Short Communication

The pseudo-dimeric tyrosyl-tRNA synthetase of *T. brucei* aminoacylates cytosolic and mitochondrial $tRNA^{Tyr}$ and requires both monomeric units for activity

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Highlights

- *Tb*-TyrRS is essential for normal growth in procyclic *T. brucei*
- *Tb*-TyrRS is the only enzyme responsible for charging of both cytosolic and mitochondrial $tRNA^{Tyr}$
- short N- or C-terminal deletions inactivate *Tb*-TyrRS

Abstract

Aminoacyl-tRNA synthetases are essential for protein synthesis. The single-copy tyrosyl-tRNA synthetase (*Tb*-TyrRS) of *T. brucei* has an unusual structure and forms a pseudo-dimer. It is therefore twice the size than tyrosyl-tRNA synthetases of most other organisms. Here we show by inducible RNAi that *Tb*-TyrRS is essential for normal growth of procyclic *T. brucei*. Furthermore we demonstrate that *Tb*-TyrRS aminoacylates cytosolic as well as mitochondrial $tRNA^{Tyr}$ indicating that it is dually localized. Finally we show that individual deletion of the 36 N- or C-terminal amino acids abolishes the function of *Tb*-TyrRS. This indicates that both monomeric units of the enzyme, the C-terminal one of which is predicted to lack enzymatic activity, are essential for *Tb*-TyrRS function. In summary our results together with previous studies support the notion that *Tb*-TyrRS might be a suitable drug target.

The parasitic protozoan *Trypanosoma brucei* is a treasure trove to investigate unusual aspects of tRNA biology. Unlike most other eukaryotes the mitochondrial genome of trypanosomes and their relatives lacks tRNA genes; as consequence all tRNAs required for organellar translation derive from nucleus-encoded cytosolic tRNAs that are imported into the mitochondrion [1]. Only two tRNAs are cytosol specific, the initiator $tRNA^{Met\text{-}i}$ and the $tRNA^{Sec}$, all the others are dually localized in the cytosol and the mitochondrion [2]. Since cytosolic and mitochondrial tRNAs derive from the same nuclear genes it would make sense if the corresponding aminoacyl-tRNA synthetases (aaRSs) would also be dually localized. The fact that 17 trypanosomal aaRSs are encoded by single copy genes supports this notion [3, 4]. However, three aaRSs have distinct genes encoding cytosolic and mitochondrial versions of the enzyme indicating that exceptions are possible [5-7].

The trypanosomal $tRNA^{Tyr}$ has been studied in detail. It is the only trypanosomal tRNA with an intron and editing of the intron is essential for splicing [8-10]. Moreover, the $tRNA^{Tyr}$ is subject to retrograde transport into the nucleus [11] and its anticodon region is differentially modified in the cytosol and the mitochondrion [12].

The *T. brucei* genome encodes a single TyrRS ortologue (*Tb*-TyrRS, Tb927.7.3620) containing both predicted anticodon-binding and catalytic domains. As expected the latter contains sequences similar to the class I aaRS signature motifs HIGH and KMSKS.

However, rather than having a single copy of each domain, the catalytic and the anticodon-binding domains of *Tb*-TyrRS are tandemly repeated which results in a protein twice the size than its counterparts in yeast and humans. Interestingly the two repeats are quite diverged and the catalytic domain in the C-terminal half of the proteins lacks class I aaRS signature motifs and thus is predicted to be inactive. Analysis of the crystal structure of the leishmanial TyrRS has shown that it forms a pseudo-dimer mimicking the structure of the homodimer formed by the canonical TyrRS where the catalytic sites pair with the anticodon binding sites [13-16].

To investigate the function of the putative *Tb*-TyrRS, we established a transgenic cell line allowing tetracycline-inducible ablation of the enzyme. The Northern blot inset in Fig. 1A shows that induction of RNAi leads to degradation of the *Tb*-TyrRS mRNAs. Most importantly, concomitant with the depletion of the *Tb*-TyrRS mRNAs, a growth arrest is observed 3 days after the addition of tetracycline. Thus *Tb*-TyrRS is essential for normal growth of insect stage *T. brucei*.

To analyze the biochemical phenotype the ablation of *Tb*-TyrRS causes, total and mitochondrial RNA was isolated from cells grown in the absence and presence of tetracycline. Subsequently, the two RNA fractions were resolved on a long acid urea polyacrylamide gel and after transfer to nitrocellulose analyzed by Northern blot hybridization. This gel system allows to separate aminoacylated from deacylated tRNAs [17]. The results in Fig. 1B show that ablation of *Tb*-TyrRS results in the accumulation of both uncharged cytosolic and uncharged mitochondrial $tRNA^{Tyr}$. However, as expected it had no influence on the aminoacylation levels of cytosolic and mitochondrial $tRNA^{Ile}$ nor on the initiator $tRNA^{Met\text{-}i}$ which serve as negative controls. The fact that the cytosol-specific initiator $tRNA^{Met\text{-}i}$ is not detected in the mitochondrial RNA fraction illustrates that it is free of cytosolic contaminants. In order to determine the migration pattern of the non-aminoacylated tRNAs an aliquot of the RNA isolated from the uninduced RNAi cell line was chemically deacylated. While this treatment resulted in the accumulation of uncharged $tRNA^{Tyr}$ and the $tRNA^{Met\text{-}i}$, the $tRNA^{Ile}$ was only partially deacylated. In summary these results show that *Tb*-TyrRS is the only enzyme responsible for aminoacylation of the cytosolic as well as imported mitochondrial $tRNA^{Tyr}$.

While full length C-terminal tagged *Tb*-TyrRS (FL-Myc) was expressed and detected in the cytosol, it was not possible to show that it is also present in the mitochondrion using biochemical methods (Fig. 2C, FL-Myc) [4, 18]. In order to exclude that this was due to

the tag that might mask a putative targeting signal, we also tested N-terminally (Myc-FL) and internally (F-Myc-L) Myc-tagged versions of *Tb*-TyrRS. The results showed that none of these variants could be detected in the mitochondrial fraction (Fig. 2C, Myc-FL and F-Myc-L). However, cutting edge proteomic methods not only detected *Tb*-TyrRS in mitochondria but also showed that its abundance was greatly reduced in organelles ablated for the main mitochondrial outer membrane protein import channel [18]. This indicates that in agreement with the RNAi analysis in Fig. 1B a fraction of *Tb*-TyrRS is imported into mitochondria but that this amount is too small to be detected by conventional biochemical methods.

Next, we tested whether intact units of both tandem repeats are required for *Tb*-TyrRS function. To that end we produced a *Tb*-TyrRS-RNAi cell line targeting the 3'-UTR of its mRNA which allows complementation with full length and truncated variants of *Tb*-TyrRS (Fig. 2A). Fig. 2B shows that complementation with either N-terminally (Myc-FL), C-terminally (FL-Myc) or internally Myc-tagged full length *Tb*-TyrRS (F-Tyr-L) restored normal growth. Mitochondrial translation is essential for both procyclic and the long slender bloodstream form of *T. brucei* suggesting that the presence of a Myc-tag at neither of the three positions interferes with the mitochondrial localization of the enzyme [19]. Expression of the *Tb*-TyrRS truncations lacking either the N- or the C-terminal 36 amino acids (ΔN36-Myc and Myc-ΔC36), on the other hand, did not restore growth even though the *Tb*-TyrRS variants are expressed in similar amounts than their full length counterparts (Fig. 2B).

Extrapolated from the structure of the leishmanial enzymes [13, 14] these results strongly suggest that the N-terminal two α-helices, which include the ELR-motif that in *Leishmania donovani* was suggested to mimic a host cytokine [15], are essential for *Tb*-TyrRS function. However, it cannot be excluded that the inability of the ΔN36-Myc variant to complement the growth arrest is caused by the lack of a putative N-terminal mitochondrial targeting signal. But this is rather unlikely, since the N-terminally Myc-tagged protein (Myc-FL) fully complements for the lack of *Tb*-TyrRS,

Moreover, TyrRS activity also requires the C-terminal 1.5 α-helices. This was surprising since deletion of this region does not affect the two anticodon binding or any other motifs. In order to confirm this result we analyzed the accumulation of uncharged $tRNA^{Tyr}$ using long acidic gels. The results show that the C-terminally truncated version

of *Tb*-TyrRS (Myc-ΔC36), in contrast to its full-length counterpart (Myc-FL) but in line with the observed growth arrest, was not able to prevent the accumulation of uncharged $tRNA^{Tyr}$ observed in total RNA fractions (Fig. 2D).

Trypanosomatidal aaRSs, including the *Tb*-TyrRS, have been suggested as targets for a chemotherapeutic attack of the diseases that are caused by these organisms. Our demonstration that the same enzyme is required for the charging of both cytosolic as well as mitochondrial $tRNA^{Tyr}$, its unusual pseudodimeric structure and the fact that both monomeric units are required for its activity underscore that *Tb*-TyrRS might be a suitable candidate for such an approach.

Acknowledgments

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Figure legends

Figure 1. *Tb*-TyrRS is essential for normal growth of procyclic *T. brucei* and is responsible for formation of tyrosyl-$tRNA^{Tyr}$ in the cytosol and the mitochondrion, respectively. A. Growth curve in the absence and presence of tetracycline (tet- and tet+) of a representative clonal *T. brucei* RNAi cell line ablated for *Tb*-TyrRS. Inset: Northern blot for *Tb*-TyrRS mRNA. The time of induction is indicated at the top. The rRNAs in the lower panel serve as loading controls. **B.** Northern blot analysis of total and mitochondrial RNA isolated under acidic conditions from the *Tb*-TyrRS-RNAi cell line. The total RNA fraction only contains ≈5% of mitochondrial RNA and, thus, essentially represents cytosolic RNA. Hours of induction (0 and 100 h) by tetracycline are indicated at the top. The blots were probed for the *T. brucei* $tRNA^{Tyr}$ (top panel) as well as for $tRNA^{Ile}$ and the cytosol-specific initiator $tRNA^{Met\text{-}i}$, which serve as controls not affected by the RNAi (bottom panels). The RNA fractions were resolved on long acid urea gels, which allow separation of aminoacylated (aa) from deacylated tRNAs (da). An aliquot of chemically deacylated RNA is loaded as control (ctrl).
The inducible RNAi cell line used was targeting nucleotides 1561-2091 of the *Tb*-TyrRS ORF. Mitochondrial RNA was isolated from the RNase-treated pellet fraction of digitonin-extracted cells as described [20]. Long acidic gels were prepared and used as indicated in [17].

Figure 2. Complementation of *Tb*-TyrRS ablated cells by tagged full length and truncated variants of the enzyme. A. Predicted domain structure of the tagged full length (Myc-FL, FL-Myc and F-Myc-L) and the truncated variants of *Tb*-TyrRS (Myc-ΔC36 and ΔN36-Myc) according to [15] (not to scale). Light grey, catalytic domains. Dark grey, anticodon domains. The ELR motif as well as the HIGH and KMSKS active site motifs common to all class 1 aaRSs are indicated. The AIDQ motif is characteristic for the ATP binding site of TyrRSs. AC1 and AC2 motifs are involved in the recognition of the anticodon stem loop of the $tRNA^{Tyr}$. The Myc-tag is indicated in blue. **B.** Growth curve of the uninduced (−Tet) and induced (+Tet) *Tb*-TyrRS 3′UTR RNAi cell line coexpressing the indicated Myc-tagged *Tb*-TyrRS variants from pLew100-based vectors. Insets, addition of tetracycline simultaneously induces ectopic expression of the Myc-tagged *Tb*-TyrRS variants (Myc) - lipoamide dehydrogenase (LDH) serves as a loading control - and downregulation of the mRNA transcribed from the endogenous *Tb*-TyrRS gene (*Tb*-TyrRS). The ethidiumbromide-stained gels showing the ribosomal rRNA region serve as a loading control for the Northern blots. The inducible RNAi cell line used for these experiments was targeting nucleotides 20-384 of the *Tb*-TyrRS 3'UTR. The growth phenotype of the uncomplemented cell lines was identical to the *Tb*-TyrRS ORF RNAi cell line. **C.** Immunoblot analysis of whole-cell (Tot), digitonin-extracted mitochondria-enriched pellet (Mit) and soluble (Cyt) fractions of cells expressing the indicated tagged *Tb*-TyrRS versions probed with anti-Myc antibodies. ATOM40 and Elongation factor 1-alpha (EF1a) serve as markers for mitochondria and cytosol, respectively. **D.** Northern blot analysis of total RNA isolated *Tb*-TyrRS 3′UTR RNAi cell line coexpressing the indicated Myc-tagged *Tb*-TyrRS variants resolved on long acid urea gels were probed for the *T. brucei* $tRNA^{Tyr}$ (left panel) as well as for $tRNA^{Ile}$ (right panel). The positions of aminoacylated (aa) and deacylated tRNAs (da) are indicated. An aliquot of chemically deacylated RNA is loaded as control (ctrl).

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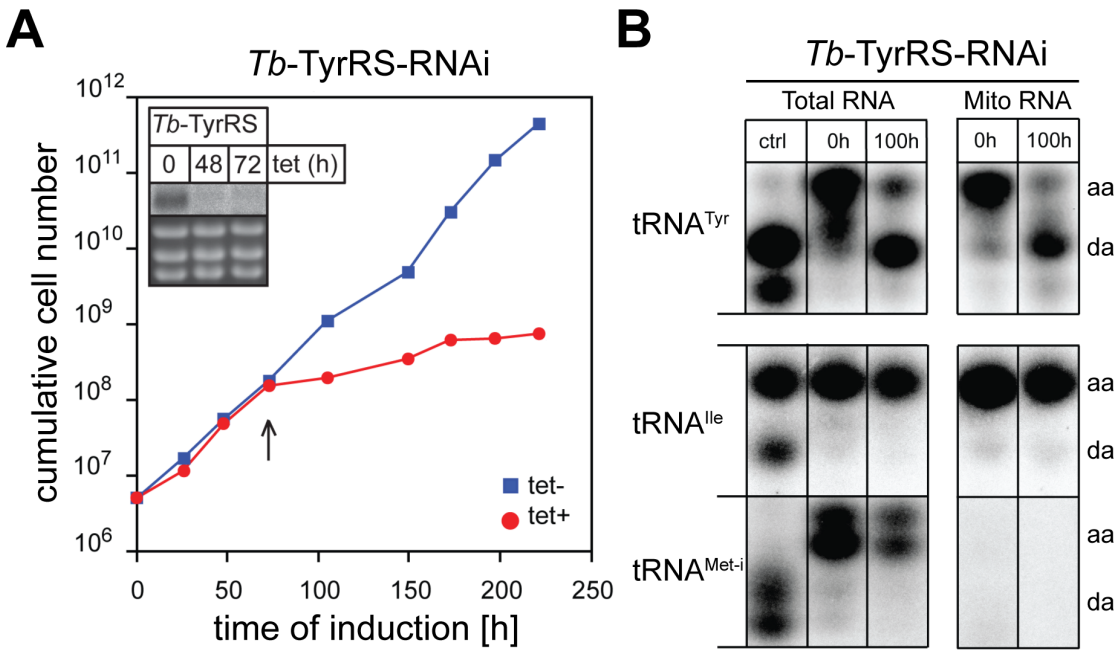
A
Tb-TyrRS-RNAi
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tRNAMet-i
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Figure 1

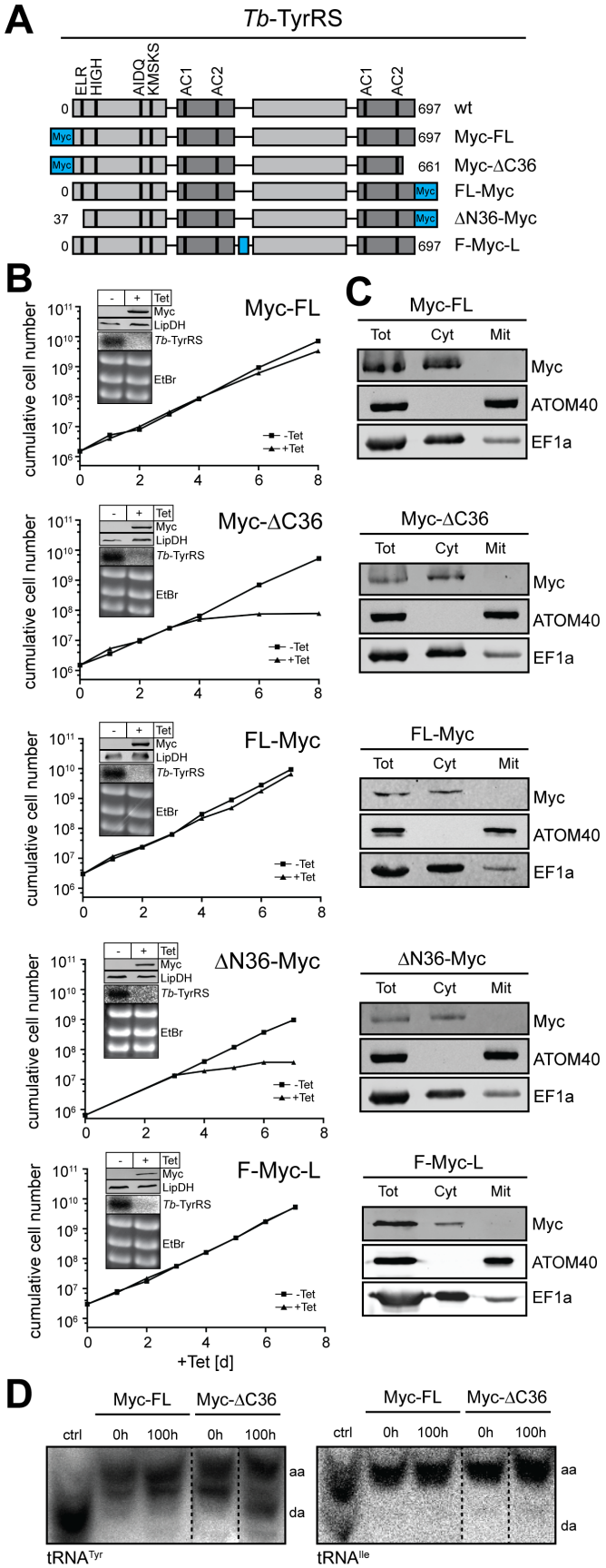
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Figure 2