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tRNA import across the mitochondrial inner membrane in *T. brucei* requires TIM subunits but is independent of protein import

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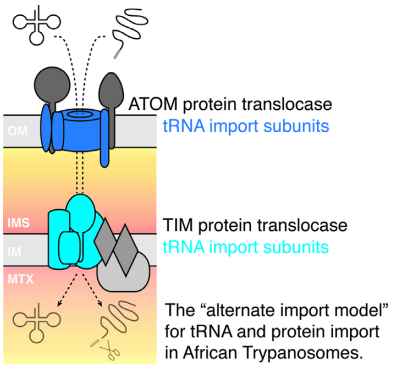
ABSTRACT

Mitochondrial tRNA import is widespread, but mechanistic insights of how tRNAs are translocated across mitochondrial membranes remain scarce. The parasitic protozoan *T. brucei* lacks mitochondrial tRNA genes. Consequently, it imports all organellar tRNAs from the cytosol. Here we investigated the connection between tRNA and protein translocation across the mitochondrial inner membrane. Trypanosomes have a single inner membrane protein translocase that consists of three heterooligomeric submodules, which all are required for import of matrix proteins. *In vivo* depletion of individual submodules shows that surprisingly only the integral membrane core module, including the protein import pore, but not the presequence-associated import motor are required for mitochondrial tRNA import. Thus we could uncouple import of matrix proteins from import of tRNAs even though both substrates are imported into the same mitochondrial subcompartment. This is reminiscent to the outer membrane where the main protein translocase but not on-going protein translocation is required for tRNA import. We also show that import of tRNAs across the outer and inner membranes are coupled to each other. Taken together, these data support the 'alternate import model', which states that tRNA and protein import while mechanistically independent use the same translocation pores but not at the same time.

GRAPHICAL ABSTRACT



INTRODUCTION

Mitochondria evolved from a single endosymbiotic event between an alpha-proteobacterium and an archaeal host cell ~2 billion years ago (1–3). During evolution the genome of the endosymbiont and subsequently the mitochondrion has been dramatically reduced to a small number of genes encoding mostly hydrophobic proteins. Consequently, essentially all eukaryotes import >95% of their complex mitochondrial proteome via the well-studied TOM–TIM pathways, the translocases of the outer and inner mitochondrial membrane (4–7).

Besides a few proteins mitochondrial genomes also encode rRNAs and tRNAs required for mitochondrial translation. However, whereas the genes for the small and the large subunit of the mitochondrial rRNAs are always encoded in the organelle, this is different for tRNAs. All plants and, many fungi and essentially all protozoa lack a variable number of apparently essential mitochondrial tRNA genes. It has been shown that in these cases the lacking mitochondrial tRNAs are compensated for by import of a small fraction of the corresponding cytosolic tRNAs (8–10). Moreover, mitochondrial RNA import may not be restricted to tRNAs but has also been suggested for the 5S rRNA (11) and RNase P RNA of mammals (12), even though their mi-

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toribosomes lack a 5S rRNA (13) and their mitochondrial tRNAs are processed by a protein-only RNase P (14).

Perhaps surprisingly mitochondrial tRNA import was discovered before protein import (15). However, while mitochondrial protein import has been studied in great detail, it is far less well understood how a polyanionic RNA is translocated across the mitochondrial outer (OM) and inner membrane (IM), the latter of which exhibits a membrane potential.

Traditionally two models have been proposed to explain mitochondrial tRNA import. The 'direct import model' suggests that tRNAs are imported as 'naked' molecules through import channels that are different from the ones used by proteins (10). It is mainly based on *in vitro* results in plants which show that tRNAs can be imported into mitochondria without the need of cytosolic factors (16). Moreover, it has been suggested that plant tRNAs use a specific voltage-dependent anion channel (VDAC) isoform as a pore to cross the OM (17), whereas IM translocation remains elusive. The 'co-import model', on the other hand, involves piggybacking of tRNAs with mitochondrial precursor proteins that are imported via the TOM-TIM pathways. It is based on results obtained in the yeast *Saccharomyces cerevisiae*, which appears to import a small fraction of a single $tRNA^{Lys}$ isoacceptor along with the precursor of mitochondrial lysyl-tRNA synthetase (18,19). An unsolved question in context of the co-import model is how a protein–tRNA complex can be maintained during translocation across the TOM and TIM import channels which can only accommodate unfolded proteins (20,21).

African trypanosomes represent an extreme case, as their mitochondrial genome is devoid of all tRNA genes (22–24). Thus, all mitochondrial tRNAs in trypanosomes have to be imported from the cytosol, making these protozoan parasites an ideal model system to study tRNA import. Trypanosomes are not only extreme when it comes to tRNA import, but they also possess a unique protein import machinery (25–27). They have an 'atypical TOM', designated as ATOM, which is composed of the Tom40 and Tom22 orthologues, ATOM40 and ATOM14 (28–30) and five trypanosomatid-specific subunits, two of which – ATOM46 and ATOM69—function as protein import receptors (28–30). Recently, we have shown that *in vivo* tRNA import requires the protein import pore ATOM40, but not the protein import receptors ATOM46 and ATOM69 (31). Thus, even though tRNAs are translocated across the ATOM40 import pore, they do not need the protein import receptors which excludes the co-import model as an explanation for OM translocation of tRNAs. Based on these results we proposed the novel 'alternate import model' which implies that in *T. brucei* the Tom40 orthologue ATOM40 acts as both a protein- and tRNA conducting pore, but that the two processes are not mechanistically linked and therefore cannot occur simultaneously at the same individual import pore (31).

The protein translocation system of the IM of *T. brucei* is even more peculiar, as it has retained only a single TIM complex (32,33). This stands in contrast to most other eukaryotes, which have two TIM complexes (27,34,35), with the TIM22 complex specializing in the insertion of proteins containing multipass transmembrane domains, such as mitochondrial carrier proteins (36) and the TIM23 complex facilitating the import of presequence-containing proteins (4,5,37). The non-canonical, singular TIM complex of *Trypanosoma brucei* has only a single integral membrane protein in common with other eukaryotes, namely TbTim17 (38–40), which despite its name is likely an orthologue of the Tim22 of the TIM22 complex (34).

The composition and function of the trypanosomal TIM complex have been dissected by combining reciprocal affinity purification of epitope-tagged putative TIM subunits and mass-spectrometry (MS) as well as RNAi-mediated gene knock-down and *in vitro*/*in vivo* protein import assays (32,33,41). Immunoprecipitation combined with SILAC-based MS analysis of import arrested intermediates that are stuck in either the presequence or the mitochondrial carrier pathway has been an especially powerful tool to define the composition of the trypanosomal TIM complex. It revealed that the trypanosomal carrier and presequence translocase consists of the same 10 subunits (33). These include the integral membrane core consisting of TbTim17, the trypanosomatid-specific TbTim42 and TbTim62 (41) as well as a putative acyl-CoA dehydrogenase homologue (ACAD). Moreover, it includes six peripheral conserved small Tim chaperones of the intermembrane space (39,42,43). The presequence translocase in addition contains the two inactive rhomboid-like proteins, TimRhom I and TimRhom II, that were not found in the carrier translocase (33). The two proteins are essential and their ablation inhibits mitochondrial protein import, however, what specific function they have in the process is not known. More recent work identified the trypanosomatid-specific J domain containing TbPam27 and the conserved mitochondrial heat shock protein 70 (mHsp70) as essential subunits of the highly unusual trypanosomal presequence-translocase associated motor (PAM) (44), a TIM module that drives ATP-dependent translocation of presequence-containing proteins (6,45,46). As expected TbPam27 was peripherally associated with TIM. These data are indicative that association of the trypanosomal TIM with TimRhom I and TimRhom II as well as with the PAM module allows for its functional specialization.

How are trypanosomal tRNAs translocated across the IM? Can the 'alternate import model', first proposed for translocation of tRNAs across the OM (31) be extended to the IM? It has been shown that TbTim17 is essential for mitochondrial tRNA import *in vivo* (47,48). Moreover, a recent study suggests that the same is the case for TbTim62 (48). Here we have investigated which of the three TIM modules (the integral membrane core/the two rhomboid-like proteins/the PAM module) are required for mitochondrial tRNA import, in order to gain insight into the mechanism of tRNA translocation across the IM.

MATERIALS AND METHODS

Transgenic cell lines

Transgenic RNAi cell lines were based on procyclic *T. brucei* strain 29–13 (49). Cells were grown in suspension culture at 27°C in SDM-79 medium (50) containing 10% (v/v) fetal calf serum (FCS). The tetracycline-inducible

RNAi constructs were derived from the pLEW100 stem-loop vector (49) using the following inserts: ATOM40 (Tb927.9.9660 ORF, nt 22–511); TbTim17 (whole ORF); mHsp70 (Tb927.6.3740, Tb927.6.3750, Tb927.6.3800 ORF, nt 1523–1975); TbPam27 (Tb927.10.13830, ORF nt 5-450); ACAD (Tb927.8.1420 ORF, nt 1027–1428); TimRhom I (Tb927.9.8260 ORF, nt 364–669), TimRhom II (Tb927.8.4150 3′-untranslated region, nt 125–364) and TbTim42 (Tb927.9.11220 ORF, nt 110–642;) as described before (29,33,39,47). Transfection and selection of clonal cell lines was done as described earlier (51).

Northern analysis for validation of RNAi cell lines

For verification of the mRNA knockdown, total RNA was prepared from cells (uninduced or induced for 48–72 h with tetracycline, final concentration 1 μg/ml) following the acid guanidinium thiocyanate RNA extraction method (52). Briefly, cells were harvested and washed in 1xPBS (phosphate buffered saline). The obtained pellets were resuspended thoroughly in 4M guanidinium thiocyanate containing 200 mM sodium acetate pH 4.0, 25 mM sodium citrate, 0.5% (w/v) sodium N-lauroyl sarcosinate and 100 mM ß-mercaptoethanol as well as 20 mg/ml oyster glycogen, followed by acid-phenol and chloroform extraction and subsequent precipitation with isopropanol. 5 μg of total RNA resuspended in loading dye (20 mM MOPS, 8 mM sodium acetate, 1 mM EDTA, 10% (v/v) formaldehyde, 50% (v/v) formamide, 0.1% (w/v) bromphenol blue, 0.05% (w/v) xylene cyanol including 0.1% (w/v) ethidium bromide was separated on a 1% (w/v) agarose gel containing 200 mM formaldehyde using the following buffer: 20 mM MOPS, 8 mM sodium acetate, 1 mM EDTA, 200 mM formaldehyde, pH 7.0. The cytosolic rRNAs at 2251, 1864 and ca. 1400nt were visualized as quality and loading control. RNA was then transferred on a charged nylon membrane (GeneScreen plus, Perkin Elmer) via capillary blot for ca. 16 h in 10× SSC (130 mM NaCl, 150mM sodium citrate, pH7.0). The membrane was UV cross-linked (150 mJ/cm^2) and dried. For northern hybridization, blots were pre-wetted in 2× SSC and pre-hybridized for 1hr at 65°C in buffer containing 0.5M Na_2HPO_4/H_3PO_4 pH 7.2, 7% (w/v) SDS, 1% (w/v) bovine serum albumin (fraction V) and 1 mM EDTA. The buffer was then replaced with an identical amount containing the corresponding probe. The northern probes were prepared with PCR amplicons of the ORF targeted by RNAi using the random hexamer–primer labelling approach based on Klenow-fragment (0.5 U/μl) in combination with α-^{32}P-dCTP and 20 μM of each dATP, dGTP and dTTP (Prime-a-Gene, Promega). Unincorporated nucleotides were separated by size-exclusion chromatography following the reaction. After hybridization for 14–18 h at 65°C the probe was recovered and the blot was washed three times for 10 min each with decreasing salt concentration: 2× SSC containing 0.1% (w/v) SDS, 1× SSC containing 0.1% (w/v) SDS and finally 0.5× SSC containing 0.1% (w/v) SDS. Blots were then plastic-wrapped and exposed to a storage phosphor screen for 24–72 h followed by analysis in a Typhoon FLA 9500 Phosphorimager.

Mitochondrial tRNA steady-state assay

Cells of the corresponding RNAi cell lines were induced in biological triplicate to quintuplicate by adding tetracycline to the cell culture medium (final concentration 1 μg/ml). Equal cell numbers (1–2 × 10^8) of uninduced and induced cells were harvested at the desired time-points, washed in 1× PBS and a mitochondria-enriched fraction was prepared using digitonin extraction followed by RNase A treatment essentially as described before (31,53). The washed cell pellets were resuspended in 500 μl 1× SoTE (0.6 M sorbitol, 20 mM Tris–HCl, pH 7.5 and 2 mM EDTA). 2.5% was removed for the whole cell (WC) protein sample analysed by immunoblotting (see below). Subsequently, 500 μl 1× SoTE containing 0.05% (w/v) digitonin were added to the remainder of the cell suspension, samples were mixed by careful inversions and incubated on ice for 10 min. Following centrifugation (6800g for 5 min at 4°C) the supernatants were discarded and the resulting pellets were resuspended in 500 μl 1× SoTE and treated with 1mg/mL RNase A for 15 min on ice. 5% of this crude mitochondrial fraction was removed for immunoblots analysis (see below) before a final centrifugation step. The obtained pellets were subjected to the acid guanidinium thiocyanate-phenol-chloroform RNA extraction (52) and precipitation as described above. 5–10 pmols of a synthetic RNA oligonucleotide (5′-GGAGCUCGCCCGGGCGAGGCCGUGC CAGCUCUUCGGAGCAAUACUCGGC-3) was added to each sample as an internal standard prior to the isolation procedure. Isolated RNA was separated in 10% (w/v) polyacrylamide gels containing 8M Urea, electrophoresis buffer 1× TBE (89 mM Tris, 89 mM boric acid 2 mM EDTA, pH 8.3), visualized by Ethidiumbromide (EthBr) staining, electro blotted onto a nylon membrane (200 mA for 14–18 h) in a tank containing 1× TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.3), subsequently UV cross-linked (250 mJ/cm^2) and dried. Blots were then probed for tRNA^{Ile} and the standard synthetic RNA oligonucleotide as described previously (31). In short, 10pmol of a complimentary short synthetic DNA oligonucleotides (tRNA^{Ile} 5′-TGCTCCCGGGCGGGTTCGAA-3; tRNA^{Lys} 5′ TGGC ACCCCCCGTGGG-GCTC 3′; tRNA^{Asn} 5′ CTCCTCCC GTTGGATTCG 3′; tRNA^{Ala} 5′ GCTCTACCACTGAG CTAC-ACG 3′ and 5′-GCCGAGTATTGCTCCGAAG -3′ for the internal standard) was radio-labelled with the polynucleotide kinase forward reaction using γ^{32}P-ATP (50 μCi specific activity 3000 Ci/mmol) followed by size-exclusion chromatography. The blots were pre-hybridized for 1 h at 55°C in buffer containing 6× SSPE (60 mM NaH_2PO_4, 0.9 M NaCl, 6 mM EDTA) and 5× Denhardt's [0.1% (w/v) Ficoll Type 400, 0.1% (w/v) Percoll, 0.1% BSA fraction V] and 0.5% (w/v) SDS after a brief re-hydration in 0.2x SSPE. For hybridization the buffer was exchanged and the probes were added simultaneously and incubated for 12–18 h at 55°C. Blots were washed three times for three minutes with wash buffer (2× SSPE, 0.5% (w/v) SDS), plastic-wrapped, exposed for ca. 12–48 h and scanned using a PhosphorImager system as described above. The tRNA^{Ile} signal was quantified and normalized to that of the internal standard, unless the average deviation of the three to five biological replicates was not more than 10%. Note: digi-

tonin is a natural product and as such the quality can be quite variable, however the RNase-treated mitochondria-enriched pellets are essentially devoid of contaminating cytosolic RNAs.

Immunoblotting

Samples for SDS-PAGE and subsequent immunoblot analysis were collected from WC and crude mitochondrial fractions as described above. Immunoblots were probed for ATOM40 (rabbit polyclonal, dilution 1:1000), TbTim17 (rat polyclonal; dilution 1:150), LDH (rabbit, dilution 1:5000), Cox4 (rabbit polyclonal, dilution 1:1000) and eEF1a (mouse, dilution 1:10.000, Merck Millipore, Product No. 05-235). All the antibodies and their working dilution have been described before (28,33,42). Commercially available secondary antibodies used for detection of the signal in a infrared scanner (LI-COR Odyssey): IRDye 680LT goat anti-mouse (LI-COR Biosciences, 1:20 000), IRDye 800CW goat anti-rabbit (LI-COR Biosciences, 1:20 000) and IRDye 680LT conjugated goat anti-rat (LI-COR Biosciences, dilution WB 1:10 000).

RESULTS

Ablation of ATOM40 or TbTim17 inhibits import of tRNAs across both membranes

The best characterized tRNA import factor in *T. brucei* is ATOM40 (29). Two independent lines of evidences indicate its important role in the process. First, inducible ablation of ATOM40, as expected should tRNA import be inhibited, results in a reduction of the steady state levels of mitochondrial tRNAs (31). Moreover, proteomic analysis of the OM in ablated cells indicates that this is a direct effect of the lack of ATOM40, and not caused by indirect downregulation of other putative OM import factors (31). Second, plugging the ATOM40 import pore by an import arrested protein abolishes mitochondrial tRNA import, very similar to when ATOM40 is removed by RNAi (31).

The time course experiment using the ATOM40 RNAi cell line confirms these results (Figure 1). In order to analyse tRNA import in the ATOM40 RNAi cell line the steady-state levels of mitochondrial tRNAs were determined. To that end mitochondrial RNA was isolated from equal cell equivalents of the uninduced and induced ATOM40 RNAi cell line. Subsequently, mitochondria-enriched fractions were produced by digitonin extraction, to eliminate cytosolic RNA contaminants the pellets were treated with RNase A (31,53). Moreover, a synthetic RNA of similar size than tRNA was added as an internal standard for the yield of the RNA isolation procedure. The resulting RNA fractions were separated on denaturing polyacrylamide gels and stained with ethidium bromide. Subsequently, the gel was blotted and processed for Northern hybridization using labelled oligonucleotides directed against the $tRNA^{Ile}$, as representative for an efficiently imported tRNA, and the internal standard. In parallel the corresponding protein extracts were analysed by immunoblots. The levels of ATOM40 were quantified to determine the efficiency of the RNAi. Dihydrolipoamide dehydrogenase (LDH), a bona-fide mitochondrial matrix protein with a long half-life that is not affected at early time points after ablation of the protein import pore ATOM40, was also tested (31). It served to show that uninduced and induced cells contain equal amounts of mitochondria and to ensure that mitochondrial integrity was maintained during the digitonin extraction and RNase A treatment. The levels of cytochrome oxidase subunit 4 (Cox4), finally, were determined to monitor the inhibition of protein import which results in the accumulation of the precursor form of the protein that carries a presequence.

All experiments were quantified using data from at least three biological replicates. The results show that ablation of ATOM40 results in a time dependent reduction of mitochondrial tRNA steady state levels (Figure 1A). Interestingly, whereas the levels of ATOM40 rapidly declined after RNAi induction (Figure 1D), the reduction in the tRNA steady state levels was only later observed at the time when the growth arrest became apparent (Figure 1B and C). This can be explained by the fact that in these *in vivo* experiment tRNA import rates cannot directly be measured, rather the decline of mitochondrial tRNA levels is used as a proxy for the inhibition of import. tRNAs are stable molecules that are only slowly turned over, which means that it takes some time before inhibition of import results in a lower level of mitochondrial tRNAs.

The only IM component known to be required for tRNA import is TbTim17 (47,48), the sole conserved subunit of the single TIM complex of trypanosomes. Thus, it was suggested that after OM translocation via the ATOM40, tRNAs are further shuttled along the ATOM–TIM pathway and ultimately traverse into the matrix using the TIM complex. In order to confirm these results, we did the same time course experiment that was done for the ATOM40 RNAi cell line for a cell line allowing inducible ablation of TbTim17. As evident from Figure 2A, mitochondrial steady-state tRNA levels declined upon TbTim17 knockdown over time. The growth phenotype of cells ablated for TbTim17 appears around ∼48 h post-induction (Figure 2B) and mitochondrial $tRNA^{Ile}$ steady-state levels are reduced by almost 50%, whereas Cox4 precursor accumulated accordingly (Figure 2A and C). This decline of mitochondrial tRNA steady-state levels is also evident in the EthBr-stained RNA gels (Figure 2A). To corroborate these data, we also quantified the mitochondrial levels of $tRNA^{Lys}$, $tRNA^{Asn}$, $tRNA^{Ala}$ (Figure 2A), which essentially behave similarly to $tRNA^{Ile}$ (Figure 2C). Thus, there is likely no bias between different tRNA species and its reasonable to assume that the quantification using $tRNA^{Ile}$ is in fact representative for all tRNA species. Interestingly, the amounts of TbTim17 dropped before a clear reduction in the levels of mitochondrial tRNAs is observed (Figure 2C and D). This is essentially identical to what is seen in the ATOM40 RNAi cell line (Figure 1C and D), whose direct involvement in tRNA import has been shown by two different experimental approaches (31). These results therefore confirm previous studies and suggest that TbTim17, as ATOM40, might be directly involved in mitochondrial tRNA import. In addition, the experiment provides further important information. Ablation of TbTim17 as expected did not affect the levels of ATOM40, however a reduction of the steady state levels of mitochondrial tRNAs is still observed identical to what happens after downregulation of ATOM40. This is

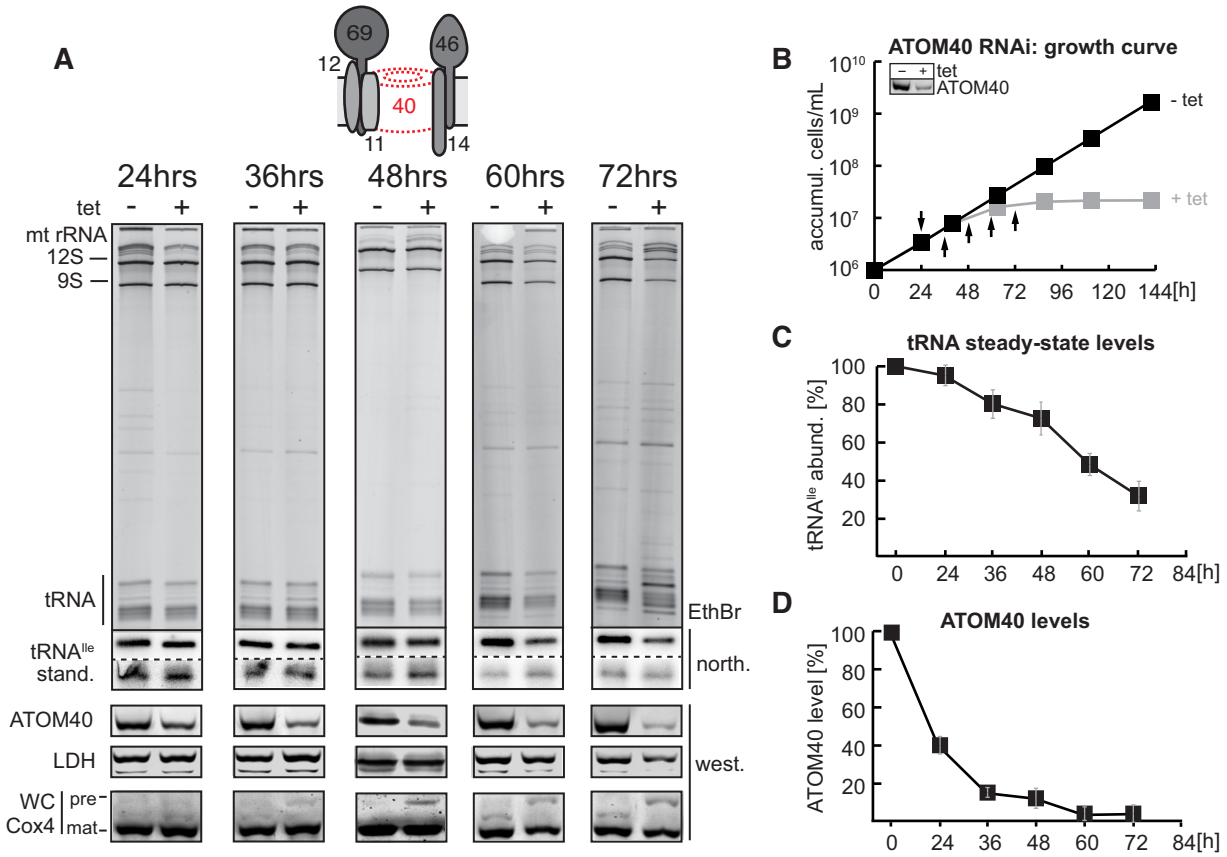

Figure 1. Ablation of ATOM40 inhibits import of tRNAs across both membranes. (**A**) The top sketch depicts the ATOM complex and its subunits with the pore-forming subunit ATOM40 targeted by RNAi knockdown (red dashed lines). RNAi was induced by tetracycline (tet) for 24–72 h. Mitochondrial-enriched fractions were produced by Digitonin extraction and RNA and protein samples were obtained accordingly. Mitochondrial RNA was separated on 8M urea/10% (w/v) polyacrylamide gels (PAGE) followed by ethidium bromide staining (EthBr). The mitochondrial rRNAs (12S and 9S) and tRNAs are indicated. The corresponding northern blots (north.) probed for $tRNA^{ile}$ and the synthetic RNA oligo (stand.) used to quality-control the RNA extraction are shown below the EthBr panel. The immunoblot panel (west.) further below show endogenous ATOM40 and LDH levels in the crude mitochondrial fractions, corroborating ATOM40 knockdown efficiency and integrity of mitochondria during the preparation. Cox4 precursor (pre) and mature (mat) protein levels were monitored in whole cell (WC) protein samples. Accumulation of Cox4 precursor is indicative of a mitochondrial protein import defect, whereas LDH serves as a control for mitochondrial integrity during the experimental procedure. (**B**) Growth curve of cells uninduced (-tet) and induced (+tet) for ATOM40 RNAi. The inset shows an immunoblot confirming the knockdown of ATOM40 48hrs post-induction. The arrows indicate the time points analysed as shown in (A). (**C**) Quantification of $tRNA^{Ile}$ signal from at least three biological replicates as depicted in (A). (**D**) Quantification of mitochondrial ATOM40 levels from the corresponding panel in (A). The error bars represent the standard deviation of the mean.

surprising, since if tRNA import across the OM and IM are independent from each other, we should see accumulation of tRNAs in the intermembrane space, where they would be protected from the RNase treatment. However, this is not observed and inhibition of tRNA import across the IM results in cytosolic rather than IMS accumulation of tRNAs. Treatment with low concentration of digitonin does not significantly disrupt the OM (54). This was confirmed by the fact that most of the soluble IMS-localized proteins Erv1 is recovered in the crude mitochondrial fraction after digitonin treatment (Supplementary Figure S1). These results therefore show that tRNA import across the OM and IM are coupled.

The integral membrane core of the TIM complex is required for tRNA import

The validation of TbTim17 as a subunit required for tRNA import lead us to investigate whether other TIM components are also involved in tRNA import. We first focused on TbTim42 and ACAD that are part of the integral membrane core of the single trypanosomal TIM complex (33).

Noteworthy, for both ATOM40 and TbTim17 RNAi, the maximal observed reduction of mitochondrial tRNA steady-state levels is ∼50% at the time when the growth arrest becomes apparent (Figures 1B and C, 2B and C). As a consequence, subsequent screening of TIM subunits focused on the same relative time point. We first tested TbTim42, which contains a single TMD. Figure 3A shows that mitochondrial tRNA steady-state levels are reduced to ∼67% at the appearance of the growth arrest 48 hours post-induction and continue to decline to ∼55% 24 h later (Figure 3B). Based on these data we conclude that TbTim42 is involved in tRNA import across the IM. Next the TIM subunit ACAD, which has at least three predicted TMDs, was tested (33). Figure 3C shows that at the time the growth arrest becomes evident (∼60 h post-induction; Figure 3D) the mitochondrial tRNA steady-state levels dropped to ∼65%,

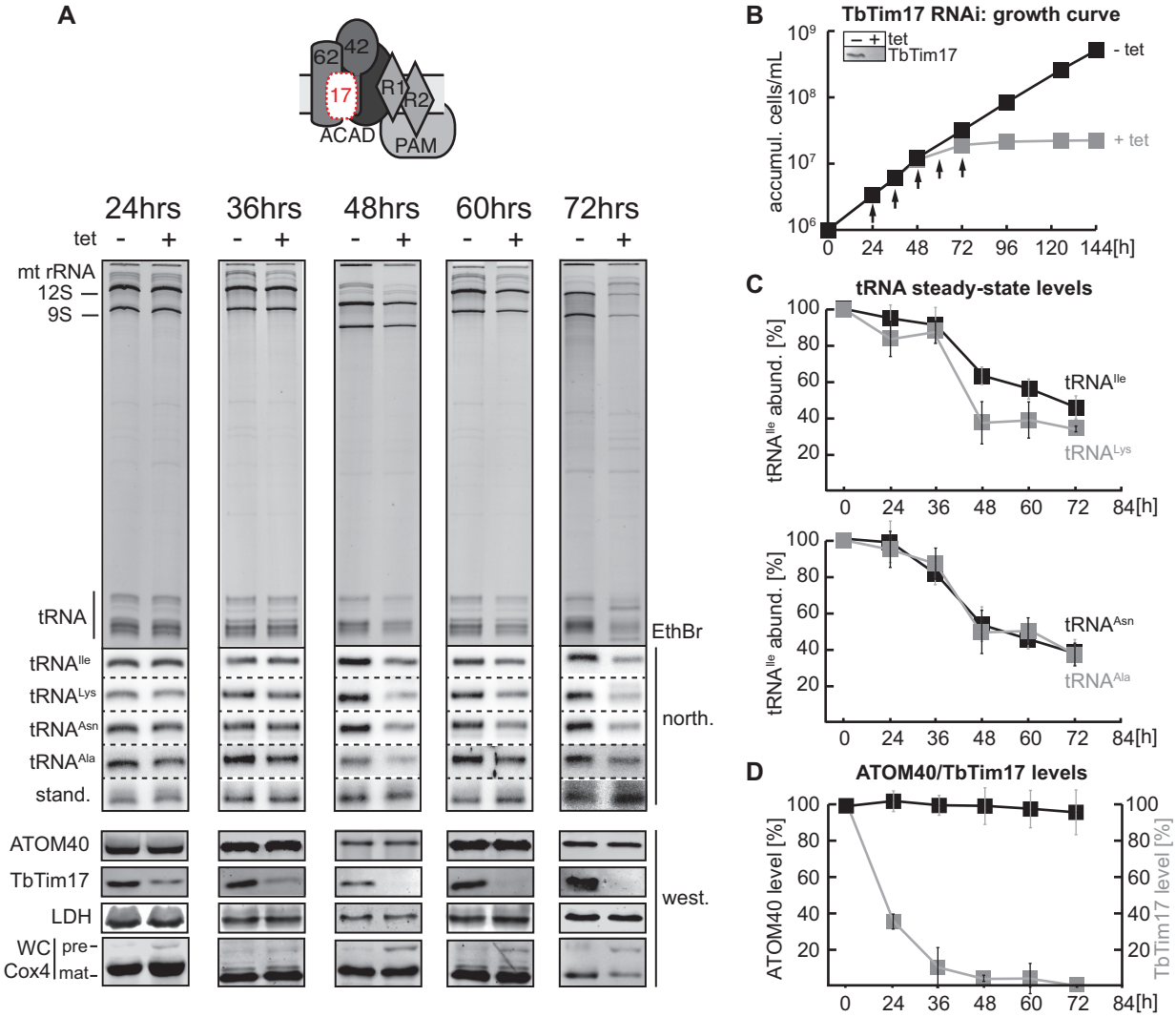

Figure 2. Ablation of TbTim17 inhibits import of tRNAs across both membranes. (**A**) Similar as in Figure 1A, but the sketch illustrates the trypanosomal TIM complex and its subunits with TbTim17 targeted for knockdown by RNAi (red dashed line). The TbTim17 time course experiment consists of the same set-up as described above (Figure 1A) showing mitochondrial RNA extracts separated in denaturing 8M urea/10% (w/v) PAGE visualized by EthBr, the corresponding northern blots (north.) probed for $tRNA^{Ile}$, $tRNA^{Lys}$, $tRNA^{Asn}$, $tRNA^{Ala}$ and the internal standard (stand.) as well as the western blot (west.) panels probed for ATOM40, LDH and TbTim17 and Cox4 precursor accumulation on WC protein samples. (**B**) Growth curve of TbTim17 RNAi cell line (as in Figure 1B). (**C**) Quantification of mitochondrial tRNA steady-state levels for the aforementioned species and (**D**) mitochondrial ATOM40/TbTim17 levels from panel (A). The error bars represent the standard deviation of the mean.

strongly suggesting that ACAD is involved in tRNA import. A recent study furthermore tested the involvement of the four predicted TMD-containing TbTim62 in tRNA import and showed that its ablation also reduced the steady state levels of mitochondrial tRNAs (48). For both, TbTim42 and ACAD, precursor accumulation starts to manifest concomitantly with the appearance of the growth phenotype (Figure 3B and D) and substantiates that tRNA import and protein import decline roughly simultaneously. However, we observed that in the TbTim42 and ACAD RNAi cell lines TbTim17 levels were also time dependently downregulated (Figure 3A and B), which is in-line with previous findings for TbTim42 (33). The same has also been observed for TbTim62 (41). Thus it appears that the four TIM subunits TbTim17, TbTim42, ACAD and TbTim62 form an interdependent integral membrane core of the single trypanosomal TIM complex that is needed for tRNA translocation across the IM. However, it is not possible to determine whether the tested TIM subunits are directly involved in mitochondrial tRNA import or whether the import inhibition is indirectly caused by the downregulation of TbTim17.

The two rhomboid-like proteins are dispensable for tRNA import

The trypanosomal TIM complex contains two more essential proteins required for mitochondrial protein import that each have multiple predicted TMDs: the two rhomboid-like proteins TimRhom I and TimRhom II. However, these two proteins are only associated with the presequence translocase and not found in the carrier translocase (33). Moreover,

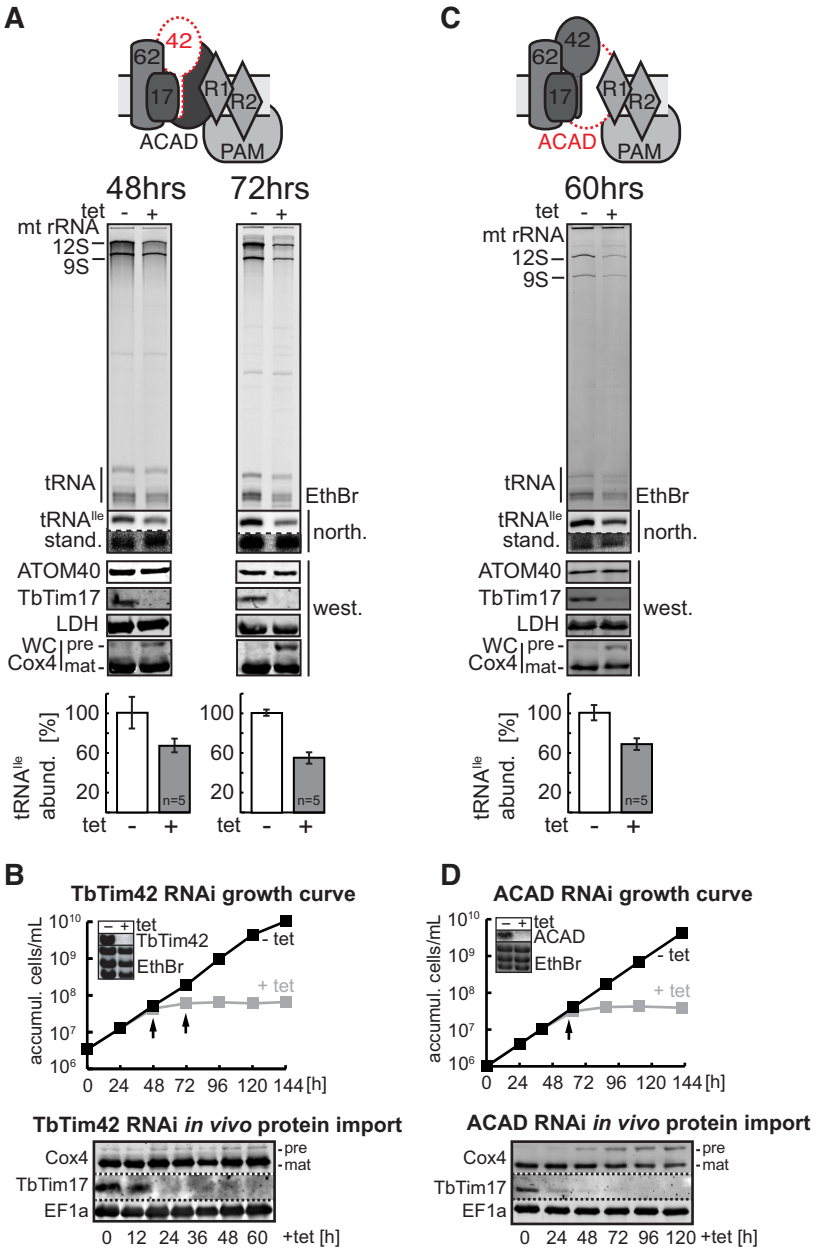

Figure 3. The integral membrane core of the TIM complex is required for tRNA import. (**A**) As in Figure 2A, but the sketch illustrates the trypanosomal TIM complex with the RNAi targeting TbTim42 (red dashed line). Two time points are shown (48 and 72hrs post-induction) depicting EthBr gels of mitochondrial RNA and the corresponding northern (north.) and western (west.) blots as described above. The graph below illustrates the abundance (abund.) of the mitochondrial steady-state levels, exemplified by $tRNA^{Ile}$ quantification for uninduced (-tet) and induced (+tet) cells. The error bars represent the standard deviation of the mean, n is the number of biological replicates. (**B**) Growth curve of the TbTim42 knockdown cell line uninduced (–tet) and induced (+tet) for RNAi. The inset shows the TbTim42 mRNA levels upon RNAi with the cytosolic rRNAs stained by EthBr serving as a loading control. The arrows indicate the two time points analyzed in (A). Immunoblots below the growth curve panel show a time course of WC protein samples probed for Cox4 to indicate precursor accumulation (pre) and mature (mat) protein and cellular TbTim17 levels. Cytosolic eEF1a serves as loading control. (**C**) Same as in (A) but for an ACAD knockdown cell line and a single time point 60 h post-induction as indicated by the arrow in the growth curve panel (**D**) for the ACAD RNAi cell line. This panel also shows the verification for the ACAD knockdown (inset) and the cellular protein levels of Cox4 (pre and mat), TbTim17 and eEF1a as described in (B), but for the ACAD RNAi cell line.

ablation of the two proteins causes an earlier and stronger growth phenotype than has been seen for all other tested translocase subunits. In order to test whether the TimRhom I and TimRhom II are required for tRNA import we analysed the steady state levels of mitochondrial tRNAs 42h post-induction which is 20 hours after the appearance of the growth arrest. The result shows that even at this relatively late time point, knockdown of the rhomboid-like proteins did not significantly change mitochondrial tRNA steady-state levels in either of the two cell lines, as evident from the EthBr-stained RNA gels and the representative quantification of the northern blots for $tRNA^{Ile}$, $tRNA^{Lys}$ and $tRNA^{Asn}$ (Figure 4A and B). This indicates that the two proteins are not involved in mitochondrial tRNA import. In line with this observation, knockdown of the TimRhom I and TimRhom II only marginally influenced TbTim17 levels (Figure 4B and D) and ∼70% and ∼80% of TbTim17 was still present 42 h post-induction. This suggests that the two rhomboid-like proteins form a TIM complex submodule that is peripherally associated with the integral membrane core of the TIM complex. Thus, while ablation of either TimRhom I or TimRhom II affects import of the presequence-containing protein Cox4, it does not inhibit mitochondrial tRNA import.

The PAM module is dispensable for tRNA import

As might be expected, the presequence translocase of *T. brucei* is associated with a PAM module. Recent work has identified two of its subunits (44). The essential integral membrane protein TbPam27, which despite having a J domain is not a homologue but rather a functional analogue of the yeast PAM subunit Pam18. Moreover, it has experimentally been verified that at least a fraction of mHsp70 of trypanosomes performs an essential function in the trypanosomal PAM (44). We therefore investigated whether TbPam27 and mHsp70 would be required for mitochondrial tRNA import using inducible RNAi cell lines (Figure 5). In both cases the induced cells abruptly stop growing after 24 h and Cox4 precursor accumulation was observed, which is indicative of an inhibition of mitochondrial protein import (Figure 5B and D). In Figure 5A, the mitochondrial tRNA steady-state levels in the TbPam27 RNAi cell line were analysed for two time points. At 24 h post-induction, when the growth arrest becomes apparent, these remained unchanged (Figure 5A). Moreover, even 48 h post induction, 24 h after the appearance of the growth phenotype, tRNA steady-state levels are only marginally reduced to ∼80%. For the PAM subunit mHsp70, which is conserved in all eukaryotes, the results look essentially identical for both time points (Figure 5C). This is surprising since we have previously reported that mHsp70 might be involved in mitochondrial tRNA import (47). However, it should be considered that in the previous study we scored reduction in the levels of a newly synthesized imported tRNA rather than the steady state levels of bulk tRNA (47). While looking at newly synthesized tRNA is the most sensitive way to analyse mitochondrial RNA import, it is also most prone to pleiotropic effects. *T. brucei* has a single mHsp70 only, which is expected to be involved in multiple essential functions (55), such as protein import, protein refolding, FeS-cluster biogenesis and maintenance of the mitochondrial DNA (56). It is therefore possible that its ablation slightly and likely indirectly affects mitochondrial tRNA import in a way that can be measured when selectively looking at newly synthesized tRNAs but not by the less sensitive but more robust assay used in the present work.

In summary these results show that two essential factors of the PAM module that are required for membrane translocation of presequence containing proteins are dispensable for mitochondrial tRNA import.

DISCUSSION

The single trypanosomal TIM complex consists of two heterooligomeric submodules which are found in both the presequence and the carrier translocase: the integral membrane core (consisting of TbTim17, TbTim42, TbTim62, ACAD) and peripheral hexameric complexes of six small Tim proteins (33,42,43). Moreover, the TIM complex contains two further submodules that are specifically associated with the presequence translocase: the TimRhom I and TimRhom II submodule and the PAM composed of TbPam27 and mHsp70 (33,44). The subunits of each TIM submodule form a highly interdependent network. It is therefore difficult to assign specific functions to specific submodule components. However, since the submodules are partially independent, each can be knocked down without immediately leading to the collapse of the others. Taking this into account we arrive at the following conclusion: only the integral membrane core is required for mitochondrial tRNA import. This is also supported by previous studies on the subject (48,57). The rhomboid submodule and the PAM on the other hand are both dispensable for the process. The peripheral small Tim submodule was not tested in the present study.

What does this reveal about the mechanism of tRNA import across the IM? The trypanosomal protein import system has been analysed in quite some detail during recent years which allows for comparative analysis of protein and tRNA import (25–27,58). All four subunits of the integral membrane core of the TIM complex are essential for both protein and tRNA import. TbTim17 is an orthologue of yeast Tim22 and therefore likely forms the protein-conducting pore in the complex. Whether this is also the case for tRNA import is not known but the similar kinetics of inhibition of protein and tRNA import after ablation of TbTim17 are at least consistent with this idea.

tRNAs need to be imported across both membranes into the mitochondrial matrix. Thus, the final destination of tRNAs is identical to soluble matrix proteins, which are exclusively imported by the mitochondrial presequence translocase. The fact that tRNA import does neither require TimRhom I and TimRhom II nor the PAM, the two modules specifically associated with the trypanosomal presequence translocase, is therefore unexpected. It indicates that even though tRNA and protein import both depend on the integral membrane core of the TIM complex the two processes must operate by different mechanisms. These results are reminiscent to what was observed for the ATOM complex in the OM (31). Electrophysiological studies and *in vivo* blocking of the ATOM40 channel indicate that the beta-

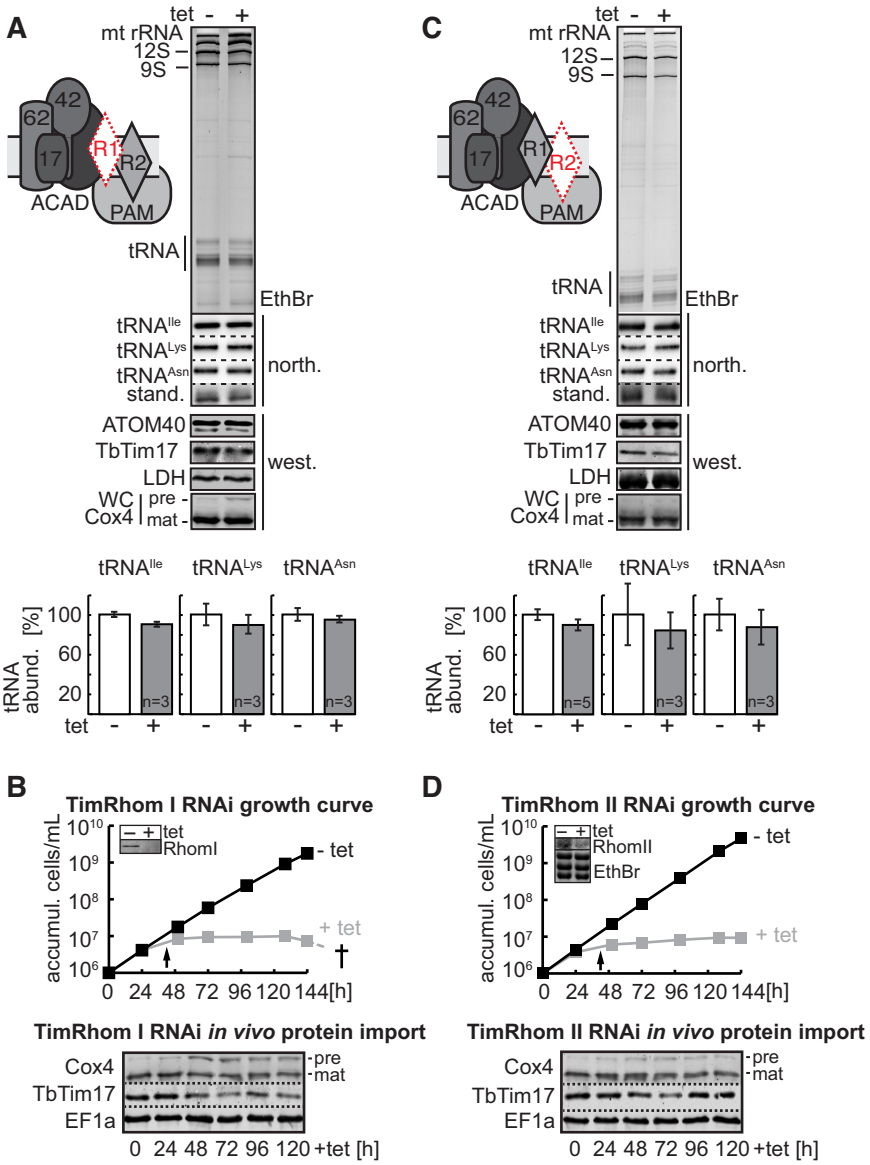

Figure 4. The two rhomboid-like proteins are dispensable for tRNA import. (**A**) and (**C**) As in the previous Figures, the sketch depicts the trypanosomal TIM complex and the knockdown of TimRhom I (R1) or TimRhom II (R2) symbolized by the red dashed line. The panels further show the EthBr gels of mitochondrial RNA for both RNAi cell lines 42hrs post-induction followed by the corresponding northern blots (north.) probed for $tRNA^{Ile}$, $tRNA^{Lys}$, $tRNA^{Asn}$ and the internal standard (stand.), as well as the respective immunoblot panels (west.) probed for ATOM40, LDH and TbTim17 and Cox4 precursor accumulation (pre) on WC protein samples. The graphs at the bottom of (A) and (C) show the quantification of $tRNA^{Ile}$, $tRNA^{Lys}$ and $tRNA^{Asn}$ as a proxy for mitochondrial tRNA steady-state levels. The error bars represent the standard deviation of the mean (n, number of biological replicates). (**B**) and (**D**) show the growth curve panels for TimRhom I or TimRhom II with the arrow indicating the time point analyzed in (A) and (C) and the insets showing the validation of the knockdown for TimRhom I and TimRhom II by western and northern analysis, respectively. The panel below the growth curves show the cellular protein levels of Cox4 (pre and mat), TbTim17 and eEF1.

barrel protein functions as a pore for both tRNA and protein import (31,59). Moreover, there is evidence that tRNAs directly bind to ATOM14 (31). Ablation of ATOM11 and ATOM12 also interferes with tRNA import possibly indirectly as the three subunits are required for the stability of the complex. The protein import receptors ATOM46 and ATOM69, however, while being essential for protein import are not required for tRNA import (31). These results exclude both the 'direct import model', since tRNAs are clearly translocated across the protein import pore, as well as the 'co-import model', because tRNA import could be uncoupled from protein import. It led us to propose a novel model termed 'alternate import model' in which tRNAs are imported across the OM along the protein import pathway but independent of ongoing protein import (31). Thus, an individual ATOM complex is either engaged in tRNA or in protein import, but not in both processes simultaneously.

Based on our results with the trypanosomal TIM complex presented in this study, the 'alternate import model' can now be extended to the MI, since here also tRNA import can be uncoupled from import of matrix proteins. Thus, unlike suggested for import of the $tRNA^{Lys}$ in yeast (19), try-

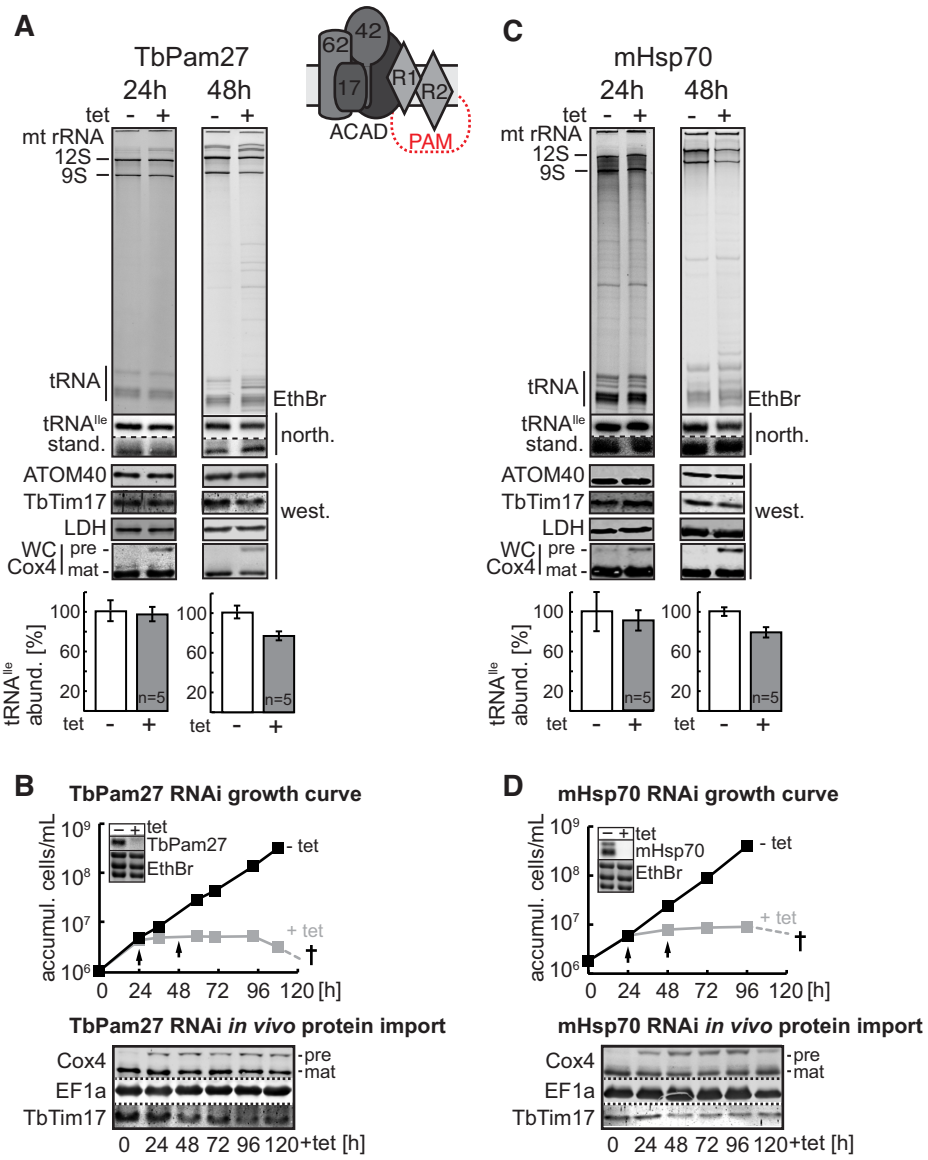

Figure 5. The PAM module is dispensable for tRNA import. The sketch depicts the trypanosomal TIM complex, with the PAM module knocked down (red dashed line) by RNAi directed against TbPam27 (**A**) or mHsp70 (**C**). For both proteins two time points were analyzed, 24 and 48 h post-induction. The northern blot panel (north.) immediately below the EthBr gels show the abundance of endogenous $tRNA^{Ile}$ in the mitochondrial RNA fractions and the synthetic RNA oligomer (stand.) as control. The immunoblot panel (west.) further below shows the abundance of endogenous ATOM40, TbTim17 and LDH in the mitochondrial fraction and Cox4 precursor (pre) and mature (mat) protein forms in the whole cell (WC) sample. The graph at the bottom shows the abundance of mitochondrial $tRNA^{Ile}$ quantified from five biological replicates ($n = 5$) as depicted in (A) and (C). The error bars represent the standard deviation of the mean. The corresponding growth curves for RNAi against TbPam27 (**B**) and mHsp70 (**D**) are shown with the insets confirming mRNA knockdown via northern blot with cytosolic rRNAs as loading control. Below the growth curves cellular protein levels of Cox4 precursor (pre and mat), TbTim17 and eEF1 are indicated.

panosomal tRNAs can be imported into the matrix across both the OM and the IM without prior binding to a mitochondrial precursor protein that would also be imported. A summary of these results and a scheme of the model is depicted in Figure 6.

Posttranslational import of mitochondrial proteins requires unfolding of the transported substrate protein (20,21). ATP hydrolysis by mHsp70 of the PAM module of the TIM generates the force to unfold and translocate proteins into the matrix of mitochondria, although by which exact mechanism is still being debated (60). Since the PAM is not required for tRNA import, this raises the question regarding the driving force for mitochondrial tRNA import. It was shown that in yeast the $tRNA^{Lys}$ is likely imported as a folded molecule (61), which is line with fact that the import pores of the yeast TOM and TIM complex can accommodate double stranded nucleic acids (62). Thus, unlike in the case of proteins, no energy would be required for unfolding of tRNAs. Moreover, different to most imported proteins, all imported tRNAs are dually localized to the cytosol and the mitochondrion. Thus, in *T. brucei* all mitochondrial tRNAs derive from a fraction of 1–10% of

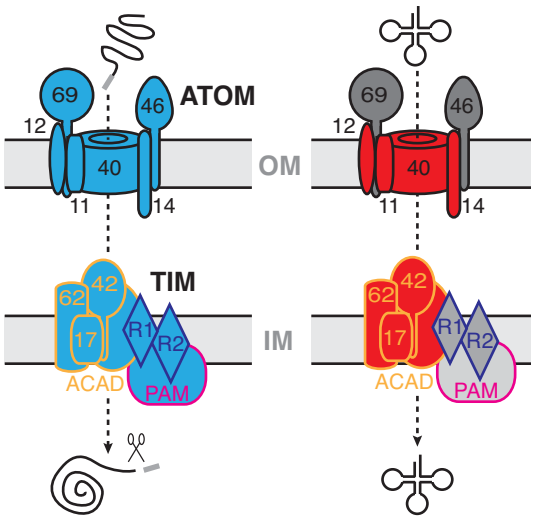

Figure 6. Model for macromolecular transport into mitochondria of *T. brucei*. The sketch depicts on the left the machinery required for the import of presequence containing polypeptides colored in blue. For the mitochondrial outer membrane (OM) this is the ATOM complex consisting of ATOM11, ATOM12, ATOM14, ATOM40 and the protein receptors ATOM46 and ATOM69. For the mitochondrial inner membrane (IM) this is the trypanosomal TIM complex consisting of the integral membrane core module TbTim17, TbTim42, TbTim62 and ACAD (golden frame and letters), the two rhomboid-like proteins TimRhom I and TimRhom II (R1 and R2, dark blue) and the presequence-associated motor submodule (PAM, cyan). The N-terminal presequence is cleaved upon import (indicated in gray). The peripheral small Tim submodule is omitted for clarity. The panel on the right depicts the components required for tRNA translocation colored in red. The dispensable submodules are shaded in gray.

cytosolic tRNAs that are imported into mitochondria (53). Since the volume of the mitochondrion in procyclic *T. brucei* amounts to 10–25% of the total cellular volume this means that the concentration of tRNAs in the matrix of the mitochondrion is lower than in the cytosol (53,63). It is therefore, in principle, possible that import of tRNAs while depending on protein import pores is driven by the concentration gradient. Should this be the case, we would expect that translocation across the OM and IM should be coupled, which is indeed the case as shown in the present study.

It has previously been shown that addition of uncouplers inhibits import of a newly synthesized tRNA in intact *T. brucei* cells indicating that the membrane potential is required for mitochondrial tRNA import (64). This seems counterintuitive since the polarity of the membrane potential would impede import of polyanionic tRNAs. However, it is possible that the negative charges of tRNAs are shielded by magnesium ions (65) which would alleviate the expected inhibitory effect of the potential. Moreover, in yeast the membrane potential has two distinct roles in mitochondrial protein import (7). It exerts an electrophoretic effect on positively charged presequences or other positively charged segments of imported proteins. This can obviously not be the role it plays in tRNA import. The other role the membrane potential has, is to activate the TIM23 or TIM22 protein translocases. It is possible that such an activation step of the TIM complex is also required for mitochondrial tRNA import in *T. brucei*. Whether *in vivo* import of tRNAs in *T. brucei* also requires ATP is unknown at present, since this is experimentally difficult to study as abolishment of the membrane potential simultaneously stops mitochondrial ATP production.

In summary, the ATOM–TIM supercomplex of *T. brucei* is the first example of a versatile macromolecule translocase, that transports either proteins or RNAs, likely through the same import pores, across the OM and IM membranes. Unexpectedly, it uses different mechanisms to do so. tRNA import has experimentally been studied in a few systems only (8–10,66), it therefore remains unclear whether the 'alternate import' model is a special case or whether this type of mechanism might be more widespread. But since in *T. brucei* tRNA import mainly depends on the few conserved subunits of ATOM and TIM complexes found in all eukaryotes, it is possible that mitochondrial tRNA import in other species (e.g. *Tetrahymena*, *Algae*, Apicomplexans etc.) might also work according to the 'alternate import model'. Moreover, it is important to note that the evidence for the co-import model in yeast, while convincing, is still indirect and that there is no experimental proof that the $tRNA^{Lys}$ is indeed imported in a complex with the precursor of mitochondrial lysyl-tRNA synthetase. Thus, even for yeast, the alternate import model cannot completely be excluded.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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