu that lacks the C-terminal motif found in trypanosomatids (see *P. falciparum* row in Fig. 6A), indicating that this subdomain cannot stringently be required to recognize eukaryotic-type tRNAs.

Another unique feature of trypanosomatids is their mitochondrial ribosomes, which are smaller than bacterial ribosomes, have very short rRNAs and hence show a very low RNA to protein ratio (Sharma *et al.*, 2009; Agrawal and Sharma, 2012). It is therefore feasible that the

trypanosomatid-specific subdomain is required for binding of EF-Tu to these unique ribosomes. A recent cryo-electron microscopy study of mitochondrial ribosomes of the trypanosomatid *Leishmania tarentolae* supports this notion, as it showed that despite the generally conserved structure, they are more porous when compared with their counterparts in mammalian mitochondria (Sharma *et al.*, 2009). Most important in the context of our study is the finding that the intersubunit space is dramatically remodelled in the mitochondrial ribosome of *L. tarentolae*, when compared with any other ribosome. Based on this distinctive intersubunit space, which accommodates the interactions of tRNAs and translational factors, the authors predicted that translational elongation factors in the *Leishmania* mitochondrion should be quite distinctive (Sharma *et al.*, 2009).

EF-Tu of *T. brucei* and *T. thermophilus* are highly homologous. A high-resolution structure of the ribosome bound to EF-Tu and aminoacyl-tRNA is available for *T. thermophilus* (Schmeing *et al.*, 2009). Thus, the overall localization of the ELKE-motif can be assigned in the *T. thermophilus* EF-Tu ribosome complex (Fig. S6). On the basis of this structural analysis the ELKE-motif would be localized in close proximity to ribosomal protein L11, which is consistent with the notion that the ELKE-motif is required for ribosome binding. L11 builds the basis of the L7/L12 stalk, a structurally flexible region of the large subunit of the ribosome that contributes to the intersubunit space. However, since the ELKE-motif would also be in close proximity to the tRNA elbow, it cannot be excluded that it is also involved in the interaction between tRNA and EF-Tu.

In summary, we believe it most likely that the trypanosomatid-specific EF-Tu subdomain may serve as an adaptation that allows the protein to fit into the distinctive intersubunit space of mitochondrial ribosomes. The observation that it can be replaced with the corresponding sequence of EF1a may be explained by the similar charge distribution that is observed in both sequences.

Trypanosomes have a unique evolutionary history. They may represent the earliest diverging eukaryotes with mitochondria capable of OXPHOS (Dacks *et al.*, 2008; Cavalier-Smith, 2010). Understanding their highly unusual mitochondrial translation system is therefore expected to provide insight into both fundamentally conserved and evolutionary flexible aspects of mitochondrial protein synthesis and perhaps also translation in general. Moreover, bacterial EF-Tu – after the ribosome itself – is the major target for antibiotics affecting protein synthesis (Krab and Parmeggiani, 1998). Thus, since mitochondrial translation is essential for the pathogenic bloodstream form of *T. brucei*, its unique features are also of interest for the hunt of novel drug targets (Brun *et al.*, 2011).

Experimental procedures

Culture of cells

Procyclic transgenic cell lines are based on *T. brucei* 29-13. They were grown at 27°C in SDM-79 supplemented with 15% fetal calf serum and the required antibiotics. Transformation and cloning of transgenic cell lines were done as described (Beverley and Clayton, 1993). The concentration of antibiotics used to select transgenic cell lines was 5 µg ml^{-1} for blasticidin and 0.1 µg ml^{-1} for puromycin. Expression of tagged proteins or double-stranded RNAs for RNAi was induced with 1 µg ml^{-1} tetracycline.

Constructs for inducible RNAi

All RNAi cell lines were stem-loop constructs based on pLew-100 carrying the blasticidin resistance gene (Wirtz *et al.*, 1999; Bochud-Allemann and Schneider, 2002). RNAi against the coding region of EF-Tu (Tb927.10.13360) was performed as previously described (Cristodero *et al.*, 2010). RNAi directed against the 3′UTR of EF-Tu (EF-Tu-3′UTR) was done using a 465 bp region immediately downstream of the stop codon. The 3′UTR of the EF-Tu mRNA is estimated to be longer than 1 kb (Kolev *et al.*, 2010). For the three remaining RNAi cell lines we used the following inserts: a 455 bp fragment (nucleotides 4–459) of the EF-Ts gene (Tb927.3.3630); a 626 bp fragment (nucleotides 1051–1677) of the EF-G1 gene (Tb927.10.5010) and a 653 bp fragment (nucleotides 3–656) of the RF1 gene (Tb927.3.1070).

Constructs for epitope tagging

Tet-inducible expression of C-terminally HA-tagged EF-Tu, EF-G1 and RF1 was achieved using pLew-100-derived constructs carrying the puromycin resistance marker (Wirtz *et al.*, 1999; Bochud-Allemann and Schneider, 2002). C-terminal *in situ* tagging of EF-Ts with the cMYC epitope was done as already described (Oberholzer *et al.*, 2005). For generation of the EF-Tu-DELKE mutant a 28-amino-acid region, comprising amino acids 372–399 of EF-Tu (encoded by: AAGGTT GCTGAGTTGAACAAAAAGTATGGGAAGGACGCGGATGA GCAAAAGAAGAAGGATGCAGAGTTGAAGGAGTTCGAAA AG), was deleted by fusion PCR. The mutant EF-Tu-1a chimera was produced by deleting 24 amino acids of EF-Tu (position 372–395) and by replacing them with 18 amino acids (position 365–382, encoded by AGATCGACCGTCG CTCTGGCAAGGAGCTGGAGAAGGCTCCCAAGTCGATCA AGT) of trypanosomal *T. brucei* EF1a.

Culture of cells for SILAC proteomics

EF-Tu RNAi cells grown in SDM-79 containing 15% FCS were washed in PBS and cultured in modified SDM-80 (Lamour *et al.*, 2005) containing 5.55 mM glucose and supplemented with the normal light (L) or heavy (H) amino acids [^{13}C/^{15}N]Arg (1.1 mM) and [^{13}C/^{15}N]Lys (0.4 mM) (Cambridge Isotope Laboratories, USA) in the presence of 20% dialysed FCS (BioConcept, Switzerland) for 2 days. Cultures were induced with tetracycline (Tet) for 4 days and uninduced and

induced cells mixed in a 1:1 ratio (L − Tet/H + Tet; L − Tet/H + Tet; L + Tet/H − Tet). Cells were extracted using digitonin to obtain a crude mitochondrial fraction (Bochud-Allemann and Schneider, 2002) for mass spectrometric analyses.

Quantitative mass spectrometry and data analysis

SILAC-labelled proteins (30 μg) of crude mitochondrial fractions ($n = 3$) resuspended in urea buffer (30 mM Tris-HCl, 7 M urea, 2 M thiourea, pH 8.5) were separated by SDS-PAGE and visualized using colloidal Coomassie Blue. Gel lanes were cut into 12 slices of equal size. Following reduction of disulphide bonds (10 mM DTT in 10 mM NH_4HCO_3, 30 min at 65°C) and subsequent alkylation of free thiol groups (55 mM iodoacetamide in 10 mM NH_4HCO_3, 30 min at RT, in the dark), proteins were in-gel digested with trypsin and the resulting peptide mixtures analysed by UHPLC-ESI-MS/MS on an LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany) online coupled to an Ultimate 3000 RSLCnano system (Thermo Scientific, Idstein, Germany) essentially as described before (Mick *et al.*, 2012).

Mass spectrometric raw data were processes with Max-Quant (version 1.3.0.5; Cox and Mann, 2008) and its integrated search algorithm Andromeda (Cox *et al.*, 2011). Proteins were identified by correlating MS/MS spectra with a database containing the entries of the *T. brucei* protein database (TriTrypDB, release 4.2) as well as the amino acid sequences of the currently known mitochondrially encoded proteins as described before (Niemann *et al.*, 2013) including carbamidomethylation of cysteine residues as fixed modification. Proteins were identified with ≥ one unique peptide of at least six amino acids and a false discovery rate of < 0.01 on peptide and protein level. SILAC-based relative protein quantification was based on unique peptides and ≥ one SILAC peptide pair. Protein abundance ratios (induced/uninduced) normalized to the median of the respective replicate were $\log_{10}$-transformed and mean $\log_{10}$ ratios across all three replicates as well as the p-value of each protein were calculated.

Immunoprecipitations

Cell lines allowing simultaneous RNAi-mediated depletion of EF-Tu (EF-Tu-3′UTR), overexpression of HA-tagged EF-Tu variants (EF-Tu-WT, EF-Tu-ΔELKE and EF-Tu-1a) and cMYC tagged EF-Ts were induced for 48 h and harvested by centrifugation. Pellets from 3.75×10^8 cells were resuspended in 470 μl of 25 mM Tris-HCl, pH 7.5, 50 mM KCl, 1× protease inhibitor mix (Roche, Switzerland), 0.25% (w/v) Nonidet-40 (NP-40) and 1 mM EDTA and lysed by incubation for 5 min at 4°C with constant mixing. Samples were cleared by centrifugation at 500 *g* for 2 min and the resulting supernatant was adjusted to 1.25% (w/v) of NP-40. After incubation for 10 min at 4°C the supernatants were centrifuged again at 16 000 *g* for 40 min. The cleared supernatants (500 μl each) were added to pre-washed beads containing covalently linked anti-HA antibodies (40 μl of 1:1 slurry) (Roche, Switzerland) and incubated for 2 h at 4°C under constant mixing. Subsequently, the beads were extensively washed in the same buffer containing 0.1% (w/v) of NP-40. Final eluates were obtained by boiling the beads in SDS sample buffer.

Miscellaneous

Digitonin extractions and ATP production assays were performed as previously described (Bochud-Allemann and Schneider, 2002; Schneider *et al.*, 2007). RNA isolation, and Northern blot analysis were performed according to Tan *et al.* (2002b).

[^{35}S]-methionine *in vivo* labelling of mitochondrially synthesized proteins was done as described (Horváth *et al.*, 2002; Hashimi *et al.*, 2013). Multiple sequence alignments were done using CLUSTAL omega from EBI (Sievers *et al.*, 2011). The following antibodies were used for immunoblots (working dilutions are indicated in parentheses): monoclonal anti-HA antiserum (HA11, Covance Research Products, Princeton, USA) (1:1000), monoclonal anti-cMYC antiserum (Invitrogen, USA) (1:1000), monoclonal anti-EF1a antiserum (Santa Cruz Biotechnology, USA) (1:10 000), polyclonal anti-mHSP70 antiserum (1:1000) (provided by R. Jensen), polyclonal anti-VDAC antiserum (1:1000), polyclonal anti-COX4 antiserum (1:1000) and polyclonal anti-Cyt C1 antiserum.

References

Acestor, N., Zíková, A., Dalley, R.A., Anupama, A., Panigrahi, A.K., and Stuart, K.D. (2011) *Trypanosoma brucei* mitochondrial respiratome: composition and organization in procyclic form. *Mol Cell Proteomics* **10:** M110.006908. doi:10.1074/mcp.M1110.006908

Agrawal, R.K., and Sharma, M.R. (2012) Structural aspects of mitochondrial translational apparatus. *Curr Opin Struct Biol* **22:** 797–803.

Alfonzo, J.D., and Söll, D. (2009) Mitochondrial tRNA import – the challenge to understand has just begun. *Biol Chem* **390:** 717–722.

Alfonzo, J.D., Blanc, V., Estevez, A.M., Rubio, M.A.T., and Simpson, L. (1999) C to U editing of anticodon of imported mitochondrial tRNA^{Trp} allows decoding of UGA stop codon in *Leishmania*. *EMBO J* **18:** 7056–7062.

Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renauld, H., Bartholomeu, D.C., *et al.* (2005) The genome of the African trypanosome *Trypanosoma brucei*. *Science* **309:** 416–422.

Beverley, S.M., and Clayton, C.E. (1993) Transfection of

Leishmania and *Trypanosoma brucei* by electroporation. *Methods Mol Biol* **21:** 333–348.

Bochud-Allemann, N., and Schneider, A. (2002) Mitochondrial substrate level phosphorylation is essential for growth of procyclic *Trypanosoma brucei*. *J Biol Chem* **277:** 32849–32854.

Brun, R., Don, R., Jacobs, R.T., Wang, M.Z., and Barrett, M.P. (2011) Development of novel drugs for human African trypanosomiasis. *Future Microbiol* **6:** 677–691.

Cavalier-Smith, T. (2010) Kingdoms Protozoa and Chromista and the eozoan root of the eukaryotic tree. *Biol Lett* **6:** 342–345.

Charrière, F., Helgadóttir, S., Horn, E.K., Söll, D., and Schneider, A. (2006) Dual targeting of a single tRNATrp requires two different tryptophanyl-tRNA synthetases in *Trypanosoma brucei*. *Proc Natl Acad Sci USA* **103:** 6847–6852.

Chiron, S., Suleau, A., and Bonnefoy, N. (2005) Mitochondrial translation: elongation factor tu is essential in fission yeast and depends on an exchange factor conserved in humans but not in budding yeast. *Genetics* **169:** 1891–1901.

Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* **26:** 1367–1372.

Cox, J., Neuhauser, N., Michalski, A., Scheltema, R.A., Olsen, J.V., and Mann, M. (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res* **10:** 1794–1805.

Crausaz-Esseiva, A., Naguleswaran, A., Hemphill, A., and Schneider, A. (2004) Mitochondrial tRNA import in *Toxoplasma gondii*. *J Biol Chem* **279:** 42363–42368.

Cristodero, M., Seebeck, T., and Schneider, A. (2010) Mitochondrial translation is essential in bloodstream forms of *Trypanosoma brucei*. *Mol Microbiol* **78:** 757–769.

Dacks, J.B., Walker, G., and Field, M.C. (2008) Implications of the new eukaryotic systematics for parasitologists. *Parasitol Int* **57:** 97–104.

Feagin, J.E. (2000) Mitochondrial genome diversity in parasites. *Int J Parasitol* **30:** 371–390.

Gray, M.W. (2012) Mitochondrial evolution. *Cold Spring Harb Perspect Biol* **4:** a011403.

Hashimi, H., McDonald, L., Stríbrná, E., and Lukeš, J. (2013) Trypanosome Letm1 protein is essential for mitochondrial potassium homeostasis. *J Biol Chem*. PMID: 23893410.

Horváth, A., Berry, E.A., and Maslov, D.A. (2000) Translation of the edited mRNA for cytochrome b in trypanosome mitochondria. *Science* **287:** 1639–1640.

Horváth, A., Nebohacova, M., Lukeš, J., and Maslov, D.A. (2002) Unusual polypeptide synthesis in the kinetoplast-mitochondria from *Leishmania* tarentolae. Identification of individual *de novo* translation products. *J Biol Chem* **277:** 7222–7230.

Kawashima, T., Berthet-Colominas, C., Wulff, M., Cusack, S., and Leberman, R. (1996) The structure of the *Escherichia coli* EF-Tu.EF-Ts complex at 2.5 A resolution. *Nature* **379:** 511–518.

Kolev, N.G., Franklin, J.B., Carmi, S., Shi, H., Michaeli, D., and Tschudi, G. (2010) The transcriptome of the human pathogen *Trypanosoma brucei* at single-nucleotide resolution. *PLoS Pathog* **6:** e1001090.

Krab, I.M., and Parmeggiani, A. (1998) EF-Tu, a GTPase odyssey. *Biochim Biophys Acta* **1443:** 1–22.

Krab, I.M., and Parmeggiani, A. (2002) Mechanisms of EF-Tu, a pioneer GTPase. *Prog Nucleic Acid Res Mol Biol* **71:** 513–551.

Lamour, N., Riviere, L., Coustou, V., Coombs, G.H., Barrett, M., and Bringaud, F. (2005) Proline metabolism in procyclic *Trypanosoma brucei* is down-regulated in the presence of glucose. *J Biol Chem* **280:** 11902–11910.

Mick, D.U., Dennerlein, S., Wiese, H., Reinhold, R., Lorenzi, I., Sasarman, F., et al. (2012) Respiratory chain assembly is linked to protein import and translational regulation via the MITRAC complex. *Cell* **151:** 1528–1541.

Niemann, M., Schneider, A., and Cristodero, M. (2011) Mitochondrial translation in trypanosomatids: a novel target for chemotherapy? *Trends Parasitol* **27:** 429–433.

Niemann, M., Wiese, S., Mani, J., Chanfon, A., Jackson, C., Meisinger, C., et al. (2013) Mitochondrial outer membrane proteome of *Trypanosoma brucei* reveals novel factors required to maintain mitochondrial morphology. *Mol Cell Proteomics* **12:** 515–528.

Oberholzer, M., Morand, S., Kunz, S., and Seebeck, T. (2005) A vector series for rapid PCR-mediated C-terminal in situ tagging of *Trypanosoma brucei* genes. *Mol Biochem Parasitol* **145:** 117–120.

Pino, P., Aeby, E., Foth, B.J., Sheiner, L., Soldati, T., Schneider, A., and Soldati-Favre, D. (2010) Mitochondrial translation in absence of local tRNA aminoacylation and methionyl tRNA formylation in Apicomplexa. *Mol Microbiol* **76:** 706–718.

RajBhandary, U.L. (1994) Initiator transfer RNAs. *J Bacteriol* **176:** 547–552.

Rorbach, J., Soleimanpour-Lichaei, R., Lightowlers, R.N., and Chrzanowska-Lightowlers, Z.M. (2007) How do mammalian mitochondria synthesize proteins? *Biochem Soc Trans* **35:** 1290–1291.

Salinas, T., Duchêne, A.M., and Maréchal-Drouard, L. (2008) Recent advances in tRNA mitochondrial import. *Trends Biochem Sci* **33:** 320–329.

Schmeing, T.M., Voorhees, R.M., Kelley, A.C., Gao, Y.-G., Murphy, F.V., Weir, J.R., and Ramakrishnan, V. (2009) The crystal structure of the ribosome bound to EF-Tu and aminoacyl-tRNA. *Science* **326:** 688–694.

Schneider, A. (2011) Mitochondrial tRNA import and its consequences for mitochondrial translation. *Annu Rev Biochem* **80:** 1033–1053.

Schneider, A., Bouzaidi-Tiali, N., Chanez, A.-L., and Bulliard, L. (2007) ATP production in isolated mitochondria of procyclic *Trypanosoma brucei*. *Methods Mol Biol* **372:** 379–387.

Sharma, M.R., Booth, T.M., Simpson, L., Maslov, D.A., and Agrawal, R.K. (2009) Structure of a mitochondrial ribosome with minimal RNA. *Proc Natl Acad Sci USA* **106:** 9637–9642.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., et al. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* **11:** 539.

Spremulli, L.L., Coursey, A., Navratil, T., and Hunter, S.E. (2004) Initiation and elongation factors in mammalian mitochondrial protein biosynthesis. *Prog Nucleic Acid Res Mol Biol* **77:** 211–261.

Tan, T.H.P., Bochud-Allemannn, N., Horn, E.K., and Schneider, A. (2002a) Eukaryotic-type elongator tRNAMet of *Trypanosoma brucei* becomes formylated after import into mitochondria. *Proc Natl Acad Sci USA* **99:** 1152–1157.

Tan, T.H.P., Pach, R., Crausaz, A., Ivens, A., and Schneider, A. (2002b) tRNAs in *Trypanosoma brucei*: genomic organization, expression and mitochondrial import. *Mol Cell Biol* **22:** 3707–3717.

Wirtz, E., Leal, S., Ochatt, C., and Cross, G.A. (1999) A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei. Mol Biochem Parasitol* **99:** 89–101.

Ziková, A., Panigrahi, A.K., Dalley, R.A., Acestor, N., Anupama, A., Ogata, Y., *et al.* (2008) *Trypanosoma brucei* mitochondrial ribosomes: affinity purification and component identification by mass spectrometry. *Mol Cell Proteomics* **7:** 1286–1296.

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