RNA BIOLOGY
https://doi.org/10.1080/15476286.2021.1940445

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A mitochondrial cytidine deaminase is responsible for C to U editing of tRNA^{Trp} to decode the UGA codon in *Trypanosoma brucei*

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ABSTRACT

In kinetoplastid protists, all mitochondrial tRNAs are encoded in the nucleus and imported from the cytoplasm to maintain organellar translation. This also applies to the tryptophanyl tRNA (tRNA^{Trp}) encoded by a single-copy nuclear gene, with a CCA anticodon to read UGG codon used in the cytosolic translation. Yet, in the mitochondrion it is unable to decode the UGA codon specifying tryptophan. Following mitochondrial import of tRNA^{Trp}, this problem is solved at the RNA level by a single C34 to U34 editing event that creates the UCA anticodon, recognizing UGA. To identify the enzyme responsible for this critical editing activity, we scrutinized the genome of *Trypanosoma brucei* for putative cytidine deaminases as the most likely candidates. Using RNAi silencing and poisoned primer extension, we have identified a novel deaminase enzyme, named here TbmCDAT for mitochondrial Cytidine Deaminase Acting on tRNA, which is responsible for this organelle-specific activity in *T. brucei*. The ablation of TbmCDAT led to the downregulation of mitochondrial protein synthesis, supporting its role in decoding the UGA tryptophan codon.

ARTICLE HISTORY
Received 6 April 2021
Revised 28 May 2021
Accepted 4 June 2021

KEYWORDS
Mitochondrion; tryptophanyl tRNA; editing; cytidine deaminase; trypanosoma

Introduction

RNA editing describes the co-transcriptional or post-transcriptional alterations of RNA sequences by insertions, deletions, and substitutions, differentiating them from their DNA template [1]. It was first discovered in African trypanosomes where four non-encoded uridine (U) residues are inserted into the mitochondrial mRNA encoding cytochrome *c* oxidase 2, thus repairing a frameshift [2]. Soon afterwards, nucleotide substitution editing was found in mammals, where cytidine to uridine (C-to-U) editing of the apolipoprotein B mRNA generates a premature stop codon, resulting in a truncated version of the protein [3]. With the increasing number of genomic and transcriptomic datasets becoming available, different types of RNA editing have been found across the prokaryotic and eukaryotic domains, with the highest incidence observed in mitochondrial and plastidial transcriptomes [4,5].

Although RNA editing is most frequently encountered in mRNAs, it also occurs in the non-coding RNA molecules such as tRNAs, mainly in the form of nucleoside conversions, such as adenosine to inosine (A-to-I) and C-to-U deaminations [6]. The A-to-I editing of eukaryotic tRNAs is catalysed by Adenosine Deaminases Acting on tRNA (ADATs) [7,8]. In the kinetoplastid parasite *Trypanosoma brucei,* responsible for serious human disease, ADAT forms the heterodimeric protein complex TbADAT2/3, which is needed for A-to-I editing at position 34 of threonyl tRNA (tRNA^{Thr}), expanding its decoding capacity [9]. Moreover, the activity of TbADA2/3 is also needed for two interconnected, sequential events involving methylation and C-to-U deamination in the anticodon loop of tRNA^{Thr} [10].

An interesting example of C-to-U editing in the anticodon has been described in the mitochondrion of kinetoplastid protists *Leishmania tarentolae* and *T. brucei* [11,12] This is illustrated and explained in Fig. 1A. In the single mitochondrion of these protists one of the stop codons, UGA, has been reassigned to tryptophan [13], which is the most common deviation from the universal genetic code, particularly frequent in organells [14]. To allow this reassignment, a non-standard tRNA^{Trp} bearing a UCA instead of CCA anticodon has to be present in the mitochondrion. The mitochondrial genome of kinetoplastid flagellates has a number of unique features, including a complete lack of the tRNA genes [15]. Consequently, a whole set of cytosolic tRNAs must be imported from the cytosol to maintain mitochondrial translation [16]. Moreover, these parasitic protists harbour in their nuclear genome only a single copy of the tRNA^{Trp} gene encoding tRNA with CCA in its anticodon, which cannot decode the predominant mitochondrial UGA codon for tryptophan. The conundrum is solved by C-to-U deamination that occurs in ~50% of tRNA^{Trp} following their import into the mitochondrion, changing CCA to UCA in the anticodon [11,12] (Fig. 1A). Consequently, within the organelle, the imported and edited tRNA^{Trp} is able to decode the UGA codon. In addition, this tRNA undergoes an unusual

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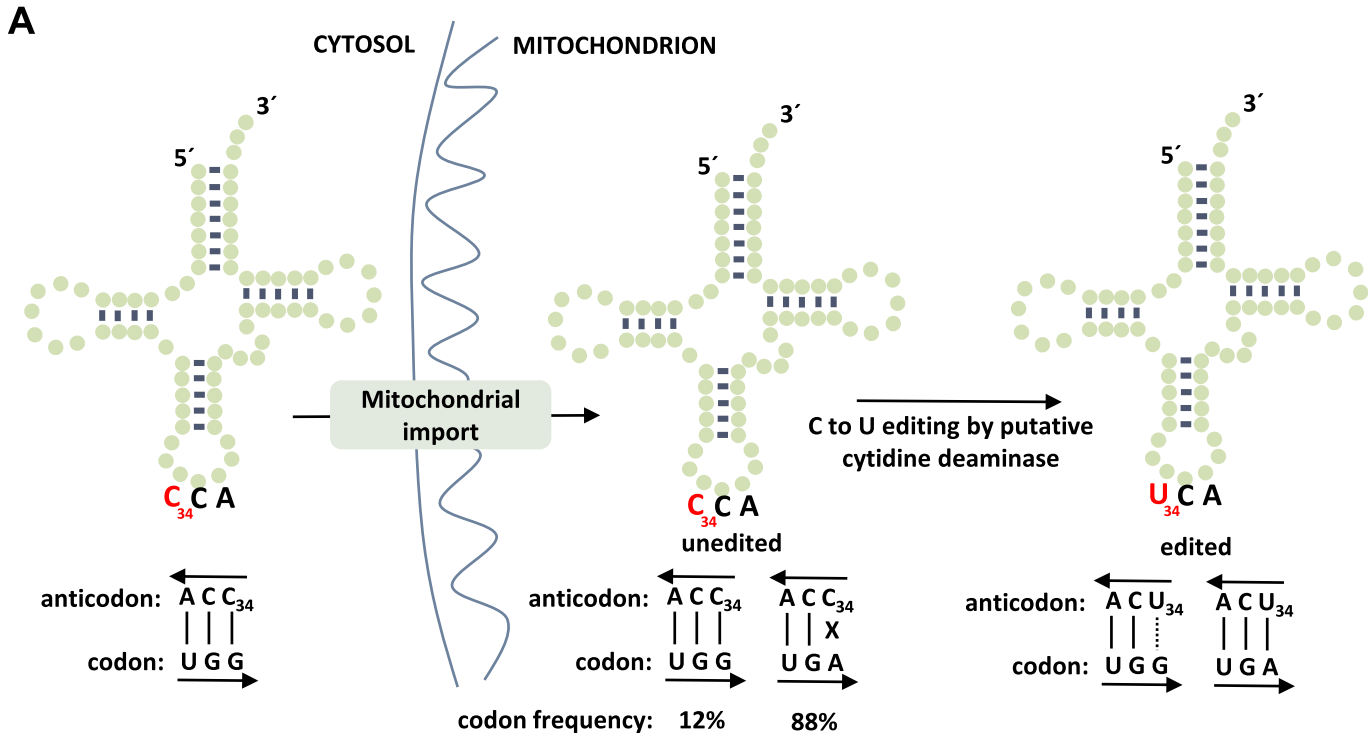

B

Gene ID	Tested by PPE*	Source	Predicted function (TriTrypDB)
Tb927.10.2200	yes	Peikert et al., 2017	hypothetical protein, conserved in *T.brucei*; PD-(D/E)XK nuclease superfamily
Tb927.10.1930	yes	Peikert et al., 2017	hypothetical protein, conserved
Tb927.10.8850	yes	Panigrahi et al. 2009	A distinct subfamily of CDD/CDA-like deaminases
Tb927.8.1590	yes	Peikert et al., 2017	ubiquitin-protein ligase, putative
Tb927.3.4530	yes	Peikert et al., 2017	hypothetical protein, conserved
Tb927.11.16940	yes	Peikert et al., 2017	MIZ/SP-RING zinc finger, putative
Tb927.9.12500	yes	Peikert et al., 2017	Present in the outer mitochondrial membrane proteome 7 (POMP7)
Tb927.9.3000	yes	Peikert et al., 2017	cytidine deaminase, putative
Tb927.11.13170	no	Peikert et al., 2017	hypothetical protein, conserved
Tb927.11.5370	no	Peikert et al., 2017	hypothetical protein, conserved
Tb927.2.4950	no	Peikert et al., 2017	hypothetical protein, conserved
Tb927.6.1680	no	Peikert et al., 2017	mitochondrial RNA binding protein 1 (MRB1680)
Tb927.7.5540	no	Peikert et al., 2017	2,4-dienoyl-coa reductase-like protein
Tb927.7.6350	no	Peikert et al., 2017	NADH-ubiquinone oxidoreductase, mitochondrial, putative
Tb927.9.12720	no	Peikert et al., 2017	predicted zinc finger protein
Tb927.9.4620	no	Peikert et al., 2017	NEDD8 activating enzyme subunit, putative
Tb927.9.8210	no	Peikert et al., 2017	Peptide deformylase 2

* poison primer extension asaay

Figure 1. **(A)** *T. brucei* encodes singe $tRNA^{Trp}$ bearing the CCA anticodon. A majority of $tRNA^{Trp}$ is used in the cytosolic translation, while a small portion is imported into the mitochondrion. Inside the organelle, $tRNA^{Trp}$ undergoes C to U editing at position 34 to decode the UGA codons, which is the predominant one specifying tryptophan. **(B)** List of putative cytidine deaminases in *T. brucei* generated based on the criteria described in Materials and methods.

thiolation at a typically unmodified position 33 prior to the C-to-U editing [17]. Interestingly, in *T. brucei*, this thio-modification functions as a negative determinant for the above-described deamination, thus maintaining in the mitochondrion an optimal ratio between the edited and unedited $tRNA^{Trp}$ [18,19].

Even though C-to-U editing at the wobble base position 34 of $tRNA^{Trp}$ was discovered already more than two decades ago [11], the enzyme responsible for this essential deamination remained unknown. Here, using a bioinformatic screen, we identified 17 putative cytidine deaminases in the *T. brucei* genome, which could be responsible for this activity. After

RNAi-mediated knock down of eight prioritized candidates and measuring the C-to-U ratio at position 34 by poison primer extension assay we have identified the responsible cytidine deaminase. To the best of our knowledge, this is the first genuine mitochondrially targeted cytidine deaminase acting on tRNA.

Results

Searching for mitochondrial tRNA cytidine deaminase in T. brucei

We reasoned that the following features should be present in the candidate mitochondrial cytidine deaminase acting on tRNA: i/a conserved deaminase (C/H*E) motif; ii/a conserved zinc-finger (PC**C) motif located in the vicinity of the deaminase domain; iii/conservation in all available kinetoplastid genomes; iv/presence in the mitochondrial proteome of *T. brucei*. The first two features were defined based on the comparative analysis of RNA deaminases [20].

We initiated the screen by scanning the *T. brucei* TREU927 predicted proteome (TriTrypDB) for the presence of deaminase (C/H*E) and zinc finger (PC**C) motifs in the same open reading frame. Our search yielded a total of 116 proteins, of which 73 and 43 proteins carried the C*E and H*E motif, respectively (data not shown). Using the same criteria, we have searched the *T. brucei* importome, a set of proteins experimentally proven to be imported into the mitochondrion of this human pathogen [21]. Overlap between these two datasets constituted 16 proteins imported into the organelle and containing both the deaminase and zinc finger domains. Additionally, we have included the Tb927.10.8850 gene, the product of which has a predicted function 'distinct subfamily of CDD/CDA-like deaminase', which was identified in another mitochondrial proteome of *T. brucei* [22]. Thus, the initial set of candidates consisted of 17 proteins (Fig. 1B).

Screening for C-to-U editing in tRNA^{Trp} in RNAi cell lines for candidate genes

For each shortlisted candidate, we generated a RNAi cell line in the procyclic stage of *T. brucei*, in which the target gene was selectively depleted upon the addition of tetracycline (Tet) to the medium. Total RNA isolated from the wild type (WT), non-induced (-Tet) and RNAi-induced cells (+Tet) was subjected to a RT-PCR reaction with gene-specific primers. When the target transcript was inducibly downregulated, the RNAi cell line was cloned and subjected to digitonin fractionation, allowing the isolation of cytosolic and mitochondrial RNAs. To assess their purity, the RNAs were analysed by Northern blot and hybridized with a probe specific for the spliced leader (SL) RNA, used as a cytosolic marker, and with a probe specific for the mitochondrial ribosomal RNA (9S rRNA), which served as a mitochondrial marker. This experiment confirmed the absence of cytosolic SL RNA in the mitochondrial fraction, demonstrating its high purity (Fig. 3B; data not shown).

Next, the mitochondrial RNA was used as a template for the poisoned primer extension assay designed to reveal the dissected C-to-U editing event in the mitochondrion-imported tRNA^{Trp}. The principle and the experimental setup is shown in Fig. 2A. In summary, a radioactively labelled primer that specifically anneals one nucleotide upstream of the tRNA^{Trp} editing site is used in the reverse transcription reaction spiked with dideoxyguanine (ddG), which functions as the chain terminator. When incorporated at the unedited position, it yields a primer +1 nucleotide product (Fig. 2A). Alternatively, the edited tRNA is terminated at the next C, resulting in a product composed of the primer +10 downstream nucleotides. As a readout for the assay, the percentage of edited tRNA^{Trp} versus the total signal of extended products (edited + unedited) was calculated per each lane.

Initially, we examined the *T. brucei* orthologue of ADAT2, which is responsible for both A-to-I and C-to-U editing in the cytosolic tRNA^{Thr} [10,23]. We wondered whether ADAT2 is possibly promiscuous and uses mitochondrial tRNA^{Trp} as a substrate despite the fact that the enzyme was exclusively localized in the nucleus and the cytosol. It turned out not to be the case as trypanosomes depleted for ADAT2 retained the mitochondrial tRNA^{Trp} editing on the wild type (WT) level, which is about 60% (Fig. 2B). Also in the other seven cell lines, each with a putative cytidine deaminase inducibly knocked-down via RNAi, only the WT level of the monitored tRNA editing event was observed (Fig. 2C–E). However, a striking phenotype was observed in cells in which the mRNA of another candidate gene, Tb.927.3.4530, was partially depleted. After 8 days following RNAi induction, products of the reverse transcription indicated the level of edited tRNA^{Trp,} dropping to 22% (Fig. 2E; framed). Based on this result, we concluded that this gene encodes the sought-after mitochondrial cytidine deaminase acting on tRNA, which we henceforth named TbmCDAT, and scrutinized the resulting phenotype further.

TbmCDAT is responsible for deamination of tRNA^{Trp} anticodon

The *T. brucei* Tb927.3.4530 protein belongs to the OG6_157091 orthogroup of the OrthoMCL specific to the kinetoplastid flagellates (https://orthomcl.org/). Due to its conservation, the gene can be easily identified in all kinetoplastid genomes available in TriTrypDB, yet no homologs can be found outside of this group of protists. There are two predicted conserved deaminase and nucleotide-binding zinc-finger domains in TbmCDAT (Fig. 3A). The mitochondrial localization of TbmCDAT was experimentally confirmed by the C-terminal tagging [24]. RNAi silencing of TbmCDAT resulted in only a very mild growth phenotype (Fig. 3D), which is probably a consequence of limited downregulation of the target transcript, as shown by Northern blot analysis (Fig. 3D, inset). The remaining TbmCDAT transcript supports a level of mitochondrial tRNA^{Trp} editing sufficient for cell survival. Repeated attempts to generate a CRISPR/Cas9 double knockout of TbmCDAT failed, suggesting its essentiality for the viability of *T. brucei* procyclics.

To closely monitor the dynamics of C-to-U editing, we have applied the poisoned primer assay described above. For this aim, RNA was isolated from mitochondria purified from

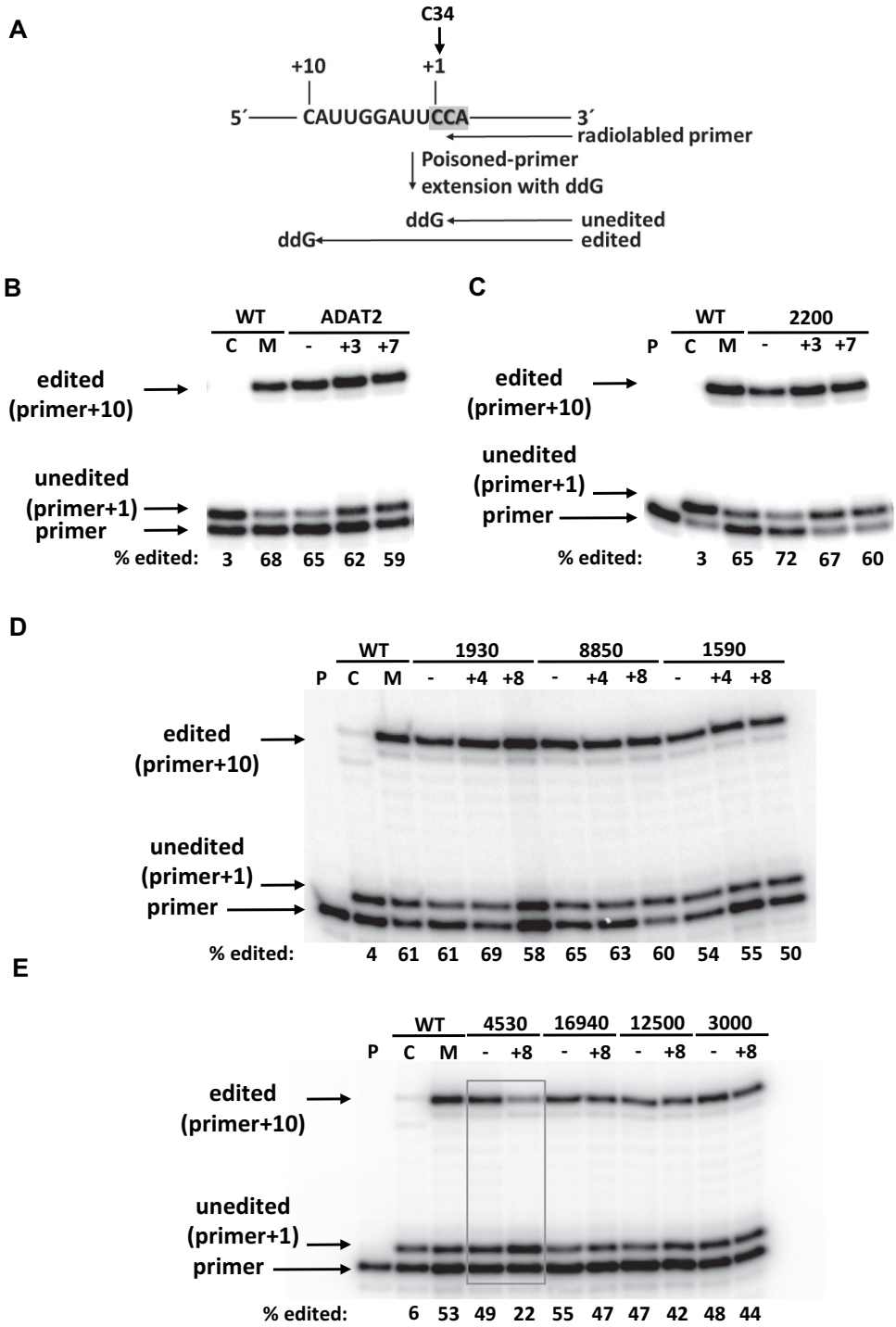

Figure 2. Poisoned primer extension assay to detect edited and unedited $tRNA^{Trp}$ in different *T. brucei* RNAi cell lines. **(A)** Diagram depicts the following applied poisoned primer extension reaction: The 5´-labelled oligonucleotide specific to $tRNA^{Trp}$ was used in the presence of ddG to extend either up to or through the editing site, yielding a primer +1 product from the unedited tRNA, or a primer +10 product from the edited tRNA. The first position of the anticodon is indicated by C34 with an arrow. Numbers +1 and +10 refer to the expected products of the reaction. **(B-E)** An autoradiograph of gels with primer extension products obtained using mitochondrial RNA isolated from cell lines, in which individual putative cytidine deaminases were targeted by RNAi. The arrows indicate the position of the observed edited and unedited products. WT stands for the wild type, (-) and (+) refer to the uninduced and RNAi-induced mitochondrial RNA fractions, with days post-induction also indicated. Cytosolic (C) and mitochondrial (M) fractions isolated from the WT cells served as a control. Each RNAi cell line is labelled by the last digits of their TriTrypDB accession number (see Fig. 1B). Primer (P) indicates lanes with primer alone. The numbers below each gel represent relative amounts of the edited $tRNA^{Trp}$ as quantified by Phosphoimager analysis. The numbers on top of each lane represent time of RNAi induction in days.

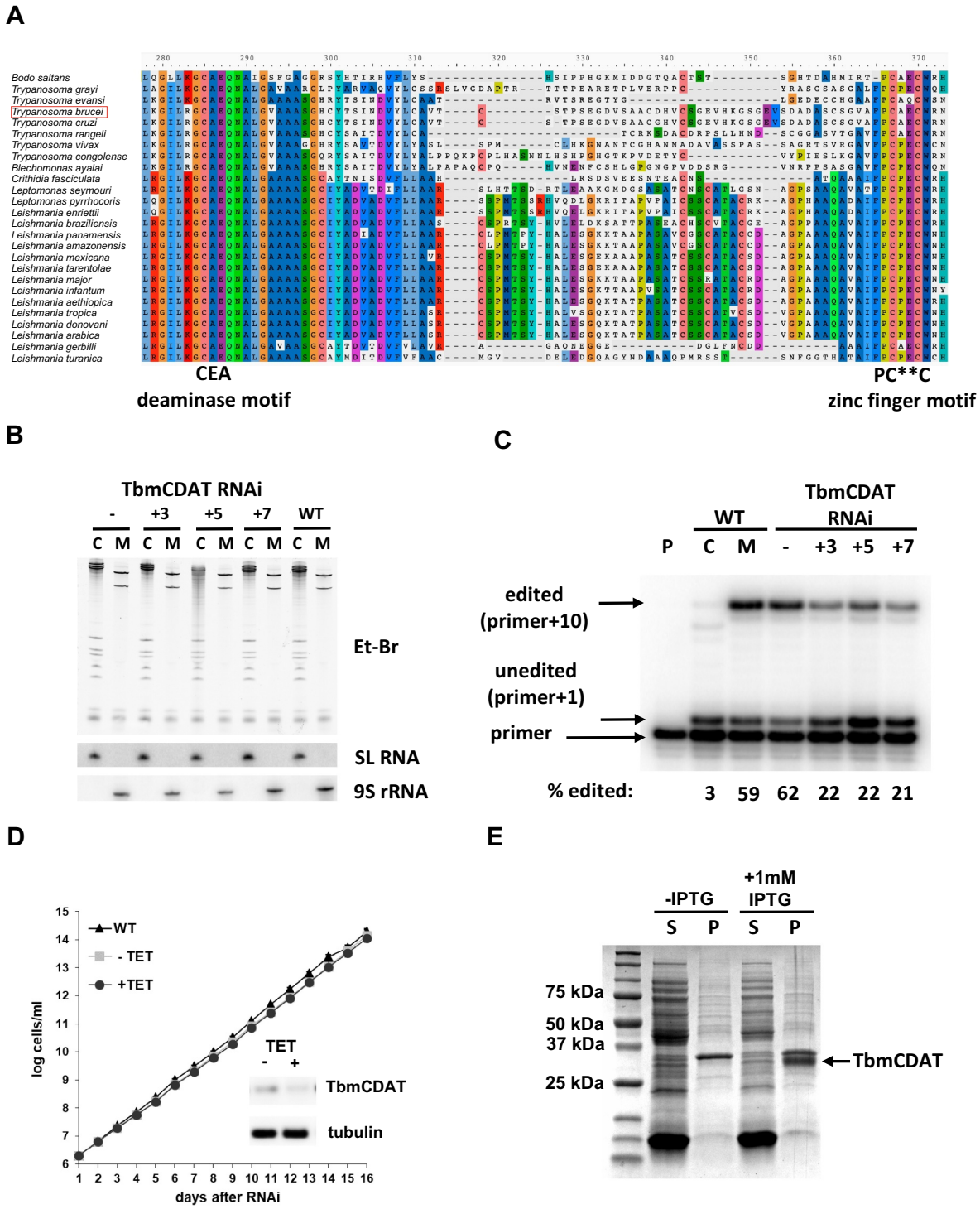

Figure 3. **(A)** Multiple protein sequence alignments of mCDAT from a range of kinetoplastid protists. Conserved deaminase (C/H*E) and zinc-finger (PC**C) motifs are highlighted. **(B)** Cytosolic (C) and mitochondrial (M) RNA from the wild type cells (WT) and the RNAi-induced cells was separated by denaturing acrylamide gel electrophoresis and the quality of RNA was confirmed by ethidium bromide-staining. The same gel was used for Northern blot analysis and probed for splice leader (SL) RNA and 9S rRNA to assess the purity of the mitochondrial fraction. (C) Autoradiograph of a gel with poisoned primer extension products obtained using mitochondrial RNA isolated from the TbmCDAT RNAi cell line. The arrows indicate the position of the product observed. (-) and (+) refer to the non-induced and RNAi-induced cells (3, 5 and 7 days post-induction). Cytosolic (**C**) and mitochondrial (M) fractions isolated from the WT cells served as a control. Primer (P) indicates lanes with primer alone. The numbers below each gel represent relative amounts of the edited $tRNA^{Trp}$ as quantified by Phosphoimager analysis. (**D**) Growth curve of the WT, non-induced (−TET), and RNAi-induced (+TET) *T. brucei* 29–13 cells transformed with a RNAi plasmid containing a fragment of TbmCDAT. RNAi was induced by the addition of tetracyclin to the medium and cell concentration was measured in 24 hrs intervals. The inset shows the reduction in TbmCDAT mRNA level as determined by RT-PCR, with tubulin mRNA serving as a loading control. **(E)** Expression of TbmCDAT in *E. coli* BL21 (DE3) cells shown in Coomassie blue-stained 10% SDS-PAGE gel. Marker in kilodalton (kDa) is shown on the left-hand side; The soluble (S) fraction and insoluble pellet (P) come from the total cell extract transformed with pET28a-TbmCDAT prior (-) and after (+) to 1 mM IPTG induction.

the WT trypanosomes, which served as a control, the non-induced TbmCDAT RNAi cells, as well as this cell line at days 3, 5 and 7 post-RNAi induction (Fig. 3B). The high purity of all analysed fractions was verified by the cytosolic SL RNA and mitochondrial 9S rRNA probes as described above (Fig. 3B). A consequence of the partial RNAi-mediated ablation of TbmCDAT was a drop of the monitored C-to-U editing to 22% already by day 3 post-induction, with a corresponding increase of the unedited molecules. RNAi cells at days 5 and 7 post RNAi-induction retained mitochondrial $tRNA^{Trp}$ editing at equally decreased levels, as compared to 59% and 62% editing levels present in the WT and non-induced cells, respectively (Fig. 3C).

To biochemically confirm the C-to-U editing activity, recombinant TbmCDAT protein with C-terminal $(His)_6$ tag was overexpressed in *E. coli*. Despite testing several induction conditions, such as temperature, time and concentration of IPTG, we always detected overexpressed TbmCDAT protein in the insoluble inclusion bodies (Fig. 3E; data not shown), preventing studies of its *in vitro* activity.

Mitochondrial translation is altered following mCDAT depletion

The majority of codons specifying tryptophan in the mitochondrial transcripts of *T. brucei* are represented by UGA. Since this codon is recognized only by the edited mitochondrial $tRNA^{Trp}_{UCA}$, these molecules are indispensable for proper mitochondrial translation. To monitor organellar translation, we have employed *in vivo* labelling with ^{35}S. In this experiment, RNAi was induced by the addition of Tet and cells were washed and incubated for 30 min with 100 μg/ml cycloheximide before the addition of ^{35}S-labelled mix of cysteine and methionine. Under these conditions, the cytosolic translation of *T. brucei* is inhibited, while the mitochondrial translation remains unaffected [25]. Total protein was extracted from the non-induced cells and those at day 7 following RNAi induction and resolved by the modified two-dimensional SDS-PAGE gel as described previously [26]. To ensure equal sample loading, the gels were stained with Coomassie Blue dye prior autoradiography (Fig. 4; insets). Due to the very hydrophobic nature of the mitochondrially-encoded proteins of *T. brucei*, only three proteins are discernible in this type of gel, namely cytochrome *c* oxidase subunit 1, apocytochrome *b* and subunit A6 of ATP synthase, identified based on their highly characteristic position in the gel [26,27]. Although the signal of all three *de novo* synthesized proteins was decreased upon TbmCDAT RNAi induction, the effect was more pronounced for ATP synthase subunit 6 and cytochrome *b*, the latter of which even had its migration affected (Fig. 4).

Discussion

As the most frequent departure from the universal genetic code, the reassignment of the UGA stop codon to tryptophan is particularly widespread in the mitochondria of both multi- and unicellular eukaryotes [14,28]. Almost all eukaryotes in which UGA is decoded by $tRNA^{Trp}$ have the UCA anticodon [13]. Along with few other groups, the kinetoplastid protists do not encode tRNAs in their mitochondrial DNA and import their complete set from the cytosol [29,30]. While the single nucleus-encoded $tRNA^{Trp}$ uses the CCA anticodon to decode UGG in the course of cytosolic translation, upon the import of this tRNA into the mitochondrion, the anticodon undergoes the C-to-U editing, allowing it to translate both UGA and UGG [11].

The same type of C-to-U editing but in different tRNA ($tRNA^{Gly}$) is known from the mitochondrion of marsupials, where it alters the GCC anticodon to GUC. About half of the unedited GCC-carrying tRNAs are aminoacylated with glycine, while the edited tRNA with GUC anticodon is aminoacylated with aspartate [31]. Thus, two distinctly charged tRNA species are produced from one tRNA gene in the marsupial mitochondria. In plants, C-to-U editing corrects a mismatched C4:A69 pair in the acceptor stem of the mitochondrial $tRNA^{Phe}_{GAA}$ into a U4:A69 [32]. However, in all

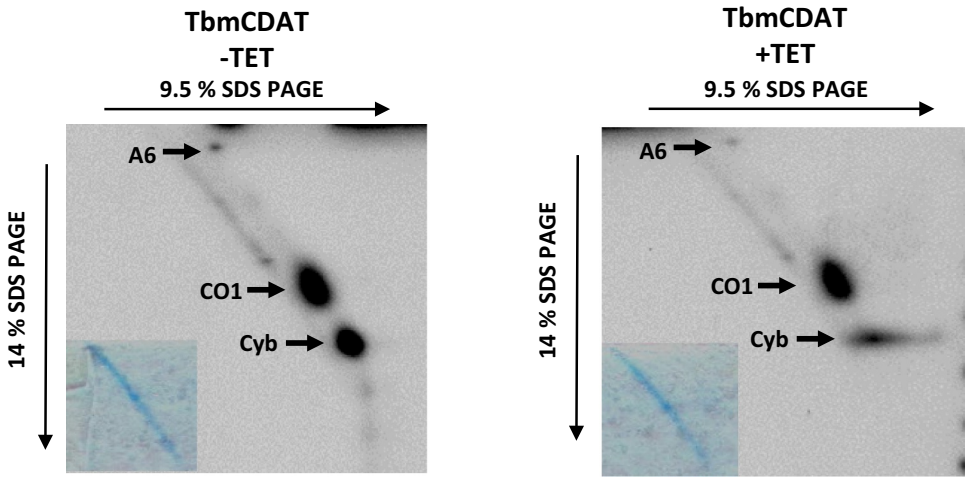

Figure 4. The downregulation of TbmCDAT leads to a decrease in mitochondrial protein synthesis. Samples obtained from the non-induced (–TET) and RNAi (+TET) cells 7 days post-induction were labelled and separated in two-dimensional gels as described in Materials and methods. The arrows mark the position of the three identifiable mitochondrial-encoded proteins – cytochrome *c* oxidase subunit 1 (CO1), apocytochrome *b* (Cyb) and ATP synthase subunit 6 (A6). The insets show Coomassie blue–stained versions of the same gels, before exposure to the X-ray film, used here to show equal loading of the samples. Arrows indicate the direction of electrophoresis, where the first and second dimensions were on 9.5% SDS-PAGE and 14% SDS gels, respectively.

these cases, the enzyme responsible for the deamination of C in the mitochondrion remains unknown. Given the similarity of this reaction with that for which the responsible enzyme has been identified in Archaea [33], it is reasonable to speculate that this type of organellar editing also proceeds by a deamination mechanism.

Consequently, using the presence of a conserved motif [20] as the key criterion in our search for the putative mitochondrial cytidine deaminase, we identified TbmCDAT as the protein responsible for C-to-U editing in kinetoplastid mitochondria. Indeed, following RNAi-mediated depletion of mCDAT in *T. brucei* deamination of C at the position 34 in the mitochondrial $tRNA^{Trp}$ was clearly affected.

Since only $tRNA^{Trp}$ carrying the edited anticodon (UCA) can decode UGA, which is the predominant tryptophan codon, TbmCDAT is supposed to be essential in *T. brucei*. We explain the lack of a significant growth phenotype in the TbmCDAT-downregulated trypanosomes as a consequence of an incomplete downregulation of the target transcript. In addition, our repeated attempts to generate a full knockout for TbmCDAT failed, strongly indicating its importance for decoding the mitochondrial transcripts. In the absence of specific antibodies against any of the *T. brucei* mitochondrially-encoded proteins, we followed their *de novo* synthesis using radioactively-labelled amino acids. As expected, RNAi silencing of TbmCDAT caused a substantial downregulation of mitochondrial translation. While the levels of ATP synthase subunit 6 and cytochrome *c* oxidase subunit 1 were decreased, albeit not dramatically, the strong effect observed for apocytochrome *b* can be explained by the highest number of the critical UGA codons in this protein. In addition, the signal corresponding to the apocytochrome *b*, exhibited atypical migration, upon RNAi of TbmCDAT (Fig. 4). This can be interpreted as a misfolding due to changes in translation dynamics caused by a lower amount of the decoding tRNA, a phenomenon described elsewhere [34,35].

Purification of the recombinant TbmCDAT overexpressed in *E. coli* was unsuccessful despite several conditions tested. This may be caused by the nature of the protein or its misfolding due to the presence of a C-terminal His-tag. Alternatively, TbmCDAT may need a binding partner for its stability, with the prime candidates being proteins involved in the thiolation of the adjacent U33, such as Nfs1, Isd11 and Mtu1 [36], there may be a tight functional dependence of these two activities. Such an interdependent regulation was described in *T. brucei* for the formation of 3-methylcytosine by a methyltransferase in complex with TbADAT2/3 [10].

Here we show, that keeping the optimal ratio of edited/unedited $tRNA^{Trp}$ may be crucial for mitochondrial physiology. In *T. brucei*, this is affected by the adjacent thiomodification of U33. RNAi silencing of the thiolation pathway increased the editing levels to almost 100% [18]. This suggests that thiolation acts as a negative determinant for deamination by TbmCDAT. As to why C to U editing needs to be limited by thiolation is not exactly clear. According to the wobble rules, the edited $tRNA^{Trp}$ should also decode the UGG codons (via a G-U pair) (Fig. 1A). However, as suggested previously and demonstrated for a different mitochondrial modification in yeast, thiolation can restrict wobble pairing, thus providing a requirement for both anticodons (C34 and U34-containing) [37,38]. Another interesting question is why the levels of the edited $tRNA^{Trp}_{UCA}$ (~60%) do not fully correlate with the number of predominant mitochondrial UGA codon (88%). This may be partly explained by the fact that the optimal ratio of edited/unedited $tRNA^{Trp}$ may also reflect a differential expression of genes with different frequencies of UGG and UGA codons during the remodelling of mitochondrial metabolism throughout the complex life cycle of these parasites. Nevertheless, in the absence of methods to efficiently explore mitochondrial translation, such as mitochondrial ribosome profiling or *in vitro* translation systems, these will remain interesting open questions.

Notably, one of the tested candidates (Tb927.9.3000), which turned out to have no role in C-to-U editing of the mitochondrial $tRNA^{Trp}$, was recently characterized as another cytidine deaminase localized in the organelle of *T. brucei*. However, it does not act on RNA but is responsible for the hydrolytic deamination of cytidine and deoxycytidine in the pyrimidine salvage pathway [39].

With the exponential growth of omics data, a large number of differences are being identified between the genome-encoded information and the corresponding transcripts. These discrepancies are often attributed to RNA editing, which has a potential to expand the amount of information hidden in individual genes. While it is relatively straightforward to describe the changes on the RNA level, it is much more challenging to identify the responsible protein machinery. However, this task is even more urgent now, when it was convincingly demonstrated that we can take advantage of editing enzymes as a tool to correct genetic disorders in humans. Very recently, a genetically engineered bacterial cytidine deaminase was used for efficient and error-free corrections of the deleterious mutations in human mitochondrial DNA [40], representing a genuine breakthrough [41]. To the best of our knowledge, a long sought-after enzyme responsible for decoding the tryptophanyl UGA codon, identified here as TbmCDAT, represents the first identified eukaryotic cytidine deaminase acting on RNA with organellar localization.

Materials and methods

Plasmids construction, transfections, RNAi induction and cell cultivation, alignments

Each amplicon was cloned into the p2T7-177 vector linearized with NotI (for genome integration) and then used to transform procyclic *T. brucei* 29–13 cells, which were subsequently selected as described elsewhere [42]. RNAi was triggered by the addition of 1 mg/mL of tetracycline to the growth medium (SDM-79). Cell density was measured every 24 hrs using the Beckman Z2 Coulter counter over a period of 16 days after the induction of double-stranded RNA synthesis. Overexpression of the full-length TbmCDAT open reading frame was carried out according to the method described previously [43]. The TbADAT2 RNAi cell line was generated as described previously [23]. The orthologous protein sequences of kinetoplastids were taken from TritrypDB, and the multiple sequence alignment was made using MAFFT [44]. The alignment was visualized using Aliview [45,46].

Oligonucleotide probes

gene	RNAi (5′ to 3′)
Tb927.10.1930	FW: CGCAAGCTTCGAAACTTATGCAGCTCGCC RV: CGCGGATCCGGTAACTGGTGACGGAGCAA
Tb927.10.2200	FW: CGCAAGCTTGCGGAGGAAGGAAGATGTGT RV: CGCGGATCCTGCCGAAGAACCCCTTTTCT
Tb927.10.8850	FW: CGCAAGCTTGCGGGGTTTTTGTTCACCTG RV: CGCGGATCCTCTGCTAGACCAAAAGCGGG
Tb927.11.16940	FW: CGCAAGCTTCGTAAGCAGCGGAAGGAAGA RV: CGCGGATCCCCTCACGGTTAAATCCCGCT
Tb927.8.1590	FW: CGCAAGCTTTGTCGTCGTGTTCAGTAGGC RV: CGCGGATCCGTCGCTTCTCGTATCCTCCG
Tb927.9.12500	FW: CGCAAGCTTGTTGGTTGGTGCTCCGCTAT RV: CGCGGATCCGAGGTTCCAGCAAGTCGGAT
Tb927.9.3000	FW: CGCAAGCTTTCATTGAAGACCGGTTGCTTG RV: CGCGGATCCCCGATACTGCTTGGTCCGAA
Tb927.3.4530	FW: CGCAAGCTTCTTTCTTCACTCCGTCCCCA RV: CGCGGATCCCGCATTACAGCCACAGACGA

gene	RT-PCR (5′ to 3′)
Tb927.10.1930	FW: GTGGGCTACACTGCTTGAGA RV: CTTCTTCGACGTTCCCACGA
Tb927.10.2200	FW: TTAGAAAAGGGGTTCTTCGGCA RV: TACGGGCATAACGGTGGATT
Tb927.10.8850	FW: CGCGTTCTACTGTGATGGCT RV: TTCTCACAGACACCGCATGG
Tb927.11.16940	FW: GATGGCGTGGTGTAGGAGTC RV: TCTCCCGGAACCACTGAGAC
Tb927.8.1590	FW: GAAGACCGGGCAAATCTCCT RV: GCGTAGTTCCTCACAACTGC
Tb927.9.12500	FW:GAAGACCGGGCAAATCTCCT RV: GCGTAGTTCCTCACAACTGC
Tb927.9.3000	FW: TGATGCCACAGTCCCTCCAT RV: CGTCCATGCCGATACTGCTT
Tb927.3.4530	FW: TGCATCTTGCAGTGGGGTAG RV: GGTAACGTCGATGGGAGGTG

The following oligonucleotides were used for the generation of RNAi cell lines and their validation by RT-PCR

Mitochondrial RNA isolation, Northern blot analysis and poisoned primer extension assay

Mitochondrial RNA isolation and Northern blot analysis were carried out as described previously [43] . Poisoned primer extension reactions were performed using SuperScript™ III reverse transcriptase (Invitrogen) as described elsewhere [11]. The reactions included either 5 µg of total cellular RNA or 1 µg of total mitochondrial RNA, and 1.25 mM ddGTP. The primer 5′-TGAGAGCTGCAGGGATTGAACCTACGACCCCTGGATTTG-3′ complementary to positions 73 thru 35 in the canonical tRNA structure was used in the primer extension reactions.

De novo synthesis of mitochondrial proteins

Analysis of *de novo* mitochondrial translation followed protocols described previously [26,47]. In brief, 1×10^7 exponentially growing procyclic cells were pelleted by centrifugation at 2,000 g for 10 min, washed twice, and resuspended in 100 µL of SoTE buffer (0.6 M sorbitol, 20 mM Tris–HCl at pH 7.5, 2 mM EDTA). Products of mitochondrial translation were labelled by incubating the cells for 2 hrs at 27°C in the presence of EasyTag EXPRESS ^{35}S protein labelling mix (100 µCi per 100 µL reaction) with the concurrent inhibition of cytosolic translation by the addition of 100 µg/mL cycloheximide. The labelled cells were analysed in denaturing two-dimensional (9.5% vs. 14%) polyacrylamide Tris–glycine SDS gels as described previously [25,26].

Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the author(s).

Acknowledgments

We thank Juan D. Alfonzo (Ohio State University, Columbus) for discussions.

Funding

This work was supported by the Czech Science Foundation grants 20-11585S to ZP, 18-15962S and 20-071856S to JL, ERC CZ (LL1601) to JL, and the ERD funds of the Czech Ministry of Education, Youth and Sports (CZ.02.1.01/0.0/0.0/16 019/0000759) to ZP and JL.

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