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THIOLATED tRNAS OF *TRYPANOSOMA BRUCEI* ARE IMPORTED INTO MITOCHONDRIA AND DETHIOLATED AFTER IMPORT

Ellen Inga Bruske, Franziska Sendfeld and André Schneider[1)]
Department of Chemistry and Biochemistry, University of Bern, Freiestr. 3, CH-3012 Bern, Switzerland

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Address correspondence to André Schneider, Department of Chemistry and Biochemistry, University of Bern, Freiestr. 3, CH-3012 Bern, Email: andre.schneider@ibc.unibe.ch

All mitochondrial tRNAs in *Trypanosoma brucei* derive from cytosolic tRNAs that are in part imported into mitochondria. Some trypanosomal tRNAs are thiolated in a compartment-specific manner. We have identified three proteins required for the thio modification of cytosolic $tRNA^{Gln}$, $tRNA^{Glu}$ and $tRNA^{Lys}$. RNAi-mediated ablation of these proteins results in the cytosolic accumulation non thio-modified tRNAs but does not increase their import. Moreover, in vitro import experiments showed that both thio-modified and non thio-modified $tRNA^{Glu}$ can efficiently be imported into mitochondria. These results indicate that unlike previously suggested the cytosol-specific thio modifications do not function as antideterminants for mitochondrial tRNA import. Consistent with these results we showed by using inducible expression of a tagged $tRNA^{Glu}$ that it is mainly the thiolated form which is imported *in vivo*. Unexpectedly, the imported tRNA becomes dethiolated after import which explains why the non thiolated form is enriched in mitochondria. Finally, we have identified two genes required for thiolation of imported $tRNA^{Trp}$ whose wobble nucleotide is subject to mitochondrial C to U editing. Interestingly, downregulation of thiolation resulted in an increase of edited $tRNA^{Trp}$ but did not affect growth.

Most protozoa, many fungi, plants and a few animals lack a variable number of mitochondrial tRNA genes. It has been shown in these organisms that the missing genes are compensated for by import of a small fraction of the corresponding cytosolic tRNAs (1,2). Among all organisms that import tRNAs trypanosomatids such as *T. brucei* and *Leishmania* are extreme in that their mitochondrial genomes have lost all tRNA genes. Trypanosomatids therefore need to import the entire set of mitochondrial tRNAs. As a consequence all mitochondrial tRNAs in trypanosomatids derive from eukaryotic-type cytosolic tRNAs that need to function in the context of the bacterial-type translation system of mitochondria (3). Two tRNAs – the initiator $tRNA^{Met}$ and the $tRNA^{Sec}$ - are cytosol-specific. It has been shown that in *T. brucei* the tRNA import specificity is mediated by binding to cytosolic translation elongation factor 1a (4). The fact that initiator $tRNA^{Met}$ and $tRNA^{Sec}$ do not bind to elongation factor 1a therefore explains their exclusive cytosolic localization.

The extent of mitochondrial localization of different trypanosomal tRNAs varies by at least an order of magnitude (5,6). The same is true for other organisms that import tRNAs (7). Cells therefore need to determine both the tRNA import specificity as well as the extent of mitochondrial localization of each imported tRNA species. How this is achieved is not known in any species.

In trypanosomatids mitochondrial tRNAs and their cytosolic counterparts derive from the same nuclear genes. However, due to compartment-specific posttranscriptional nucleotide modifications, the cytosolic and the corresponding imported tRNAs are often physically different. Mitochondria-specific nucleotide modifications (probably methylations) were found at the cytidine 32 of $tRNA^{Lys}$(CUU), $tRNA^{Leu}$(CAA) and $tRNA^{Tyr}$ (8,9). The $tRNA^{Trp}$ of trypanosomatids is subject to extensive mitochondria-specific modifications which include methylation of the pseudouridine at position 32, 2-thiolation of the uridine at position 33 as well as methylation and C to U editing of the wobble nucleotide (10-12). The wobble nucleotide of $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) of *Leishmania tarentolae*

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consists of 5-methoxycarbonylmethyl uridine (mcm^5U). In addition to this modification the same nucleotide also contains a cytosol-specific thio modification at position 2 of the uracil (resulting in mcm^5S^2U) and a mitochondria-specific 2'-*O*-methyl group in the ribose (resulting in mcm^5Um) (13). Little is known about the function of these compartment-specific modifications. It has been shown that the cytidine 32 modifications are not required for import of the affected tRNAs but rather are a consequence of the mitochondrial localization of the tRNAs (9). Moreover, C to U editing of the imported $tRNA^{Trp}$ is required for correct decoding of the UGA codon, since in trypanosomatid mitochondria this codon has been re-assigned to tryptophan (11,12). Finally, it has been proposed that in *L. tarentolae* the cytosol-specific thio modifications of the $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) might be antideterminants for mitochondrial import (13). According to this model the extent of import of the two tRNAs would be regulated by the extent of the thiolation. Evidence for this hypothesis is based on the essentially exclusive cytosolic localization of the thio-modified molecules. Moreover, it has been shown that isolated thio-modified $tRNA^{Glu}$(UUC) is less efficiently imported into isolated mitochondria than the corresponding *in vitro* transcript lacking any modifications (13).

2-Thiolation of the wobble nucleotide of cytosolic $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) is not restricted to trypanosomatids but occurs in all eukaryotes. Moreover, the same appears to be the case for the corresponding mitochondrial encoded tRNAs (14). Recent work in the yeast *Saccharomyces cerevisiae* has shown that thiolation of tRNAs depends on components of the Fe/S cluster (ISC) biogenesis pathway (15-17). ISCs of iron sulfur proteins are essential for a wide variety of cellular processes and their assembly requires complex and interconnected machineries that are localized in both mitochondria and the cytosol (17). Interestingly, thiolation of cytosolic tRNAs required components of both the cytosolic and the mitochondrial ISC assembly pathways whereas thiolation of mitochondrial tRNAs needed only components of the mitochondrial ISC assembly pathway (15).

Recently, there has been great progress in the identification and characterization of the numerous factors that are required for 2-thiolation of eukaryotic tRNAs (18-21). Of special importance in the context of this study is the identification of yeast and human Mtu1, a mitochondria-specific tRNA 2-thiouridylase, responsible for the last step in the cascade leading to thiolation of mitochondrial tRNAs (22).

Here we show by using individual RNAi-mediated ablation of three components of the ISC biogenesis pathway that reduction of the thio-modified fraction of $tRNA^{Gln}$(UUG), $tRNA^{Glu}$(UUC) and $tRNA^{Lys}$(UUU) does not lead to increased import of the unmodified tRNA species. Moreover, *in vitro* import experiments indicate that both thio-modified and non thio-modified tRNAs are efficiently imported into mitochondria. We present evidence that the enrichment of non thio-modified $tRNA^{Glu}$ in mitochondria can best be explained by a mitochondrial dethiolation activity that removes the thio modification from the imported tRNAs. Finally, we identify two gene products required for thiolation of imported $tRNA^{Trp}$. We show that thiolation of the $tRNA^{Trp}$ is not essential for normal growth of *T. brucei* and that its extent is negatively correlated with the extent C to U editing.

Experimental procedures

Cell culture - *T. brucei* 427 and *T. brucei* 29-13 were grown at 27°C in SDM79 supplemented with 5% and 15% of fetal calf serum, respectively. In the case of *T. brucei* 29-13 the medium was supplemented with 25 μg/ml hygromycin and 15 μg/ml G-418.

Transgenic cell lines - All transgenic cell lines used in this study are based on *T. brucei* strain 29-13. RNAi directed against the Tb-Nbp35 (Tb10.70.6210), Tb-Cfd1 (Tb927.7.1500), Tb-Nfs1 (Tb11.55.0013) and Tb-Mtu1 (Tb927.8.1830) mRNAs was performed using pLew-100 (23) based stem loop constructs containing the puromycine

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resistance gene as described (24). As inserts we used a 505 bp fragment (nucleotides 217-722) for Tb-Nbp35, a 475 bp fragment (nucleotides 345 to 820) for Tb-Cfd1, a 952 bp fragment (nucleotides 107-1059) for Tb-Nfs1 and a 492 bp fragment (nucleotides 577 to 1069) for Tb-Mtu1. The cell line allowing inducible expression of a tagged $tRNA^{Glu}$ (Fig. 6) was established using the same strategy that had been successfully used in the case of the tagged elongator $tRNA^{Met}$ (4). Two tetracycline operators were fused in tandem to the immediate 5'-end of the tagged $tRNA^{Glu}$ gene. The tetracycline operator-$tRNA^{Glu}$ gene cassette was flanked on the 5'-side by 261 nt corresponding to the 5'-flanking region of the $tRNA^{Leu}$ gene, a sequence shown to be compatible with high levels of tRNA expression (25), and on the 3'-side by 25 nt of its endogenous 3'-flanking region. Sequence alignments of the trypanosomal $tRNA^{Glu}$ with other eukaryotic $tRNAs^{Glu}$ allowed to identify conservative nucleotide replacements of which A72 to G72 and G5:C67 to G5:U67 were chosen as tags (Fig. 6A). The tags were introduced by PCR directed mutagenesis and verified by sequencing. All constructs were linearized with NotI prior to transfection, selection with puromycine, cloning and induction with 1 μg/ml tetracycline were done as described previously (26).

Northern analyses - Isolated RNA fraction were separated and analyzed on a short 8 M urea/10% polyacrylamide sequencing gel containing 0.5% of [(N-acryloylamino)phenyl] mercuric chloride (APM) in order to separate non thio-modified from thio-modified tRNAs. Northern hybridizations using radioactively labeled oligonucleotide probes were done as described before (5). The following tRNAs were detected using the indicated oligonucleotide probe: $tRNA^{Glu}$(UUC), 5′GTGGTTCCGGTACCGGGA3'; $tRNA^{Gln}$(UUG), 5′GTGGTGGTCCTACCAGGAT3′; $tRNA^{Trp}$(CCA), 5′TGAGGACTGCAGGGATTG3′; $tRNA^{Lys}$(UUU), 5′GTGGCGCCTTCCGTGGGGATC3′. The tagged $tRNA^{Glu}$(UUC) could be specifically detected using the oligonucleotide 5′TGGCTCCGATACCGGGA3′. Imported $tRNA^{Phe}$ in the *in vitro* import assays was detected using oligonucleotide 5'GTGGTGCGAATTCTGTGGATC3'.

In vitro import of tRNAs - Import of tRNAs into isolated mitochondria was done as described (4). Mitochondria from uninduced and induced (72 hours) Tb-Nbp35 RNAi cell lines (Fig. 6A) or from wildtype *T. brucei* 427 (Fig. 6B) were isolated using the isotonic lysis procedure as described (27). For the experiments shown in Fig. 6A 4.2 pmol of *in vitro* transcribed $tRNA^{Phe}$ from yeast was used substrate. For the experiments shown in Fig. 6B the native $tRNAs^{Glu}$ present in the isolated total tRNA fraction from uninduced and induced (72 hours) Tb-Nbp35 RNAi cells were used as import substrates. In order to be able to detect the imported $tRNA^{Glu}$ it was specifically labeled at its 3'-end with radioactive dCTP using the oligonucleotide-directed 3'-splint labeling technique (9,28). The oligonucleotide that was used, 5′GTGGTGGTCCTACCAGGAT3′, hybridizes to the 3'-end of the $tRNA^{Glu}$ and leaves a 5'-G overhang. Using this technique the $tRNA^{Glu}$ could be highly efficiently labeled. The labeled $tRNA^{Glu}$ was then either gelpurified or directly used in the import assay yielding identical results.

Results

Thiolation of cytosolic tRNAs of T. brucei requires Tb-Nbp35, Tb-Cfd1 and Tb-Nfs1. To investigate the role thio modifications may play in regulating mitochondrial tRNA import in *T. brucei* we produced cell lines allowing inducible RNAi-mediated ablation of components of the trypanosomal ISC biogenesis pathways. The selected factors were the orthologues of yeast Nbp35 and Cfd1, components of the cytosolic Fe/S protein assembly (CIA) machinery, and of yeast Nfs1, a component of the mitochondrial ISC biogenesis pathway (17). Fig. 1 shows that ablation of any of these three factors leads to a growth arrest 3 days after induction of RNAi. This is expected since ISC biogenesis is essential in all eukaryotes.

The wobble nucleotide of $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) of

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Leishmania, a close relative of *T. brucei*, contains a thio group at position 2 of the uridine (13). To investigate whether this base modification requires the ISC biogenesis pathway we isolated total RNA from uninduced and induced RNAi cell lines and analyzed them by Northern blots. The RNA was separated on acrylamide gels containing [(N-acryloylamino)phenyl] mercuric chloride (APM) that causes a retardation in the migration of tRNAs that contain a thio carbonyl group (29,30). Fig. 2 shows that approx. 80% of total trypanosomal $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) is thio-modified (Fig. 2, -Tet, tot samples). Moreover, downregulation of either Tb-Nbp35, Tb-Cfd1 or Tb-Nfs1 led to a 2 to 5-fold increase of non thio-modified $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) (Fig. 2, +Tet, tot lanes) indicating that, as expected based on results in yeast (15), thiolation of cytosolic tRNAs requires all three proteins. The wobble base of $tRNA^{Lys}$(UUU) is thought to be universally modified by a variety of modifications all of which include a thio group. Northern analysis shows that also in *T. brucei* the $tRNA^{Lys}$(UUU) is thio-modified and that this modification depends on Tb-Nbp35, Tb-Cfd1 and Tb-Nfs1. This is different from the $tRNA^{Lys}$(UUU) of the closely related *L. tarentolae* which was reported to lack the thio modification (13).

Accumulation of non thio-modified tRNA does not increase import. Similar to the situation in *L. tarentolae* (13), the thio-modified $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) of *T. brucei* are highly enriched in the cytosolic fraction (Fig. 2, -Tet, tot lanes) and essentially absent in mitochondria (Fig. 2, -Tet, mt lanes). Thus, in order to test whether thio modifications act as anti import determinants in *T. brucei* we analyzed the localization of— the non thio-modified tRNAs that accumulate after downregulation of Tb-Cfd1, Tb-Nbp35 and Tb-Nfs1. Should the thio modification indeed function as antideterminant for mitochondrial tRNA import one would expect an increase in the import of the non thio-modified tRNA fraction. The cell fractionation in Fig. 2 (+Tet, tot and mt lanes) shows that this is not the case: in all three cell lines the non thio-modified tRNAs that accumulate after induction of RNAi remain in the cytosol. Moreover, the same is the case for the non thio-modified fraction of the $tRNA^{Lys}$(UUU). Northern analyses of enriched nuclear and cytosolic RNA fractions (4) showed that the non thio-modified tRNA accumulated in the cytosol and was not retained in the nucleus (data not shown).

Thiolation of mitochondrial $tRNA^{Trp}$ of T. brucei requires Nfs1 and Mtu1. The fraction of leishmanial and trypanosomal $tRNA^{Trp}$ that is imported into mitochondria is thiolated at the ultimate nucleotide before the anticodon (10). Thus, we decided to test whether Tb-Nbp35, Tb-Cfd1 or Tb-Nfs1, three components of Fe/S protein assembly machinery, besides being required for 2-thiolation of cytosolic tRNAs (Fig. 2) are also needed for thiolation of the imported mitochondrial $tRNA^{Trp}$. Moreover, we did the same test for the trypanosomal Mtu1 orthologue. Mtu1 is a highly conserved mitochondria-specific tRNA 2-thiouridylase whose bacterial MNMA homolog functions in 2-thiolation of bacterial tRNAs (22).

Accumulation of non-thiomodified $tRNA^{Trp}$ in induced RNAi cell lines shows that mitochondria-specific thiolation of $tRNA^{Trp}$ depends on Tb-Nfs1 and Tb-Mtu1 but unlike thiolation of the cytosolic tRNAs and in line with results from yeast (16) does not require Tb-Nbp35 or Tb-Cfd1 (Fig. 3). However, unlike Tb-Nfs1, Tb-Mtu is not involved in thiolation of cytosolic tRNAs (data not shown). Interestingly, ablation of Tb-Mtu1 in contrast to the other three tested proteins does not impair growth (Fig. 1, right most panel). Since it is known that mitochondrial translation is essential in insect stage *T. brucei* this shows that functional mitochondrial proteins can be synthesized in the absence of thiolated $tRNA^{Trp}$.

Removal of the thiolation stimulates $tRNA^{Trp}$ editing. The fraction of leishmanial and trypanosomal $tRNA^{Trp}$ that is imported into mitochondria is not only thiolated at ultimate nucleotide before the anticodon but also undergoes C to U RNA editing at the wobble position in order to recognize the UGA codon that has been reassigned to tryptophane (11). RNAi-mediated downregulation of Tb-Nfs1 and Tb-Mtu1

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results in an accumulation of non-thiomodified $tRNA^{Trp}$ (Fig. 3). The two RNAi cell lines can therefore be used to investigate whether and how the mitochondria-specific thiolation is connected to the C to U editing. The extent of RNA editing was as analyzed by HinfI restriction digests of RT-PCR amplified $tRNA^{Trp}$. Fig. 4 shows that there is 1.5-1.6 fold increase of the percentage of edited $tRNA^{Trp}$ in induced Tb-Nfs1 and Tb-Mtu1 RNAi cells. This increase is due to accumulation of non-thiomodified $tRNA^{Trp}$ since it is not seen in the induced Tb-Nbp35 and Tb-Cfd1 RNAi cell lines (data not shown). Thus, it appears that thiolation at position 33 has an inhibitory effect on the C to U editing of the adjacent uridine. These results are in complete agreement with the recently published study by Wohlgamuth-Benedum et al. (31) which demonstrated the same inhibitory effect of thiolation on editing of trypanosomal $tRNA^{Trp}$ by using a poisoned primer extension assay. Moreover, the results obtained with the Tb-Mtu1 RNAi cell line extend this study by showing that the degree of $tRNA^{Trp}$ editing does not influence the growth rate of *T. brucei* (Fig. 1, right most panel).

Tb-Nbp35 RNAi does not affect the tRNA import machinery. It would in principle be possible that the ISC machinery itself, or an unknown ISC-protein depending on it, is directly involved in mitochondrial tRNA import. Should this be the case the cytosolic accumulation of non thio-modified tRNA observed in Fig. 2 could still be consistent with a putative role of thio modifications as antideterminants for mitochondrial tRNA import. In order to exclude this possibility we performed *in vitro* import assays: isolated mitochondria from the uninduced and induced Tb-Nbp35-RNAi cell line were incubated with *in vitro* transcribed substrate tRNA in the presence and absence of ATP. As a model substrate we were using *in vitro* transcribed $tRNA^{Phe}$ of yeast, since it had been shown by using transgenic cells that heterologous tRNAs of yeast and human origin are efficiently imported into *T. brucei* mitochondria *in vivo* (32).

Fig. 5A shows that *in vitro* transcribed $tRNA^{Phe}$ was efficiently imported into mitochondria isolated from uninduced cells and from cells induced for 72 hours, the time point cytosolic accumulation of non thio-modified tRNAs was observed. This shows that the cytosolic accumulation of non thio-modified tRNAs caused by downregulation of Tb-Nbp35 is not due to an impaired tRNA import machinery.

Thio-modified tRNAs are efficiently imported in vitro. If the thio modifications in cytosolic tRNAs do not function as import antideterminants both thio-modified and non thio-modified tRNAs should be imported into isolated mitochondria with equal efficiency. In order to test this, we prepared tRNAs from induced and uninduced Tb-Nbp35 RNAi cell lines. The $tRNA^{Glu}$ present in the two fractions was specifically labeled by oligonucleotide-directed 3'-splint labeling (9,28). Subsequently the two tRNA fractions were resolved on a polyacrylamide gel, the labeled tRNA was visualized on the wet gel, cut out and eluted. The eluted radioactive $tRNA^{Glu}$ (UUC) was then used as substrate for *in vitro* import assays. Fig. 5B shows that the ratio of thio-modified to non thio-modified $tRNA^{Glu}$ (UUC) as monitored on APM gels did not significantly change during import into isolated wild-type mitochondria.

Our *in vitro* import assay faithfully reproduces the membrane translocation step of mitochondrial tRNA import. However, as described before, it does not show the same specificity that is observed *in vivo* (4). This can be explained by the absence of eukaryotic elongation factor 1a which has been shown to mediate the specificity of tRNA import in *T. brucei*. Thus, our results show that at the level of the membrane translocation step there is no selectivity for either thio-modified or non thio-modifed tRNAs.

Imported $tRNA^{Glu}$ is subject to dethiolation. If thio-modified tRNAs can be imported into mitochondria *in vitro* (Fig. 5B) why do they accumulate in the cytosol *in vivo*? A possible explanation would be that thio-modified tRNAs are indeed imported into mitochondria *in vivo* but that after import the thio modification is removed. Such a scenario could explain the highly enriched cytosolic localization of the

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thio-modified $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) that is seen at steady state.

The dynamics of the modification can be studied by following the fate of the thiolation on newly synthesized $tRNA^{Glu}$(UUC). For this purpose we produced a cell line that allows inducible expression of a tagged $tRNA^{Glu}$(UUC). This was achieved by transfection of *T. brucei* 29-13, which expresses the tetracycline repressor, with a construct containing the tetracycline operator 5' of the tagged $tRNA^{Glu}$(UUC) gene. We have previously used the same approach to study import of newly synthesized tagged $tRNA^{Met}$ (4). The two nucleotide replacements that were used as tags for the $tRNA^{Glu}$(UUC) (Fig. 6A) are not expected to interfere with the function of the tRNA since they occur naturally in $tRNAs^{Glu}$ of other eukaryotes (33). Figure 5B shows that addition of tetracycline induces expression of the tagged $tRNA^{Glu}$(UUC) in a time-dependent manner. Moreover, analysis of digitonin-extracted mitochondrial fractions showed that the tagged $tRNA^{Glu}$(UUC) is thiolated and imported into mitochondria.

The RNA samples were separated on an APM-containing gel to determine the ratio of thiolated versus non thiolated tagged $tRNA^{Glu}$(UUC) at different time points. As expected the extent of thiolation of the newly synthesized tagged $tRNA^{Glu}$(UUC) in the total RNA fraction was essentially identical to the one observed for the endogenous wild-type $tRNA^{Glu}$(UUC) and remained constant over time (Fig. 6B, top and middle panel). However, for the newly synthesized tagged $tRNA^{Glu}$(UUC) that was imported into mitochondria the situation was different. Whereas shortly after synthesis a large extent was thio-modified this fraction decreased at longer induction times (Fig. 6B, upper panel, compare 6 h and 24 h).

In order to analyze the dynamics of the thiolation more precisely we quantified its extent in total and mitochondrial $tRNA^{Glu}$(UUC) 6 and 72 hours after induction in three independent experiments. The extent of thiolation of the newly synthesized $tRNA^{Glu}$(UUC) in the total RNA fraction was the same at 6 and 72 hours of induction. The tagged $tRNA^{Glu}$(UUC) that was imported into mitochondria, however, was thiolated to the same extent than its cytosolic counterpart when analyzed six hours after induction but shows a significant lower level of thiolation when analyzed 72 hours after induction. This lower level is close to the one of the endogenous imported $tRNA^{Glu}$(UUC) (Fig. 6C, right panel). At this time point the tagged $tRNA^{Glu}$(UUC) is at steady state and therefore does not represent the newly synthesized $tRNA^{Glu}$(UUC) anymore.

In principle there are three ways of how to explain the time-dependent decline of the thiolation level of mitochondrial $tRNA^{Glu}$: i) higher import efficiency for non-thiolated $tRNA^{Glu}$, ii) intramitochondrial degradation that is specific for the thiolated species or iii) removal of the thiomodification after import. Different import efficiencies for the two $tRNA^{Glu}$ species can be excluded based on the in vitro import assays shown in Fig. 5B as well as based on data shown by Paris et al. (34) . Moreover, should selective intramitochondrial degradation of the thiolated tagged $tRNA^{Glu}$ be responsible for the observed accumulation of non-thiolated tRNA in mitochondria, we would expect the percentage of the tagged mitochondrial $tRNA^{Glu}$ to decrease with time, since a with time an increasingly larger fraction of the molecules would be degraded. The bottom right panel of Fig. 6B shows that this is not the case since the fraction of tagged tRNA that is present in mitochondria is very similar at the 6, 8 and 24 hours, respectively. However, during that time period we clearly see a shift in the ratio of thiomodifed to non-modified $tRNA^{Glu}$ (Fig. 6B, top panel).

Thus the most parsimonious explanation for our results is that *in vivo* the $tRNA^{Glu}$(UUC) is imported in the thiolated state and that after import it becomes partially dethiolated explaining the enrichment of dethiolated $tRNA^{Glu}$(UUC) that is observed in mitochondria.

Discussion

The involvement of components in the ISC biogenesis pathway for thio modification of eukaryotic tRNAs has so far mainly been analyzed in yeast (15,16). In the present study we have extended this analysis

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to the parasitic protozoa *T. brucei*. We show that Tb-Nbp35 and Tb-Cfd1, components of the CIA machinery, as well as Nfs1, a component of the mitochondrial ISC biogenesis machinery, are required to thiolate cytosolic $tRNA^{Glu}$(UUC), $tRNA^{Gln}$(UUG) and $tRNA^{Lys}$(UUU) of *T. brucei*. Thiolation of the mitochondrially imported $tRNA^{Trp}$, however, required Tb-Nfs1 and Tb-Mtu1 but was independent of Tb-Nbp35 and Tb-Cfd1. According to the recently revised eukaryotic phylogeny eukaryotes are divided into six supergroups (35,36). *S. cerevisiae* together with humans belongs to the *Ophistokonta*, whereas *T. brucei* belongs to the *Excavata*. The fact that tRNA thiolation in trypanosomes requires the same components as in yeast indicates that the tRNA thio modification pathway is conserved within the two supergroups and strongly suggests that it was already present in the common ancestor of all eukaryotes.

L. tarentolae and *T. brucei* are closely related and most aspects of mitochondrial tRNA import that have been investigated in the two species are highly similar. Both lack mitochondrial tRNA genes (37,38), show identical import specificity and edit the imported $tRNA^{Trp}$ in order to accommodate the variant genetic code (11,12). Whether the actual tRNA import machinery is also identical is unclear at present. In *Leishmania tropica* the tRNA import machinery has been reported to consist of a heterooligomeric protein complex composed mainly of components from different respiratory complexes (39), whereas in *T. brucei* it has not been characterized yet. Taking into account all the similarities described above and their shared evolutionary history we would expect the tRNA import mechanism to be conserved between the two groups of trypanosomatids.

However our experiments in *T. brucei* show (i) that increasing the fraction of non thio-modified tRNA *in vivo* does not increase the extent of mitochondrial partition of any of the three tested tRNAs and (ii) that thiolated tRNAs can be efficiently imported into mitochondria both *in vitro* and *in vivo*. This demonstrates that in *T. brucei* the thio modification of the wobble nucleotide in cytosolic tRNAs does not function as an antideterminant for mitochondrial tRNA import.

The same conclusion was reached in a recent publication by Paris et al. (34). They were able to show that ablation of Tb-Nfs1 by RNAi does not change the extent of mitochondrial localization of thio-modified or non thio-modified trypanosomal tRNAs. Further experiments in the same study showed that a chemically dethiolated $tRNA^{Glu}$ was as efficiently imported into isolated mitochondria as its fully modified counterpart (34). These results are fully consistent with our results and support the conclusion that in *T. brucei* thio modifications do not act as antideterminants for mitochondrial tRNA import.

Thus, it appears that while thio modifications mediate tRNA sorting in *Leishmania* they play no such role in *T. brucei*. However, in the light of the fact that *T. brucei* and *Leishmania* are closely related it should also be considered that the situation might be the same in both organisms. In fact, a detailed comparison of the *in vitro* import experiments done in *T. brucei* and *Leishmania* shows that the apparent contradiction between the *in vitro* import experiments can be explained. In the original study in *L. tarentolae* it was shown that *in vitro* import of the native fully modified $tRNA^{Glu}$(UUC) was less efficient than the corresponding *in vitro* transcribed $tRNA^{Glu}$(UUC) lacking any nucleotide modifications (13). However, an average tRNA has 13 modified residues. It is therefore possible that the difference in the *in vitro* import efficiencies of the tested substrates is not a consequence of the thiomodified uridine but of one or more of the other modifications. The experiments performed in our study and in the one by Paris et al. (34), on the other hand, compared *in vitro* import efficiencies of two $tRNAs^{Glu}$(UUC) that differ only by the wobble nucleotide thio modification. Interestingly, in these cases both substrates were imported with equal efficiency.

The fact that thio-modified $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) in *L. tarentolae* are essentially specific for the cytosol and that their non thio-modified counterparts are highly enriched in mitochondria has been taken as evidence

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that thio-modified tRNAs are not imported *in vivo* (13). The thio-modified $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$ (UUG) of *T. brucei* show essentially the same intracellular distribution than the corresponding tRNAs in *L. tarentolae*. However, we show that in *T. brucei* the mitochondrial enrichment of the non thio-modified $tRNA^{Glu}$(UUC) can best be explained by post import removal of the thio modification rather than by selective import of the non thio-modified tRNA.

Dethiolation could either be due to non-enzymatic intramitochondrial oxidation or it could be an enzyme-mediated reaction. Our results do not allow to distinguish between the two possibilities. However, the fact that a large fraction of mitochondrial $tRNA^{Trp}$ is permanently 2-thio-modified (10,12) shows that dethiolation is specific for a subset of tRNAs which suggests that it is enzyme-catalyzed.

2-thiolation is highly conserved in eukaryotes and occurs in tRNAs that read two degenerate codons ending in purine of split codon boxes where the pyrimidine- and purine-ending codons code for different amino acids. The 2-thiolation together with a methoxycarbonylmethyl group on position 5 (mcm^5S^2) were proposed (i) to restrict the wobble base pairing to purines and (ii) to stabilize the correct codon anticodon interaction (40-42). The fully modified wobble uridine (mcm^5S^2U) of cytosolic tRNAs was shown to be essential in *S. cerevisiae* (43). In nematodes and in fission yeast 2-thiolation of tRNAs has been implicated in maintaining genome integrity (18). Finally there is a link between 2-thiolation of tRNA and human diseases: a mutation in the human mitochondrial $tRNA^{Lys}$ gene which abolishes the modification of the wobble uridine including the 2-thiolation causes myoclonus epilepsy associated with ragged-red fibers (MERRF) by preventing efficient binding of the mutant tRNA to the ribosome (44).

As expected for eukaryotic tRNAs the wobble uridine of leishmanial $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) is modified to mcm^5U (13). Moreover, the cytosolic fraction of the wobble uridine contains a thio modification at position 2 of the uracil resulting in mcm^5S^2U. It is reasonable to assume that also in trypanosomatids these modifications serve to restrict wobble base pairing and to stabilize codon anticodon interactions. However, the wobble nucleotide of the mitochondrial fraction of trypanosomatid $tRNA^{Glu}$(UUC) and $tRNA^{Gln}$(UUG) lacks the thio group and contains instead a 2'-*O*-methyl group on the ribose yielding mcm^5Um (13). Thus, the mitochondrial 2'-*O*- methyl group probably serves an analogous function as the cytosolic 2-thio modification in the cytosol.

In addition to modulating decoding, thio modifications may have regulatory functions. It has been suggested that in *T. brucei* cytosol-specific thio modifications influence the stability of $tRNA^{Glu}$ and $tRNA^{Gln}$ (31). We could confirm this observation in our RNAi cell lines, however, the downregulation was more variable and less pronounced. Wohlgemuth-Benedum et al. (31) furthermore showed that downregulation of thiolation by ablation of Nfs1 causes stimulation of tRNA editing. Based on this result they proposed that the mitochondria-specific thiolation of the $tRNA^{Trp}$ plays a role in regulating the extent of editing. Tight regulation of tRNA editing might be required if unedited and edited $tRNA^{Trp}$ have non-overlapping functions in translation assigned to UGG and UGA codons, respectively. We have shown that ablation of Mtu1 essentially abolishes thiolation of mitochondrial $tRNA^{Trp}$ which in agreement with the results by Wohlgemuth-Benedum et al. (31) leads to an increase in C to U editing of the wobble nucleotide. However, ablation of Mtu1 did not effect growth of insect stage *T. brucei* even though the energy metabolism of these cells depends on oxidative phosphorylation and therefore requires mitochondrial gene products. These results show that efficient synthesis of mitochondrial proteins tolerates significant changes in the ratio of unedited to edited $tRNA^{Trp}$. However, this is in cell culture and the situation might well be different in vivo and/or in the different stages of the life cycle.

In summary, we have shown that thio modification of the wobble nucleotide in cytosolic tRNAs of trypanosomatids, unlike previously suggested, are not

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antideterminants for mitochondrial tRNA import. Moreover, we demonstrate that thio-modified tRNAs can readily be imported both *in vitro* and *in vivo*. Finally, we provide evidence that the thio group gets removed after import, illustrating an unprecedented dynamics of a tRNA nucleotide modification.

REFERENCES

1. Salinas, T., Duchêne, A. M., and Maréchal-Drouard, L. (2008) *Trends Biochem. Sci.* **33**, 320-329
2. Schneider, A., and Marechal-Drouard, L. (2000) *Trends Cell Biol.* **10**, 509-513
3. Schneider, A. (2001) *Int. J. Parasitol.* **31**, 1403-1415
4. Bouzaidi-Tiali, N., Aeby, E., Charrière, F., Pusnik, M., and Schneider, A. (2007) *EMBO J.* **26**, 4302-4312
5. Tan, T. H. P., Pach, R., Crausaz, A., Ivens, A., and Schneider, A. (2002) *Mol. Cell. Biol.* **22**, 3707-3717
6. Kapushoc, S. T., Alfonzo, J. D., Rubio, M. A. T., and Simpson, L. (2000) *J. Biol. Chem.* **275**, 37907-37914
7. Vinogradova, E., Salinas, T., Cognat, V., Remacle, C., and Maréchal-Drouard, L. (2009) *Nucleic Acids Res.* **37**, 1521-1528
8. Schneider, A. (1996) *Nucleic Acids Res* **24**, 1225-1228
9. Schneider, A., McNally, K. P., and Agabian, N. (1994) *Nucleic Acids Res.* **22**, 3699-3705
10. Crain, P. F., Alfonzo, J. D., Rozenski, J., Kapushoc, S. T., McCloskey, J. A., and Simpson, L. (2002) *RNA* **8**, 752-761
11. Alfonzo, J. D., Blanc, V., Estevez, A. M., Rubio, M. A. T., and Simpson, L. (1999) *EMBO J.* **18**, 7056-7062
12. Charrière, F., Helgadóttir, S., Horn, E. K., Söll, D., and Schneider, A. (2006) *Proc. Natl. Acad Sci. USA* **103**, 6847-6852
13. Kaneko, T., Suzuki, T., Kapushoc, S. T., Rubio, M. A., Ghazvini, J., Watanabe, K., Simpson, L., and Suzuki, T. (2003) *EMBO J.* **22**, 657-667
14. Suzuki, T. (2005) *In: Grosjean H, editor. Fine-Tuning of RNA Functions by Modification and Editing.* NY: Springer, pp. 24–69.
15. Nakai, Y., Nakai, N., Lill, R., Suzuki, T., and Hayashi, H. (2007) *Mol Cell Biol.* **27**, 2841-2847
16. Nakai, Y., Umeda, N., Suzuki, T., Nakai, M., Hayashi, H., Watanabe, K., and Kagamiyama, H. (2004) *J. Biol. Chem.* **279**, 12363-12368
17. Lill, R., and Mühlenhoff, U. (2008) *Annu. Rev. Biochem.* **77**, 669-700
18. Dewez, M., Bauer, F., Dieu, M., Raes, M., Vandenhaute, J., and Hermand, D. (2008) *Proc. Natl. Acad. Sci. USA* **105**, 5459-5464
19. Nakai, Y., Nakai, M., and Hayashi, H. (2008) *J. Biol. Chem.* **283**, 27469-27476
20. Huang, B., Lu, J., and Byström, A. S. (2008) *RNA* **14**, 2183-2194
21. Noma, A., Sakaguchi, Y., and Suzuki, T. (2009) *Nucleic Acids Res.* **37**, 1335-1352
22. Umeda, N., Suzuki, T., Yukawa, M., Ohya, Y., Shindo, H., Watanabe, K., and Suzuki, T. (2005) *J. Biol. Chem.* **280**, 1613-1624
23. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. (1999) *Mol. Biochem. Parasitol.* **99**, 89-101
24. Bochud-Allemann, N., and Schneider, A. (2002) *J. Biol. Chem.* **277**, 32849-32854
25. Crausaz-Esseiva, A., Marechal-Drouard, L., Cosset, A., and Schneider, A. (2004) *Mol. Biol. Cell.* **15**, 2750-2757

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26. McCulloch, R., Vassella, E., Burton, P., Boshart, M., and Barry, J. D. (2004) *Methods Mol. Biol.* **262**, 53-86
27. Schneider, A., Charrière, F., Pusnik, M., and Horn, E. K. (2007) *Methods Mol. Biol.* **372**, 67-80
28. Hausner, T. P., Giglio, L. M., and Weiner, A. M. (1990) *Gen. Dev.*. **4**, 2146-2156
29. Igloi, G. L., and Kössel, H. (1987) *Methods Enzym.* **155**, 433-448
30. Igloi, G. L. (1988) *Biochem.* **27**, 3842-3849
31. Wohlgamuth-Benedum, J. M., Rubio, M. A., Paris, Z., Long, S., Poliak, P., Lukes, J., and Alfonzo, J. D. (2009) *J. Biol. Chem.* **284**, 23947-23953
32. Hauser, R., and Schneider, A. (1995) *EMBO J.* **14**, 4212-4220
33. Jühling, F., .Mörl, M., Hartmann, R. K., Sprinzl, M., Stadler, P. F., and Pütz, J. (2009) *Nucleic Acids Res.* **37**, D159-D162
34. Paris, Z., Rubio, M. A., Lukes, J., and Alfonzo, J. D. (2009) *RNA* **15**, 1398-1406
35. Adl, S. M., Simpson, A. G., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R., Bowser, S. S., Brugerolle, G., Fensome, R. A., Fredericq, S., James, T. Y., Karpov, S., Kugrens, P., Krug, J., Lane, C. E., Lewis, L. A., Lodge, J., Lynn, D. H., Mann, D. G., McCourt, R. N., Mendoza, L., Moestrup, O., Mozley-Standridge, S. E., Nerad, T. A., Shearer, C. A., Smirnov, A. V., Spiegel, F. W., and Taylor, M. F. (2005) *J. Eukaryot. Microbiol* **52**, 399-451
36. Simpson, A. G., and Roger, A. J. (2004) *Curr. Biol.* **14**, R693-R696
37. Hancock, K., and Hajduk, S. L. (1990) *J. Biol. Chem.* **265**, 19208-19215
38. Simpson, A. M., Suyama, Y., Dewes, H., Campbell, D. A., and Simpson, L. (1989) *Nucleic Acids Res.* **17**, 5427-5445
39. Adhya, S. (2008) *Int. J. Biochem. Cell. Biol.* **12**, 2681-2685
40. Johansson, M. J., Esberg, A., Huang, B., Björk, G. R., and Byström, A. S. (2008) *Mol. Cell. Biol.* **28**, 3301-3312
41. Numata, T., Ikeuchi, Y., Fukai, S., Suzuki, T., and Nureki, O. (2006) *Nature* **442**, 419-424
42. Ashraf, S. S., Sochacka, E., Cain, R., Guenther, R., Malkiewicz, A., and Agris, P. F. (1999) *RNA* **5**, 188-194
43. Björk, G. R., Huang, B., Persson, O. P., and Byström, A. S. (2007) *RNA* **13**, 1245-1255
44. Yasukawa, T., Suzuki, T., Ishii, N., Ohta, S., and Watanabe, K. (2001) *EMBO J.* **20**, 4794-4802

FOOTNOTES

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The abbreviations used are: mcm^{5}U, methoxycarbonylmethyl uridine; ISC, Fe/S cluster; APM, [(N-acryloylamino)phenyl] mercuric chloride; CIA, cytosolic Fe/S protein assembly; MERRF, myoclonus epilepsy associated with ragged-red fibers.

FIGURE LEGENDS

Fig. 1. Tb-Nbp35, TbCfd1 and Tb-Nfs1 but not Tb-Mtu1 are essential for normal growth of procyclic *T. brucei*. Growth curve in the presence and absence of tetracycline (+, -Tet) of representative clonal Tb-Nbp35, Tb-Cfd1, Tb-Nfs1 and Tb-Mtu1 RNAi cell lines. Insets: Northern blots of the corresponding mRNAs. The RNA from induced cells was isolated at 72 hours, the time of the growth arrest (arrows). The rRNAs in the lower panel serve as loading controls.

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Fig. 2. Ablation of Tb-Nbp35, Tb-Cfd1 and Tb-Nfs1 causes accumulation of non thio-modified cytosolic tRNAs but does not influence mitochondrial import. (**A**) RNA isolated from total cell (tot) and digitonin-extracted mitochondrial pellets (mt) (5) of uninduced (-Tet) and induced (+Tet) Tb-Nbp35-RNAi cell line were separated on APM-containing polyacrylamide gels that allow to separate thio-modified (thio-tRNA) from non thio-modified tRNA (non-thio-tRNA). Top panel shows the ethidiumbromide stained gel. Bottom panel shows the corresponding Northern blot probed for the indicated tRNAs. The numbers at the bottom indicate the percentage (± standard error) of non-thio-modified cytosolic tRNA before (-Tet) and after (+Tet) induction of RNAi for 72 hours. The graph compares the extent of mitochondrial localization of the non thio-modified tRNAs in uninduced cells (-Tet, set to 100%) to the one in induced cells (+Tet). Standard errors are indicated. (**B**) Same analysis as in (A) but for the Tb-Cfd1 RNAi cell line. (**C**) Same analysis as in (A) and (B) but for the Tb-Nfs1 RNAi cell line.

Fig. 3. Ablation of Tb-Nfs1 and Tb-Mtu1 but not of Nbp35 and Tb-Cfd1 causes accumulation of non thio-modified mitochondrial $tRNA^{Trp}$. RNA isolated from total cell (tot) and digitonin-extracted mitochondrial pellets (mt) of the indicated uninduced (-Tet) and induced (+Tet, 72 hours for Tb-Nfs1, Tb-Nbp35, Tb-Cfd1 and 120 hours for Tb-Mtu1) RNAi cell lines were separated on APM-containing polyacrylamide gels. Positions of thio-modified (thio-tRNA) and non thio-modified tRNAs (non-thio-tRNA) are indicated. Top panel shows the ethidiumbromide stained gel. Bottom panel shows the corresponding Northern blot probed for the $tRNA^{Trp}$. The numbers at the bottom indicate the percentage (± standard error) of non thio-modified mitochondrial $tRNA^{Trp}$ before (-Tet) and after (+Tet) induction of RNAi.

Fig. 4. Downregulation of thiomodified $tRNA^{Trp}$ stimulates C to U editing. Extent of $tRNA^{Trp}$ editing in induced and uninduced Tb-Nfs1 and Tb-Mtu1 RNAi cell lines was quantified by an RT-PCR based assay as described (12). The top panel shows that the cytosolic and mitochondrial RNA fractions that were used as templates for RT are free of DNA. The extent of RNA editing was analyzed by using a HinfI restriction digest (lower panel). RNA editing destroys a HinfI site that is only present in the cDNA derived from the unedited $tRNA^{Trp}$ (14). Introduction of a synthetic HinfI at the 5' end of the 5' RT-PCR primer provides an internal control for the HinfI digestion. cDNA amplified from unedited $tRNA^{Trp}$ contains two HinfI sites and, thus, will be digested into three fragments (46, 22, and 21 nt; unedited). The cDNA derived from edited $tRNA^{Trp}$ contains the synthetic HinfI site only and will be digested into two fragments (68 and 21 nt; edited). Measuring the intensities of the diagnostic bands for non edited (46 nt) and edited (68 nt) $tRNA^{Trp}$ allows, after correcting for their different molecular mass, determination of the fraction of edited $tRNA^{Trp}$ in *T. brucei* mitochondria. The mean of the increase including standard error of $tRNA^{Trp}$ editing in induced RNAi cells is indicated. The restriction digests have been analyzed on a single gel for each RNA cell line but the lanes have been rearranged electronically for clarity (dashed lines).

Fig. 5. *In vitro* import assays. (**A**) Nbp35 is not required for tRNA import. *In vitro* import assays in the presence and absence of ATP using mitochondria isolated from the uninduced (-Tet) and induced (+Tet) Nbp35-RNAi cell line and *in vitro* transcribed yeast $tRNA^{Phe}$ as a substrate. The input lane (IP) depicts 1% of the added substrate. The imported $tRNA^{Phe}$ was detected by Northern blot and specific oligonucleotide hybridization. The percentages of the added substrate that is imported are indicated. (**B**) *In vitro* import does not show preference for thio or non thio-modified $tRNA^{Glu}$. *In vitro* import assay in the presence and absence of ATP using wildtype mitochondria and substrate $tRNA^{Glu}$ isolated from the uninduced (-Tet) and induced (+Tet) Nbp35-RNAi cell line into wildtype mitochondria. The substrate $tRNA^{Glu}$ was radioactively labeled using the 3'-end splint labeling technique (9,28) and detected by using a phosphorimager. The samples were analyzed on a APM gel. The input lane (IP) depicts 1% of the added substrate. The percentage of non thio-modified

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tRNA^{Glu} before and after import is indicated. All reactions shown in (A) and (B) were treated with micrococcus nuclease. The ethidium bromide stained panels show the two mitochondrial rRNAs and serve as loading controls.

Fig. 6. Evidence for dethiolation of tRNA^{Glu} after import. (**A**) Predicted secondary structure of the trypanosomal tRNA^{Glu}. The nucleotide changes that were introduced to tag the tRNA^{Glu} that can be specifically detected by oligonucleotide hybridization are indicated. **(B)** Inducible expression of tagged tRNA^{Glu}. Appearance of tagged tRNA^{Glu} (upper panel) as well as the endogenous wildtype tRNA^{Glu} (middle panel) in the cytosol (tot) and in digitonin-extracted mitochondria (mt) was monitored by Northern analysis. The RNA samples were separated on APM-gels in order to distinguish thio-modified from non thio-modified tRNAs^{Glu}. The bottom panel shows the corresponding ethidiumbromide-stained gel (EtBr). Positions of the mitochondrial rRNAs (mt rRNA) and the cytosolic (cyt rRNA) as well as the tRNA region are indicated. Graph, bottom left: quantitative analysis of four independent experiments of the type shown on the top panels. The signal corresponding to the tagged tRNA^{Glu} in the total RNA fraction at 24 hours of induction was set to 100%. Graph, bottom right: comparison of relative amounts of tagged tRNA^{Glu} (both thio-modified and non thio-modified) that are recovered in mitochondria after 4, 6 and 24 hours of induction, respectively. The fraction of the mitochondrial tagged tRNA^{Glu} after 24 hours of induction was set to 100%. Standard errors are indicated. **(C)** Percentages of thiolated tRNA^{Glu*} (light grey bars) and of thiolated endogenous wildtype tRNA^{Glu} (dark grey bars) were determined 6 and 72 hours after induction in both the cytosolic (tot, left panel) and the mitochondrial fractions (mt, right panel). The graphs depict the mean of three independent experiments (standard errors are indicated).

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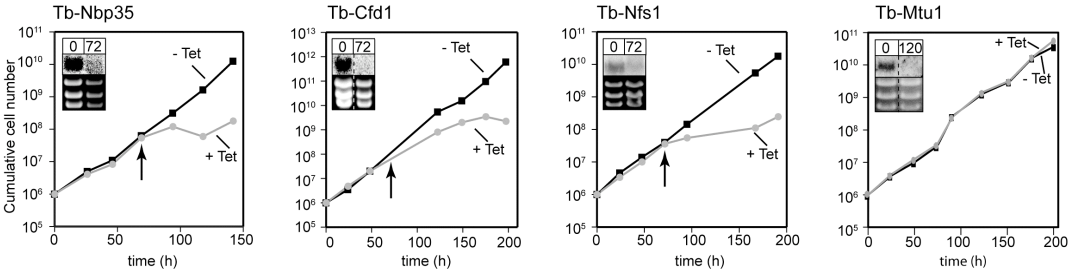

Fig. 1

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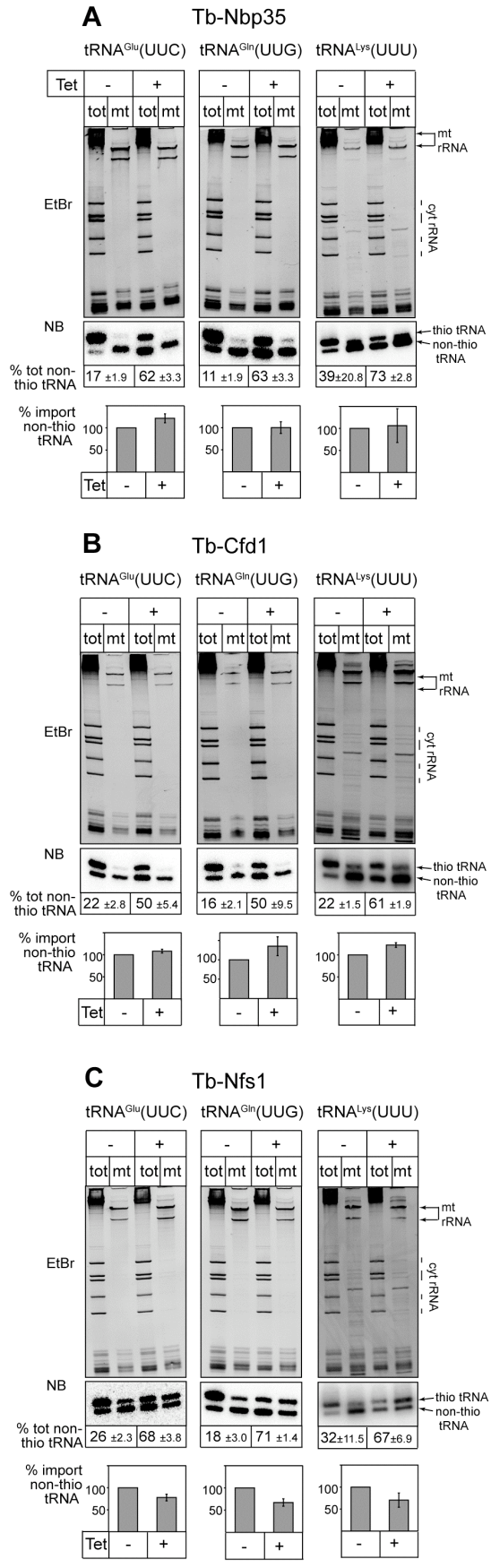

Fig. 2

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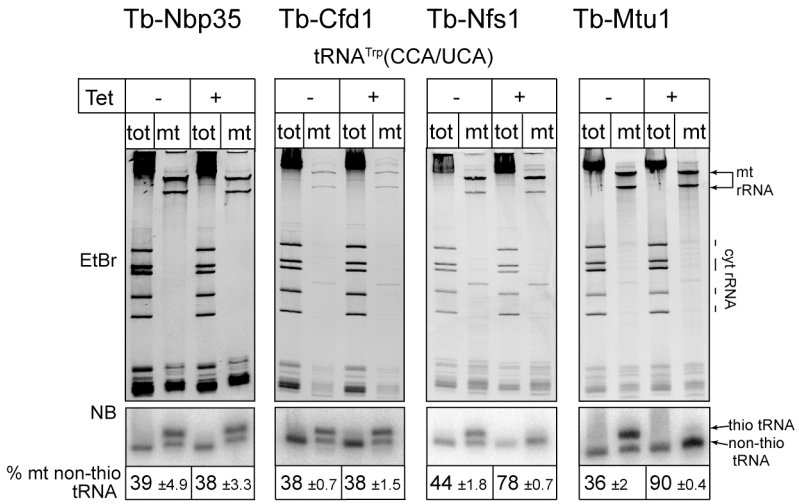

Fig. 3

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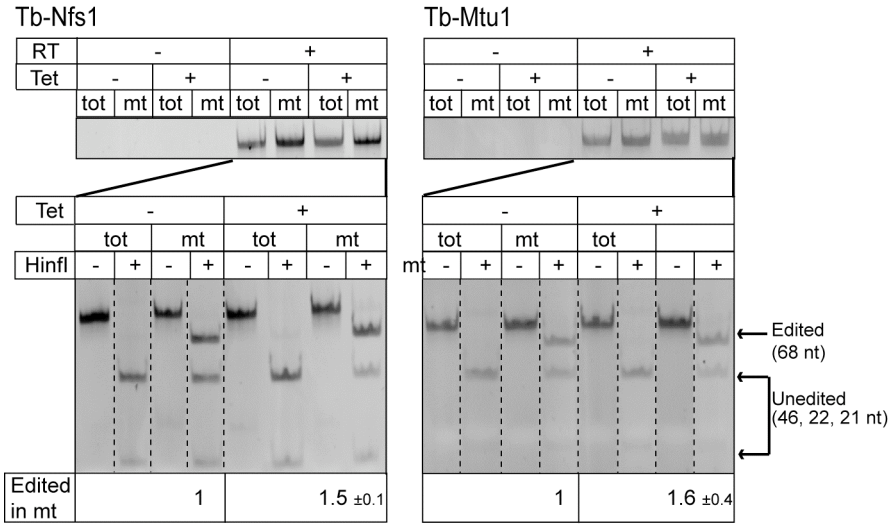

Fig. 4

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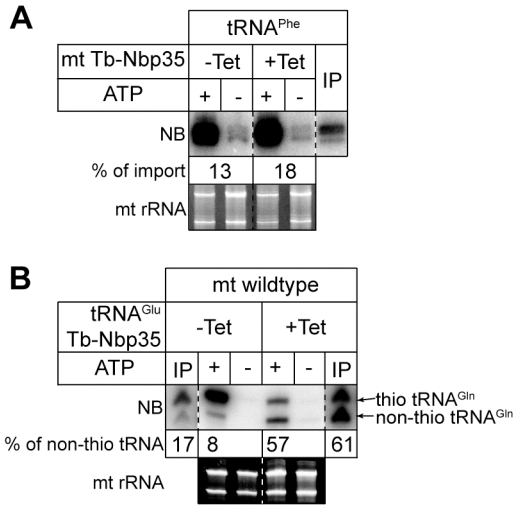

Fig. 5

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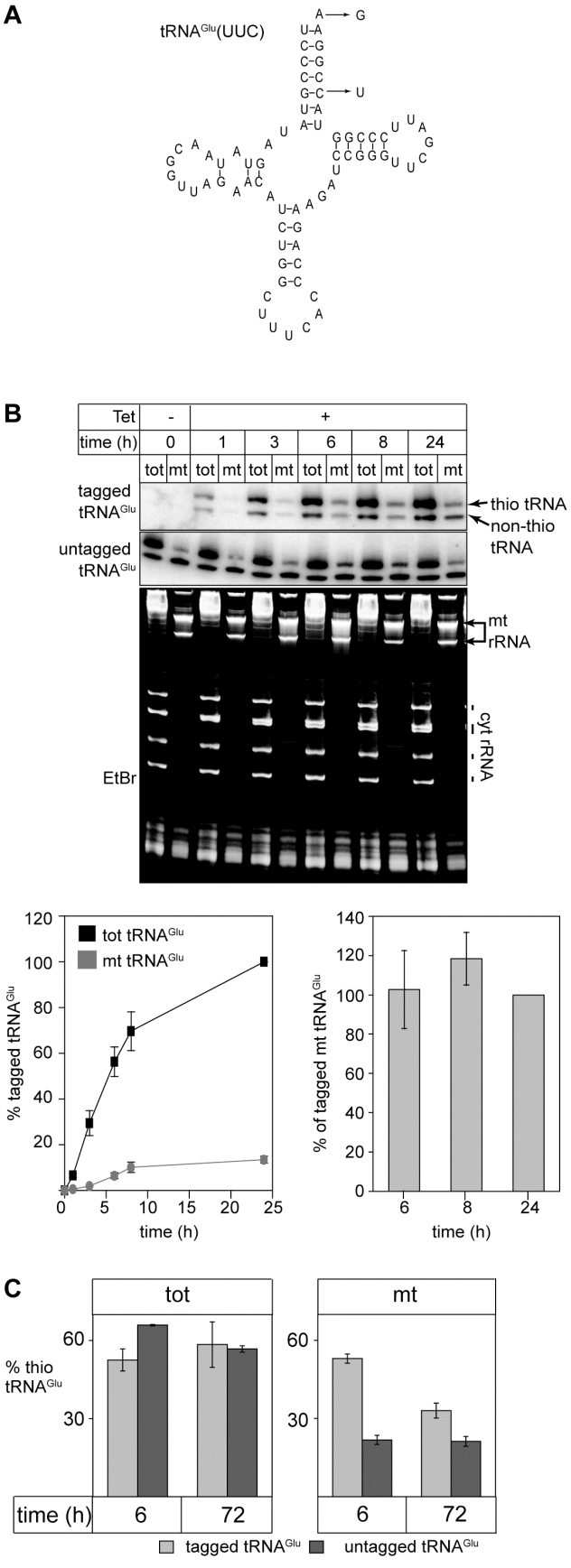

Fig. 6

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