

PROTEIN AND TRANSFER RNA ARE IMPORTED INTO THE MITOCHONDRIA
OF TRYPANOSOME BRUCEI BY USE OF A SHARED MITOCHONDRIAL
MEMBRANE PROTEIN COMPLEX

by

DAVID SEIDMAN

(Under the Direction of Dr. Stephen L. Hajduk)

ABSTRACT

Unlike most other eukaryotic organisms, the mitochondrial genome of *Trypanosoma brucei* does not contain genes coding for tRNAs; nuclear encoded tRNAs must be imported in order to maintain active translation within the mitochondria. Translocation of tRNA into the mitochondria requires ATP hydrolysis, an active membrane potential as well as proteinaceous membrane components. Although much is known about the biophysical requirements for mitochondrial tRNA import, very little is known about the proteins that are involved in this translocation process. Similar to tRNA import, the identity of the mitochondrial protein import machinery of *Trypanosoma brucei* is unknown as the canonical Translocase Outer Membrane and Translocase Inner Membrane have yet to be identified. Through the work presented in this dissertation, a mitochondrial protein complex that is functional in the translocation of tRNA and protein into the mitochondria has been identified. By use of affinity purification and mass spectrometry, we were able to identify mitochondrial membrane complexes that

specifically bind tRNA. Candidate proteins were then characterized for their role in mitochondrial tRNA import by use of an *in vivo* tRNA import assay in tandem with RNA interference for specific candidates. Dual affinity tagged proteins involved in tRNA import were pulled down and their binding partners were identified by mass spectrometry. From this analysis, we were able to identify shared components between tRNA and protein import machinery (Tim17, mHSP70, mHSP60 and mHSP20). Further analysis of the complex by *in vivo* protein import assays, showed that the identified protein complex is required for both the mitochondrial import of tRNA and protein. In addition, we are also able to show that mitochondrial tRNA and protein import utilize the same biophysical requirements for import. These results suggest that tRNA and protein are imported into the mitochondria of *Trypanosoma brucei* by use of the same mitochondrial membrane protein complex and that this pathway may represent a universal import mechanism for all eukaryotic organisms.

INDEX WORDS: *Trypanosoma brucei*, Mitochondrial tRNA Import, Mitochondrial Protein Import

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David Seidman

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by

David Seidman

Major Professor: Stephen L. Hajduk

Committee: Robert Sabatini
Michael Terns
Ron Orlando
Zac Wood

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2012

DEDICATION

To my wife.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The Disease

Trypanosoma brucei brucei is the causative agent of African sleeping sickness in primates and Nagana (wasting disease) in non-primates. This parasitic protist is divided into three main sub-species; *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* are responsible for the primate sleeping sickness infection, while *T. brucei brucei* causes the non-primate infections. The parasite is spread from the bite of the tsetse fly (*Glossina, spp.*) an insect vector to the host. In the advanced stage of infection, *T. brucei* is able to cross the blood brain barrier where it then invades the central nervous system resulting in numerous neurological symptoms and eventually results in coma. If left untreated, all cases of infection become fatal. As of 2009, the number of reported new cases of human African trypanosomiasis (HAT) had dropped below 10,000 for the first time in over 50 years, though the true number is believed to be significantly higher due to unreported cases (1). Although the number of new cases in humans has dropped, this is very similar to decline in cases seen during the 1970's, which was followed by the reemergence of high levels of infection during the 1990's (2). Trypanosomiasis also poses a major socioeconomic problem, causing an estimated loss of \$4.75 billion due to the loss of livestock and land where infected tsetse flies inhabit (3).

Life Cycle and Mitochondrial Biogenesis

The life cycle of *T. brucei* consist of multiple life stages that are each unique to the developmental cycle of the parasite. As the parasite progresses throughout the life cycle from the blood stream (BS) infective stage to the procyclic (PC) insect form, many morphological and biochemical changes occur including, but not limited to the repositioning of the kinetoplast, changes in cellular shape and size, lose of the variable surface glycoprotein (VSG) antigen coat, a reduction in endocytosis and the loss of host infectivity (4-8). During the differentiation process, the single mitochondrion of *T. brucei* undergoes the most dramatic changes in structure and metabolism (Figure 1.1). While in the PC life stage, the mitochondrion of *T. brucei* forms a large branched, plated cristae structure, which encompasses approximately 25% of the cells total volume. Whereas, the mitochondrion of the BS form has a relative cellular volume of 5%, forming a non-branching tubular cristae (4, 9). Changes in mitochondrial volume and structure correlate with changes in cellular metabolism and mitochondrial protein content. Studies examining nuclear encoded proteins that are imported into the mitochondria have shown that expression levels are down regulated anywhere from 10-100 fold in the BS forms (10-14). Transcription, mRNA stability and protein levels of components essential for oxidative phosphorylation and electron transport are developmentally regulated during differentiation from the PC to BS form (4, 10, 11, 15, 16). The developmental regulation of components required for mitochondrial biogenesis in BS forms leads dramatic changes in energy metabolism and mitochondrial function. Where the PC form utilizes oxidative phosphorylation to generate energy from amino acid sources, the BS generates energy through the non-cytochrome mediated oxidation of glucose by use glycolysis (17, 18).

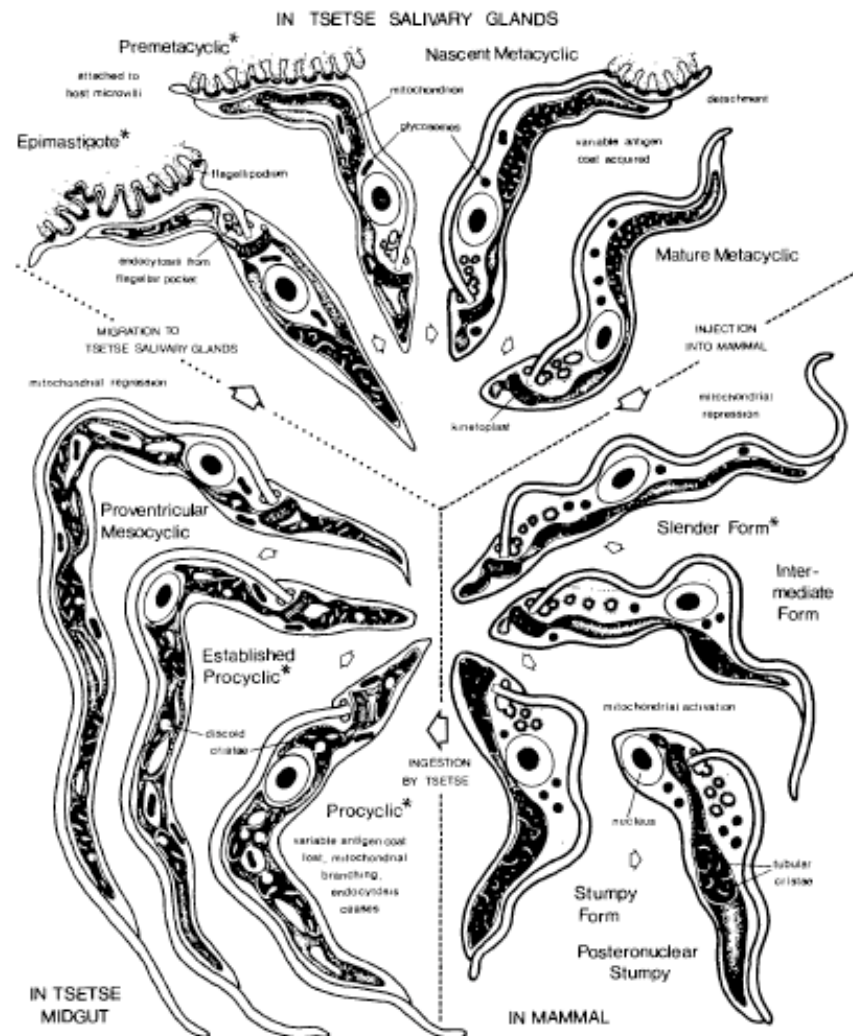


FIGURE 1.1 Developmental life cycle of *T. brucei*, highlighting mitochondrial biogenesis, morphological and biochemical changes. When in the mammal, the bloodstream (BS), “slender” form contains a long, tubular mitochondrion that is repressed in oxidative phosphorylation. As the BF form transition to the procyclic (PC) stage in the tsetse midgut, the mitochondrion is enlarged, forming branched cristae and is active in oxidative phosphorylation. The PC then transitions from the tsetse midgut to the salivary glands metacyclic stage, where the mitochondrion regresses in size and structure. Finally, when a mammalian host is bitten by the tsetse, the metacyclic form is injected into the bloodstream of the host, where it then transitions to the BS, completing the life cycle. BS and PC represent stages, which can be maintained in culture outside of the tsetse. * Dividing life stages. (Adapted from Vickerman, *Br Med Bull* 1985)

Further, in absence of a functional electron transport chain in BS forms, mitochondrial membrane potential is maintained by an alternative function of the ATP-synthase complex, which has been proposed to hydrolyze ATP in order to pump protons across the membrane, generating the potential (19, 20). Although there are many morphological and biophysical changes to the BS mitochondrion, mitochondrial translation, maintenance of mitochondrial DNA as well as protein and tRNA import are required in order for the BS form to continue progression through the life cycle (21, 22).

Trypanosoma brucei Mitochondrial Genome

The mitochondrial DNA (mtDNA) of *T. brucei* is unique. Much like most other eukaryotic organisms, the mitochondrial genome of *T. brucei* is a circular DNA and contains a minimal set of genes required for mitochondrial biogenesis. However, there are many factors that differentiate the mitochondrial genome of *T. brucei* from that of other eukaryotic organisms. Trypanosomes are of the class *Kinetoplastida*, for which they were initially characterized based on their mtDNA arrangement, which forms a structure termed the kinetoplast (23). The kinetoplast (kDNA) is disk-like structure that is formed by a network of concatenated DNA mini and maxicircles (Figure 1.2). Each cell contains from 5,000-10,000 minicircles and approximately 50 maxicircles (5). Minicircles encode for guide RNAs, which are functional in mitochondrial RNA editing (24, 25). Whereas the maxicircles resemble mtDNA from most other eukaryotic organisms in that they contain the genes that encode for proteins that are functional in electron transport as well as rRNA (Figure 1.3).

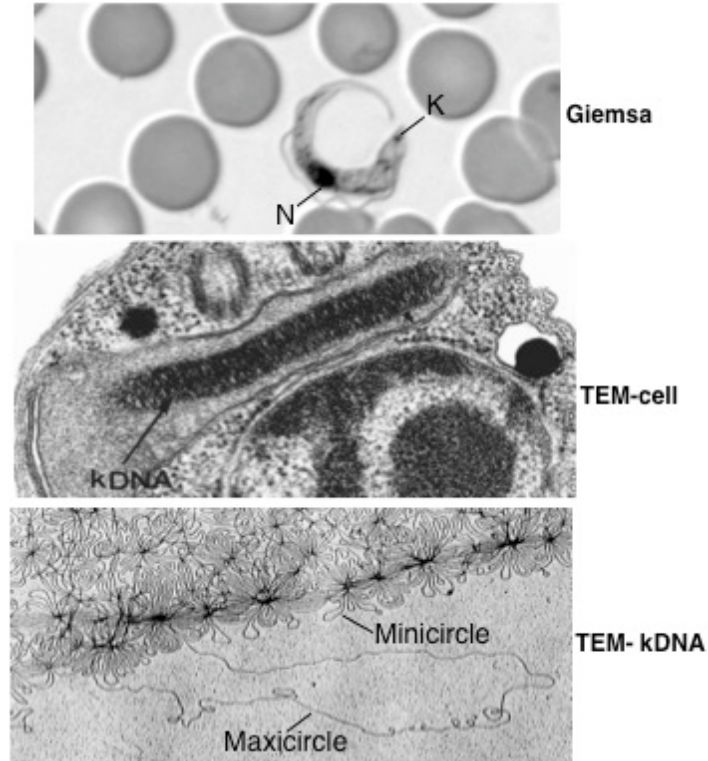


FIGURE 1.2. Mitochondrial DNA of *T. brucei*. mtDNA forms organized network (Top panel) that forms a disk like structure (Middle panel). N, Nucleus; K, Kinetoplast/kDNA. kDNA is formed by a network of concatenated Mini- and Maxicircles (Bottom panel). (Images courtesy of Stephen L. Hajduk).

There are numerous characteristics that make the maxicircle genome unique from other eukaryotic mtDNA. First, many gene transcripts must be extensively modified by RNA editing in order to create the appropriate open reading frames (26, 27). Second, most other eukaryotic organisms encode for a minimal, yet complete set of tRNAs on their mtDNA, however, this is not the case for the kinetoplastid or apicomplexa protozoa

as there are no tRNA genes encoded by the maxicircle. To make up for the lack of tRNA genes, all of the tRNAs found within the mitochondria are nuclear encoded and must be imported from the cytosol (28-31). It has been estimated that ~5% of the entire cellular pool of tRNA is imported into the mitochondria, with each individual tRNA species mitochondrial level ranging from ~1-8% (29, 32). As the mitochondrial genome of *T. brucei* is limited in the number of encoded genes, the mitochondrion must import nuclear encoded proteins and RNAs in order to maintain its normal function.

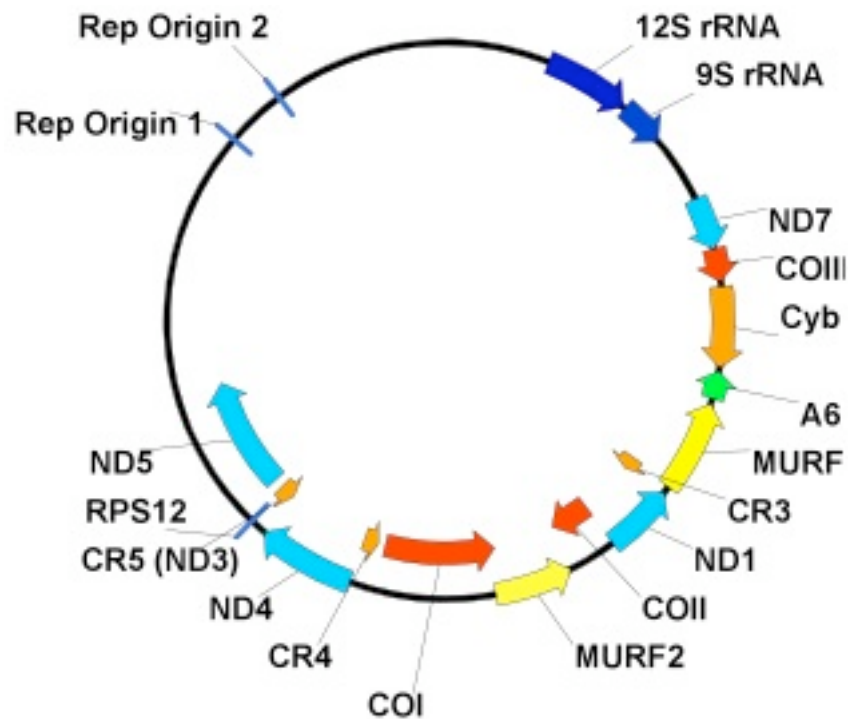


FIGURE 1.3. Maxicircle genome of *T. brucei*. Gene arrangement resembles that of other eukaryotic organisms, including genes encoding for rRNAs and a limited number of electron transport chain protein components. To date, no genes encoding for tRNAs have been identified on the maxicircle.

Mitochondrial RNA Import

Although kinetoplastids and apicomplexa must import all of their tRNAs from the cytosol in order to maintain normal mitochondrial function, most other eukaryotic organisms import tRNA to some degree. Mitochondrial tRNA import was initially described over 40 years ago in *Tetrahymena pyriformis*, but the diversity and mechanisms of import were not realized until decades later (33). In *S. cerevisiae* and in humans, it has been shown that 1-2 tRNAs are imported into the mitochondria, whereas *T. pyriformis*, an organism which only encodes 7 tRNAs on its genome, imports at least 15 tRNAs (34-37). In almost every case, the nuclear encoded tRNA that is imported into the mitochondria provides for a required codon that is not found within the mitochondrial encoded tRNA pool. Further, eukaryotes import numerous other RNA species into their mitochondria, including 5S rRNA, micro RNAs and the RNA components of RNase P and RNase MRP (35, 38-45). Most of these RNAs are large, negatively charged macromolecules that must be translocated into the mitochondrial matrix against the membrane potential. Although there is much diversity among all eukaryotic organisms, by closer examination, the mechanisms for RNA import appear to be similar.

tRNA Import in *S. cerevisiae* – The mechanisms for tRNA import have been thoroughly described in *S. cerevisiae* over the past two decades (34, 46-51). Although the mitochondrial genome of *S. cerevisiae* encodes for a complete set of tRNA, studies have found that two different tRNAs are imported into the mitochondria. Each of these two tRNAs are imported into the mitochondria by different mechanisms.

The first tRNA that was described as being imported into the mitochondria of *S. cerevisiae* was tRNA^{Lys} (CUU) (52, 53). Through these experiments, nuclear encoded tRNA^{Lys} (CUU) was identified as the only tRNA within the mitochondrial pool that was not encoded by mtDNA. The mitochondria of *S. cerevisiae* encodes for tRNA^{Lys} (UUU), which can be thiolated at its wobble base pair and accommodate for the non-mitochondrial encoded CUU lysine anticodon. However, when the import of tRNA^{Lys} (CUU) is impaired, it was found that mitochondrial translation of mRNA substrates containing the AAG codon were significantly inhibited at higher temperatures (37°C). This was shown to be due to the inability to thiolate mitochondrial encoded tRNA^{Lys} (UUU) at its wobble base pair, inhibiting its ability to recognize the AAG codon (54). In addition to demonstrating that the nuclear encoded tRNA^{Lys} (CUU) is essential to mitochondrial function, the mechanism by which tRNA^{Lys} (CUU) is imported has been thoroughly described by the use of both *in vitro* and *in vivo* tRNA import assays (Figure 1.4A).

tRNA^{Lys} (CUU) was shown to be specifically imported into the mitochondria by use of an *in vivo* import system in which labeled tRNA substrates were electroporated into intact *S. cerevisiae* cells and mitochondrial import was monitored. From this assay, it was found that from the pool of over 20 different tRNAs that were used, only tRNA^{Lys} (CUU) was imported into the mitochondria (48). *In vitro* import of tRNA^{Lys} (CUU) was found to require ATP, an active mitochondrial membrane potential and mitochondrial membrane protein components (47, 48). In order for the tRNA to be

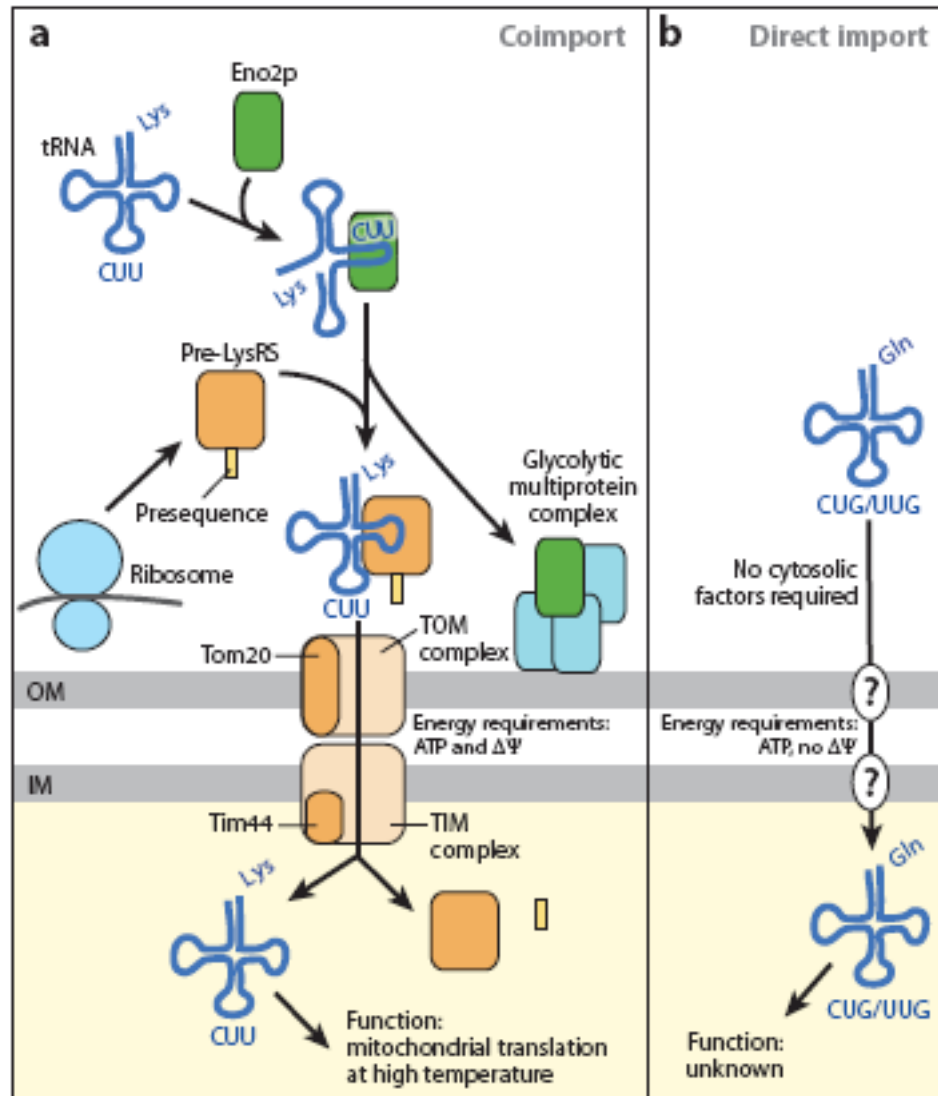


FIGURE 1.4 Mechanisms for tRNA import in *S. cerevisiae*. (A) Import pathway for tRNA^{Lys} (CUU). Requires cytosolic cofactors (Eno2p), protein chaperone (Pre-LysRS), components of the protein import machinery as well as ATP and Membrane potential. tRNA^{Lys} (CUU) is essential for translation at high temperatures (37°C) (B) Import pathway for tRNA^{Gln} (CUG/UUG). Proteins required for tRNA import have not been characterized. Requires ATP hydrolysis but does not require mitochondrial membrane potential. Unknown mitochondrial function (Adapted from Schneider, *Annu. Rev. Biochem* 2011).

imported into the mitochondria of *S. cerevisiae*, it was found that two nuclear encoded proteins aid in the targeting and translocation steps.

The first factor required is the cytosolic localized glycolytic enzyme enolase (Eno2p). Eno2p is a soluble cytosolic factor that functions in the final step of glycolysis and also associates at the surface of the mitochondrial membrane (50). However, it was found that only the soluble, non-mitochondrial associated form of the Eno2p enzyme binds and directs aminoacylated tRNA^{Lys} (CUU) to the mitochondria. It has been postulated that Eno2p then transfers tRNA^{Lys} (CUU) to the second nuclear encoded component required for tRNA import, the precursor of mitochondrial lysyl-tRNA synthetase (preMsk1p) (51). The tRNA^{Lys} (CUU) – preMsk1p complex is then co-transported across the mitochondrial membrane, into the matrix. Using an *in vitro* import assay, this tRNA translocation was shown to require ATP, an active mitochondrial membrane potential as well as functional components of the mitochondrial membrane protein import machinery (46, 47). Through a series of genetic screens, as well as inhibition of components of the protein import channel, it was found that a component of the Translocase of the Outer Membrane (TOM), TOM20 as well as a component of the Translocase of the Inner Membrane (TIM), TIM44 are required for both mitochondrial tRNA and protein import *in vitro* (47, 51). How tRNA and protein are co-transported through the complexes has yet to be determined in *S. cerevisiae*, though it has been shown that the tRNA is not unfolded and that protein import channels have the ability to co-import precursor proteins bound to nucleic acid substrates (49, 55). Together, these results show that tRNA and protein import may be linked in *S. cerevisiae*.

The second tRNA imported into the mitochondria of *S. cerevisiae* is the tRNA^{Gln} (UUG and CUG) (Figure 1.4B) (34). Using *in vitro* and *in vivo* assays, these two nuclear encoded tRNAs were found to neither require cytosolic chaperones nor a mitochondrial membrane potential for import, suggesting that tRNA import was independent from mitochondrial protein import. Previously though, it had been shown that the only nuclear encoded tRNA that was found within the mitochondria of *S. cerevisiae* is the tRNA^{Lys} (CUU) (52, 53). Further, recent *in vitro* and *in vivo* studies examining the biogenesis and function of mitochondrial encoded tRNA^{Gln} (UUG) were unable to detect nuclear encoded tRNA^{Gln} (CUG) within the mitochondria of *S. cerevisiae*, but were able to detect the presence of nuclear encoded tRNA^{Lys} (CUU) (56). These findings show that tRNA^{Gln}(CUG) is not imported into the mitochondria of *S. cerevisiae*. The researchers were also able to show that tRNA^{Gln}(CUG) is not essential for mitochondrial biogenesis, further indicating that this tRNA is not imported. Through a series of genetic screens, the authors were able to show that in their system, a mitochondrial-encoded tRNA^{Gln} (UUG) is first mischarged by an imported glutamyl-tRNA synthetase to glutamate-tRNA^{Gln}. This process is then followed by a transamidation step to convert the charge to the correct glutamine-tRNA^{Gln}. Further, mitochondrial encoded tRNA^{Gln} (UUG) contains a cmnm⁵s²U modification at the first nucleotide position of the anticodon, which in turn allows for the recognition of both CAA and CAG codons (57, 58). By these processes, the mitochondrial encoded tRNA^{Gln} (UUG) is able to function in the recognition of CAG codons (56). Based on these findings, it is unknown what the function of nuclear encoded tRNA^{Gln} (CUG or UUG) would be within the mitochondria of *S. cerevisiae* as they should not be required for mitochondrial translation and have not been detected

within the mitochondria by multiple different studies (52, 53, 56). In summary, the mitochondrial import of tRNA^{Lys} (CUU) has been validated by multiple different studies and is functionally required within the mitochondria, whereas the import of tRNA^{Gln} remains questionable as further studies have shown that this tRNA is not present in the mitochondria, nor is it required for mitochondrial biogenesis.

tRNA Import in Plants – The import of nuclear encoded tRNAs from the cytosol into the mitochondria in plants has been difficult to study due to the diversity of imported tRNA substrates. Although closely related, each plant species has been found to not only import different tRNAs, but also differ in the number of nuclear encoded tRNAs that are imported (59). Based on this, extrapolating findings in one plant species to another may be difficult as each plant species may have developed their own mechanism for sorting and import. The mitochondrial genomes of plants have been found to vary in the number of encoded tRNA genes, ranging from 3 to 29 tRNA genes (60-62). The green alga *Chlamydomonas reinhardtii* represents the most extreme case in the plantae kingdom, as its genome was found to only encode 3 tRNAs, yet nearly 34 nuclear encoded tRNAs share a dual localization between the cytosol and mitochondria. Further, the steady state distribution of tRNAs correlates well with cytosolic and mitochondrial codon usage, suggesting there is a sorting mechanism that accommodates this (62, 63).

Thus far, the only plant system in which a mitochondrial membrane import system has been identified is *Solanum tuberosum* (Figure 1.5A) (64). For this organism, an *in vitro* import assay was developed, which allowed for the import of radiolabeled tRNA substrates into isolated mitochondria (65). From these studies, it was found that tRNA import required ATP, an active mitochondrial membrane potential as well as

mitochondrial membrane protein exposed to the cytoplasm. However, it must be noted that the specificity of the system was lacking as tRNAs that exclusively localize to the cytosol in plants were imported into the mitochondria in an ATP independent fashion *in vitro* (65). It is possible that this represents a flaw with the *in vitro* system as the bioenergetic requirements differed for mitochondrial targeted tRNAs and non-mitochondrial tRNAs. In lieu of this, it was found that tRNA import in *S. tuberosum* requires components of the protein import machinery in order to be translocated into the mitochondria, although not directly through the protein import channel (64). tRNA, as well as other nucleic acid substrates, were found to interact *in vitro* with the voltage dependent anion channel (VDAC) of *S. tuberosum*. To test for the function of the VDAC in tRNA import, antisera against the VDAC, as well as inhibitors (Ruthenium Red) of the channel were used to show that tRNA is imported through the VDAC of *in vitro* isolated mitochondria. To determine if components of the protein import machinery function in mitochondrial tRNA import, TOM20 and TOM40 antisera was used to test for tRNA VDAC binding and import *in vitro*. When either TOM20 or TOM40 antisera were used on isolated mitochondria, binding to the VDAC as well as tRNA import was inhibited *in vitro*. In light of this, the researchers propose that although tRNA binds to components of the TOM complex, it is not transported through the protein import channel, as inhibition of protein import does not inhibit mitochondrial tRNA import (64). It would be interesting to determine if other components of the TOM complex, such as if either of the other preprotein receptors (TOM22 and TOM70) function in the binding and or translocation of tRNA into the mitochondria. By pinpointing which receptors interact

with tRNA and protein, this may help elucidate the mechanisms by which these macromolecules are imported into the mitochondria of *S. tuberosum*.

RNA Import in Mammalian Systems – Studies examining mammalian mitochondrial RNA import have found that a variety of different RNA substrates are imported into the mitochondria, including 5S rRNA, tRNA, microRNAs as well as the RNA components of RNase P and RNase MRP (35, 38, 42-45). Much controversy surrounds the import of microRNAs as well as the RNA components of RNase P and RNase MRP. In the case of microRNAs, no study has been able to determine if these substrates are physically imported and function has yet to be determined (42, 43). Further, studies examining the activity of RNase P showed that it can function in an RNA independent fashion (66). The function of RNase MRP RNA has been questioned since the mitochondrial level of this RNA may not be sufficient to maintain function (39, 67). However, recent *in vitro* and *in vivo* studies have been able to show that depletion of a mitochondrially intermembrane space targeted enzyme polynucleotide phosphorylase (PNPASE), significantly inhibits the mitochondrial import of the RNA components of RNase MRP and RNase P, which resulted in an impairment of tRNA processing (45). PNPASE was also shown to be involved in the binding and import of the RNA components of RNase MRP and RNase P, as well as 5S rRNA. This import, monitored *in vitro* and *in vivo*, required ATP, mitochondrial membrane protein as well as the mitochondrial membrane potential (Figure 1.5B) (45). Examining the effect of PNPASE *in vitro* displayed that RNA import does not require cytosolic chaperones, in contrast to previous studies which have identified specific cytosolic factors required for 5S rRNA import (40, 44). In these *in vitro* studies, it was shown that both tRNA and 5S rRNA are imported

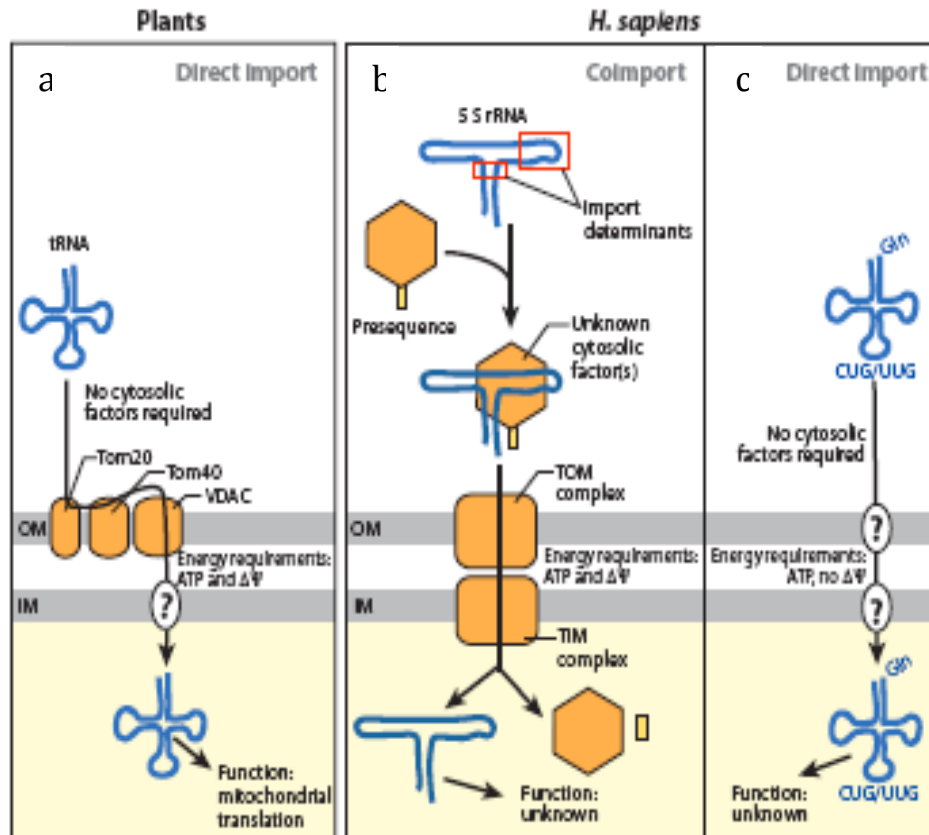


FIGURE 1.5 RNA import mechanisms in plants and *H. sapiens*. (A) tRNA has been found to be imported *in vitro* without cytosolic cofactors, but requires binding to components of the TOM complex as well as ATP hydrolysis and an active membrane potential. Imported tRNAs are required for mitochondrial translation. (B) Mitochondrial import of 5S rRNA is depicted in Humans. 5S rRNA import requires the binding to cytosolic cofactors as well as translocation through the protein import channels. Import was found to also require ATP hydrolysis as well as the mitochondrial membrane potential. Recently, 5S rRNA was found to assemble into mitochondrial ribosomes (68). (C) *In vitro* import of tRNA^{Gln} in humans. Import was found to not require cytosolic chaperones or a mitochondrial membrane potential. The mechanism by which tRNA^{Gln} is imported as well as its mitochondrial function is unknown (Adapted from Schneider, *Annu. Rev. Biochem* 2011).

through human mitochondrial protein import channels and also require the mitochondrial membrane potential (Figure 1.5B) (40). However, independent studies examining the mitochondrial import of tRNA^{Gln} into human mitochondria have found that there is no requirement for either cytosolic chaperones or membrane potential, suggesting alternate mechanisms may be used for different RNAs (Figure 1.5C).

tRNA Import in Protozoa – The discovery of mitochondrial tRNA import was first described in *T. pyriformis* by Suyama in 1967 (33). Since then, it has been found that numerous other protist require mitochondrial tRNA import in order to maintain mitochondrial translation. Whereas *Tetrahymena* encode a limited number of tRNAs on their mitochondrial genome, the parasitic protist, represented by the kinetoplastids and apicomplexa, do not encode for tRNAs on their mitochondrial genome (30, 31, 36, 37, 69). Instead, all of the imported tRNAs are nuclear encoded and must be imported from the cytosol (28, 29, 70). The two best-studied protozoan systems, which examined the mitochondrial import of nuclear encoded tRNA, are the kinetoplastids *T. brucei* and *Leishmania* (Figure 1.6). Since the discovery that all of the tRNAs contained within the mitochondria are nuclear encoded, numerous experimental studies have been able to validate that dozens of these tRNAs are imported into the mitochondria of *T. brucei* and *Leishmania* (28, 29, 32, 71). A number of protist studies have focused on the sequence elements as well as the biophysical requirements for mitochondrial tRNA import, yet there is very little consensus as to what these elements and conditions are. For example, in *T. pyriformis* it has been shown that the tRNA anti-codon provides a signal that targets tRNAs for mitochondrial import, whereas in *T. brucei* and *Leishmania* different signals have been found (72). Studies examining import determinants in *Leishmania* have

found that there are two types of tRNA (Type I and Type II) which each contain unique signals for mitochondrial import within their D-Arm/Anticodon and T-Stem respectively (73). Results examining import signals in *T. brucei* have also provided differing results. Previously it had been shown that precursor, dicistronic tRNAs are found within the mitochondria and that the mitochondria also possesses RNase P activity (74-76). In support of this, it has been found that dimeric tRNA transcripts that are preferentially imported into the mitochondria over mature tRNA substrates *in vitro*, and that dicistronic tRNAs can be found within the mitochondria *in vivo* (77). However, recent *in vivo* studies have been able to show that the import signal appears to be contained within the T-stem of mature tRNA transcripts (78, 79). These results suggest that the specificity for recognition and import of tRNA may rely on multiple sequence elements, rather than one specific import determinant.

Studies examining the biophysical requirements for tRNA import have also provided mixed results. All studies have shown that mitochondrial tRNA import requires ATP and mitochondrial membrane protein components, there is controversy over whether or not an active mitochondrial membrane potential is required. The membrane potential is required for the binding and insertion of pre-protein targeting sequences, which are generally highly positively charged (80, 81). However, it would be thought that the flow of protons out of the mitochondrial matrix, would oppose the import of a large, negatively charged molecule like a tRNA. *In vitro* import studies in *Leishmania* and *T. brucei* gave mixed results in regards to the requirement for the membrane potential (82-85). However, results obtained in *T. brucei* have shown that *in vitro* mitochondrial

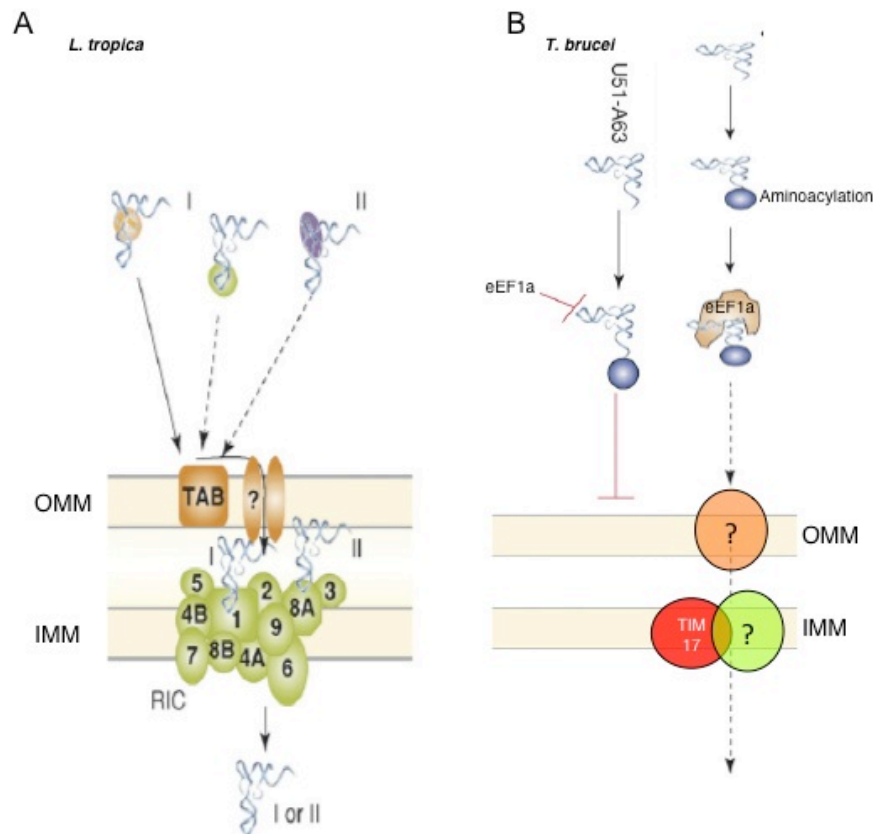


FIGURE 1.6 tRNA import pathways in *L. tropica* and *T. brucei* (A) Import of Type I (Orange – D-Arm and Green – Anticodon import signals) and Type II (Purple-T-Stem import signal) in *L. tropica*. Each tRNA binds to a putative Tubulin Antisense Binding (TAB) protein at the outer mitochondrial membrane (OMM) where they are then transferred through an unknown outer membrane complex. An eleven-subunit inner mitochondrial membrane (IMM) RNA Import Complex (RIC) has been identified as functioning in tRNA import. Type I tRNAs bind to RIC 1, whereas Type II tRNAs bind to RIC 8A. Both are then transported into the matrix through the pore formed by RIC 6 and RIC 9. (B) T-stem mediated tRNA import in *T. brucei*. tRNAs containing the U51:A63 base pair combination do not interact with eEF1a and are retained in the cytosol. Elongator tRNAs are aminoacylated and bound by eEF1a where they are then transported to the mitochondrial surface by an unknown mechanism. tRNA is then translocated through OMM (Orange) and IMM (Green) protein complexes. Tim17 has been shown to function in tRNA import across the IMM, but whether it is a component of the IMM tRNA import complex has yet to be determined. (Adapted from Salinas *et al.*, Trends in Biomedical Science 2008).

import can be nonspecific as tRNA substrates that are known to be retained within the cytosol are imported into the mitochondria (79, 86). Therefore, results obtained by use of *in vitro* import assays should be carefully interpreted as isolating active mitochondria that allow for the specific import of mitochondrially targeted RNA appears to vary between studies.

The best-defined mitochondrial tRNA import complex has been characterized in *Leishmania tropica* (Figure 1.6A). Import of tRNA in *L. tropica* was initially described as requiring the interaction with a 15 kDa outer mitochondrial membrane (OMM) protein. This protein has been characterized as a putative tubulin antisense binding (TAB) protein, but the actual identity of the protein has yet to be determined (87). An inner mitochondrial membrane (IMM) protein complex was also identified. The RNA Import Complex (RIC) was identified through a series of RNA pull down assays as well as by native gel protein extractions (88-90). The RIC was found to be an eleven-subunit complex of approximately 600 kDa, which is functional for *in vitro* tRNA import into isolated mitochondria and phospholipid vesicles (88, 89). Further, the authors were able to determine the identity of necessary protein components of the RIC through a series of *in vivo* antisense RNA assays (90). However, it must be noted that recent concerns about the identity of the RIC complex, as well as the function of its components in mitochondrial tRNA import have been raised leading to a recommendation by the editors of the Proceedings of the National Academy of Sciences, USA, to view these results with caution as an investigation is underway (91-93). Five of the eleven subunits of the RIC are known components of the electron transport chain of the IMM (RIC 1, 5, 6, 8A and 9) with four of the six subunits (1, 6, 8A and 9) identified as essential for tRNA import also

being components of the electron transport chain (90). Interestingly, RIC 1 (ATP Synthase Subunit α) and RIC 8A (Complex III Subunit 6b) have been identified as the tRNA Type I and II binding receptors respectively, while RIC 6 (Rieske Iron Sulfur Protein (ISP)) and RIC 9 (Complex IV Subunit VI) form the translocation pore (88, 89, 94, 95). The dual function of these highly expressed mitochondrial respiratory proteins in mitochondrial tRNA raised many concerns as to whether this complex would be present in the closely related kinetoplastid *T. brucei*. Firstly, the mitochondria of *T. brucei* is developmentally regulated as the parasite progresses through its life cycle (Figure 1.1), and mitochondrial translation and tRNA import occur in both the procyclic and bloodstream life stages (21). If a protein complex were to function in mitochondrial tRNA import, then its components should be constitutively expressed throughout the entire life cycle. This is not the case for numerous components of the RIC complex as components of the respiratory chain, specifically the cytochromes, are developmentally regulated and not detected in the *T. brucei* bloodstream form (10, 11, 15, 16). Further, it has been experimentally shown in *T. brucei* that RIC 1 (ATP Synthase Subunit α) and RIC 8A (Complex III Subunit 6b) are not required for mitochondrial tRNA import (92, 93). Thus, out of the six RIC components that were found to be essential for mitochondrial tRNA import in *L. tropica*, five of these subunits are either developmentally regulated or are not expressed in *T. brucei*, raising the question as to what the components of the *T. brucei* import complex are.

Identification of *T. brucei* mitochondrial membrane proteins that are functional for tRNA import has been less fruitful (Figure 1.6B). Recently an *in vivo* tRNA import assay was developed in which the mitochondrial import of a newly synthesized tRNA

could be monitored at the exact same time as the RNAi depletion of a target protein (79). Using this system, it was found that the eukaryotic elongation factor 1a (eEF1a) functions in mitochondrial tRNA import. eEF1a is an essential cytosolic protein that binds to all elongator tRNAs, except for selenocystine tRNA (tRNA^{Sec}) as well as the initiator methionine tRNA (tRNA^{Met-i}). Further, all of the tRNAs that interact with eEF1a are imported into the mitochondria, whereas tRNA^{Sec} and tRNA^{Met-i} remain in the cytosol. Based on these findings, it was determined that the T-stem pair U51:A63 of tRNA^{Met-i} as well as the U8:A66 pair of tRNA^{Sec} are anti-determinants for binding to eEF1a, and that by not being able to bind to eEF1a, these two tRNAs remain cytosolic (79). By mutating the T-stem of tRNA^{Met-i} to a nucleotide pair that restored binding to eEF1a, the researchers were then able to show that this tRNA is imported into the mitochondrial in an aminoacylated form (79). In these studies the *in vivo* import assay used showed that tRNA import required mitochondrial membrane potential. However, when these studies were conducted *in vitro* on isolated mitochondria, in the absence of cytosolic cofactors (i.e. eEF1a), it was found that tRNA^{Sec} and tRNA^{Met-i} were non-specifically imported into the isolated mitochondria, again highlighting problems associated with *in vitro* import into isolated mitochondria (79).

Although there are numerous studies examining the biophysical requirements for mitochondrial tRNA import in *T. brucei*, little is known about the mitochondrial membrane proteins that are involved in the translocation process. Thus far, the only mitochondrial membrane protein that has been identified as being functional in mitochondrial tRNA import is Tim17, a component of the IMM protein import complex (Figure 1.6B) (93). Using the *in vivo* tRNA import assay, in combination with RNAi, researchers

were able to show that the membrane associated Tim17 as well as the mitochondrial matrix localized Heat Shock Protein (mHSP) 70 are functional in tRNA import. However, it is not clear whether this effect on tRNA import is due to an inhibition of tRNA import, or if inhibiting the import of essential mitochondrial proteins is also affecting tRNA import levels. Interestingly though, these studies suggest that there may be a linkage between tRNA and protein import in *T. brucei*.

Mitochondrial Protein Import in *Trypanosoma brucei*

Eukaryotic organisms import protein into the mitochondria by use of the TOM and TIM mitochondrial membrane protein complexes. Typically, preproteins bind to the TOM receptors Tom20, Tom22 or Tom70 at the outer mitochondrial membrane where they are then shuttled through the import channel formed by Tom40, Tom22, Tom7, Tom6 and Tom5 (96-99). It has also been found that proteins that are targeted to the outer membrane, including subunits of the TOM complex, contain a unique C-terminal mitochondrial localization signal (MLS) that allows for direct insertion into the membrane (100). Most proteins that are targeted for the IMM or the mitochondrial matrix generally contain an N-terminal MLS that is required for interactions with TIM and TOM protein receptors. After passage through the TOM complex, preproteins then interact with the TIM receptors Tim23 and Tim17. From this point, proteins that are targeted to the matrix are translocated through the pore formed by Tim23 in an energy dependent fashion that also requires the mitochondrial membrane potential (101). Once the preprotein sequence is transferred through the Tim23 pore, it is bound by mHSP70 which then acts in conjunction with Tim44 as an ATP dependent motor to drive protein import across the inner membrane (102, 103). Although the canonical mitochondrial

protein import pathway has been well defined, the attempts to identify homologues of the TIM and TOM protein complexes in *T. brucei* have not been fruitful.

To date, only two components (Tim17 and mHSP70) of the mitochondrial TIM complex as well as two putative components of the TOM complex (Sam55 and ATOM) have been identified and characterized for the function in protein import. *In vitro* protein import analysis in *T. brucei* has shown that the process is ATP dependent, requires mitochondrial membrane protein and also depends on the mitochondrial membrane potential, which was further corroborated by studies examining protein import in Tim17, mHSP70 and ATOM RNAi cell lines (93, 104-106). Trypanosomal mHSP70 has high sequence homology to mHSP70 from most other eukaryotic organisms and has recently been experimentally shown to function in mitochondrial protein import in *T. brucei* (93). Beyond mHSP70, the identification of other components of the protein import machinery has not yielded many results as there has been much difficulty identifying homologues through bioinformatic techniques (105-108). The amino acid sequence of Tim17 and Sam55 only display 25-26% and 17-22% sequence identity to other eukaryotic systems, while ATOM displays no homology to eukaryotic TOM components, instead ATOM displays high sequence homology to the Omp85 protein family which is essential for outer membrane protein insertion and translocation in bacteria (105, 106, 108). The essential function of ATOM in protein import in *T. brucei* suggests that the trypanosome mitochondrial protein import machinery may be an ancient relic of early divergent eukaryotic protein transport systems. The inability to confidently identify homologous components of the canonical TIM and TOM protein complexes further suggest this may be the case.

A Shared Mechanism for Mitochondrial tRNA and Protein Import?

Eukaryotes are highly diverse yet have many universal features. Mitochondrial biogenesis requires the import of nuclear encoded protein and RNA in order to maintain function and although various *in vitro* and *in vivo* import assays in multiple systems appear to describe divergent mechanisms for import, closer analysis potentially reveals a universally conserved mechanism for RNA and protein import. Biophysically, the import of protein and tRNA require both ATP and membrane protein, however results obtained as to whether the mitochondrial membrane potential is essential for RNA import remain mixed (34, 35, 40, 47, 82-84, 92, 104, 109). Further, every *in vivo* study has shown a dependency on cytosolic cofactors for delivery of RNA to the mitochondria (44, 51, 79). Even though these cytosolic cofactors have been shown to be essential for import, most *in vitro* assays are conducted in the absence of added cytosolic proteins using a synthetic, “naked” tRNA, suggesting that specificity for recognition of imported RNAs may be lost (34, 35, 82, 83). In lieu of this, in every eukaryotic system, it has been shown that components of the mitochondrial protein import translocons (TIM and TOM) are essential for both the import of protein and tRNA, suggesting eukaryotic organisms may have evolved a universal mechanism for the import of these macromolecules. (40, 45, 47, 64, 93).

The goals of this dissertation were set out to identify putative components of a mitochondrial tRNA import complex in *T. brucei* as well as to determine if there was a relationship between tRNA and protein import. Initially this work focused on identifying mitochondrial membrane proteins that specifically interacted with *T. brucei* specific tRNA substrates and affected mitochondrial import. Through the course of the study, it

was found the identified complex is functional in both mitochondrial tRNA and protein import. Together, the results of this dissertation show that the identified protein complex is functional in both mitochondrial protein and tRNA import and that the biophysical requirements for import are the same for both macromolecules. In combination with the results obtained from other eukaryotic organisms, these results further support the hypothesis that RNA and protein are imported into the mitochondria by use of a common import machinery.

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CHAPTER 2

A MITOCHONDRIAL MEMBRANE COMPLEX THAT CONTAINS PROTEINS

NECESSARY FOR tRNA IMPORT IN *TRYPANOSOMA BRUCEI*¹

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Abstract

The mitochondrial genome of *Trypanosoma brucei* does not contain genes encoding tRNAs, instead this protozoan parasite must import nuclear encoded tRNAs from the cytosol for mitochondrial translation. Previously, it has been shown that mitochondrial tRNA import requires ATP hydrolysis and a proteinaceous mitochondrial membrane component. However, little is known about the mitochondrial membrane proteins involved in tRNA binding and translocation into the mitochondrion. Here we report the purification of a mitochondrial membrane complex using tRNA affinity purification and have identified several protein components of the putative tRNA translocon by mass spectrometry. Using an *in vivo* tRNA import assay, in combination with RNA interference, we have verified that two of these proteins, Tb11.01.4590 and Tb09.v1.0420, are involved in mitochondrial tRNA import. Using PTP-tagged Tb11.01.4590, additional associated proteins were identified including Tim17 and other mitochondrial proteins necessary for mitochondrial protein import. Results presented here identify and validate two novel protein components of the putative tRNA translocon and provide additional evidence that mitochondrial tRNA and protein import have shared components in trypanosomes.

Introduction

The mitochondrial genomes of most eukaryotes contain genes encoding tRNAs, which function in maintaining active mitochondrial translation of proteins vital for mitochondrial function. However, some mitochondrial genomes do not encode complete sets of tRNA and the cell must import tRNAs from the cytosol into the mitochondria (1, 2). Studies in mammals (3), fungi (4), plants (5) and protozoa (6-11) have examined the bioenergetic and sequence requirements for mitochondrial tRNA import. Although diverse mechanisms for translocation of tRNA have been identified, recent studies examining tRNA import in trypanosomes suggest that involvement of components of the mitochondrial protein translocation machinery may be a conserved feature of tRNA import in all organisms (12).

The mitochondrial genomes of the kinetoplastid protozoa, *Leishmania tropica* and *Trypanosoma brucei*, are completely devoid of mitochondrial tRNA genes. Instead, all of the mitochondrial tRNAs are encoded within the nucleus and show dual localization with approximately 90% of each tRNA species in the cytosol and 10% in the mitochondrion, the exceptions being the selenocysteine and initiator methionyl-tRNAs which are exclusively cytoplasmic (6, 13-15). The mechanism by which these tRNAs are imported into the trypanosome mitochondria is unknown, however, *in vitro* studies, with isolated organelles have shown that translocation of tRNAs into the mitochondrion requires ATP hydrolysis and a proteinaceous membrane component (9, 16, 17). In *L. tropica*, it has been reported that an outer mitochondrial membrane (OMM) protein (8, 18), as well as an eleven-subunit inner mitochondrial membrane (IMM) RNA Import

Complex (RIC) are required for tRNA import (19-22). Many of the essential subunits of this *Leishmania* IMM RIC complex are proposed to function both in tRNA import and as components of the mitochondrial respiratory complexes including the cytochrome c reductase (Complex III), cytochrome c oxidase (Complex IV) and the a-subunit of the F₁F₀-ATP synthase. However, the identification of several components of the RIC, including the Reiske iron sulfur protein (ISP) and the a-subunit of the F₁-ATP synthase, have recently come into question (12, 21, 23, 24). Furthermore, it is unlikely that the RIC of *T. brucei* has the same protein composition as *Leishmania* since the bloodstream form (BF) of trypanosomes do not express the cytochrome c oxidase or cytochrome c reductase complexes, yet are able to import tRNAs into their mitochondria (25).

While little is known about the mitochondrial tRNA import machinery of African trypanosomes a cytosolic protein, eukaryotic elongation factor 1a (eEF1a), has recently been shown to play a role in mitochondrial tRNA import in *T. brucei* (17). In addition, the canonical Translocase Inner Membrane 17 (Tim17) of the mitochondrial protein import complex has been shown to function in *T. brucei* protein and tRNA import (26). Here, we report the biochemical purification and characterization of a mitochondrial membrane complex in *T. brucei* involved in the translocation of tRNAs from the cytosol into the mitochondria. Two hypothetical proteins, Tb11.01.4590 and Tb09.v1.0420, were shown to function in mitochondrial tRNA import. The putative tRNA translocon also contains the Tim17 protein, and other proteins proposed to function in mitochondrial protein import. these findings suggest that mitochondrial tRNA and protein import machinery in trypanosomes may either share protein components or that there is a common translocon for both proteins and tRNAs.

Results

Identification and characterization of tRNA binding mitochondrial membrane protein complexes

Previous studies on *T. brucei* suggested that mitochondrial membrane proteins were necessary for tRNA import (9). As a first step in the identification of the protein components necessary for tRNA import into mitochondria, we asked whether mild non-ionic detergent (1% Triton X-100) extraction of mitochondrial membranes released protein complexes that could bind tRNAs. Mitochondria were isolated from PF *T. brucei* following hypotonic lysis and differential centrifugation. Transmission electron microscopy revealed that 87% (n = 100) of the mitochondria in these preparations contained both outer and inner mitochondrial membranes (Figure 2.1A). A small fraction of mitochondria appeared to have a single membrane, consistent with the formation of mitoplasts lacking the outer membrane that is formed by hypotonic lysis and shearing. Detergent extracted mitochondrial protein complexes were incubated with radiolabeled *T. brucei* tRNA^{Leu(CAA)} followed by separation of protein complexes using blue native gel electrophoresis (27). Under these conditions, tRNAs bound two high molecular weight protein complexes. The sizes of the tRNA binding complexes were estimated based on the known molecular weights of the *T. brucei* mitochondrial cytochrome c oxidase, cytochrome c reductase and the ATP synthase which were identified by LC-MS/MS in these preparations (Sykes and Hajduk, personal communication)

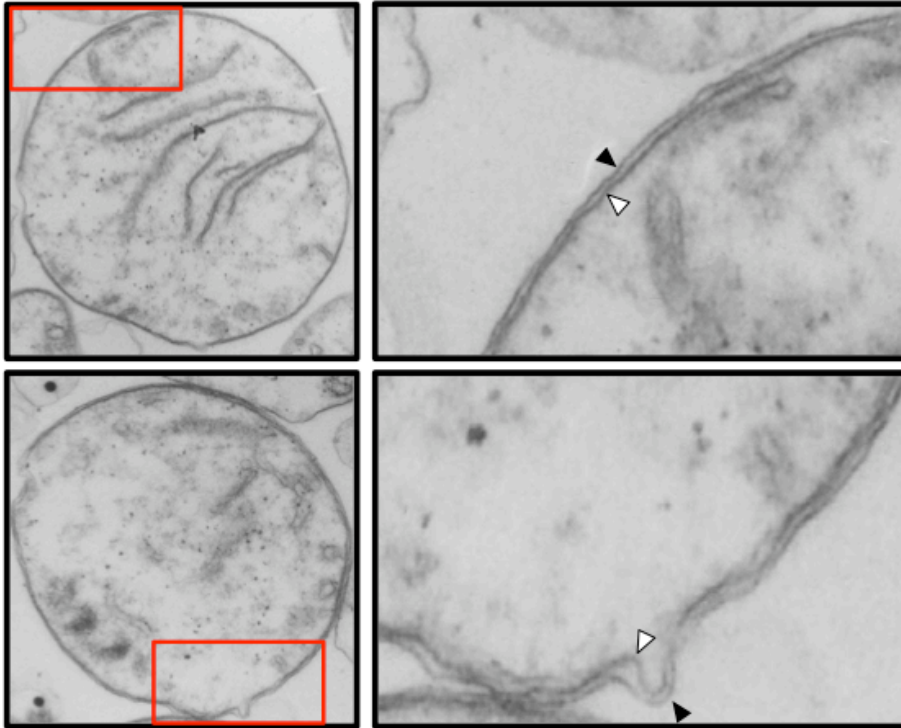
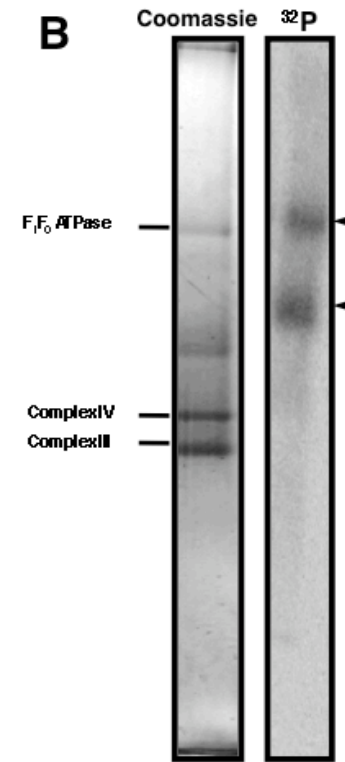
A**B**

FIGURE 2.1. Identification of tRNA binding complexes in extracts from *T. brucei* mitochondria. **(A)** Isolated mitochondria were characterized by transmission electron microscopy. Most mitochondria had intact outer (black arrowhead) and inner membranes (white arrowheads). A small fraction of mitochondria appear to have lost the outer membrane due to the hypotonic swelling and shearing protocol used in cell lysis (not shown). The areas designated by the red boxes in the lower magnification images were enlarged to better visualize the mitochondrial membranes (right panels). Total magnification - Left Panels = 27,272x Right Panels = 89,089x. **(B)** Radiolabeled tRNA^{Leu(CAA)} binds two high molecular weight mitochondrial protein complexes (black arrowheads). Detergent extracts of *T. brucei* mitochondrial membrane were incubated with ³²P- tRNA^{Leu(CAA)} and fractionated by Blue Native Gel (BNG) electrophoresis. The major mitochondrial membrane complexes visualized on stained BNG (left panel) were identified by LC-MS/MS as F₁F₀ ATP synthase (F₁F₀ATPase), cytochrome c oxidase (Complex IV) and cytochrome c reductase (Complex III) and were used as internal size standards (Sykes and Hajduk, unpublished results). The tRNA binding complexes were detected by autoradiography (right panel).

and by previous analysis of the ATP synthase (28). The larger complex is >820kDa while the lower tRNA binding complex is approximately 500kDa (Figure 2.1B).

In order to purify these mitochondrial membrane complexes, we developed a tRNA affinity purification method using a synthetic *T. brucei* tRNA^{Leu(CAA)}, biotinylated at the 5' end and then anchored to a streptavidin paramagnetic resin. Using this as a ligand, we were able to pull down a specific subset of proteins from intact mitochondria and detergent extracts (1% Triton X-100) containing mitochondrial membranes (Figure 2.2). Since the native tRNA translocon must be able to recognize and bind tRNAs presented on the cytosolic face of the mitochondrion, we reasoned that tRNA substrates must be able to bind mitochondrial membrane proteins on the purified, intact organelle. To test this, purified mitochondria were incubated with the tRNA affinity resin, followed by treatment with non-ionic detergent (1% Triton X-100) then washed extensively with a low ionic strength buffer to remove non-specifically bound proteins. tRNA binding proteins were released by stepwise salt elution. A sub-fraction of mitochondrial proteins was reproducibly recovered following treatment with 0.25 - 0.50 M NaCl (Figure 2.2A). A similar set of proteins was seen when mitochondrial membrane proteins were fractionated using the biotinylated tRNA affinity method (Figure 2.2B, middle and right panels), but not when using proteins extracted from the mitochondrial matrix (Figure 2.2B, left panel). Protein profiles from the intact mitochondria and solubilized mitochondrial membranes were similar. This suggests that the biotinylated tRNA ligand interacts with a mitochondrial membrane complex that contains a tRNA binding domain exposed to the trypanosome cytoplasm. These results are consistent with a potential role for this complex as a tRNA translocon.

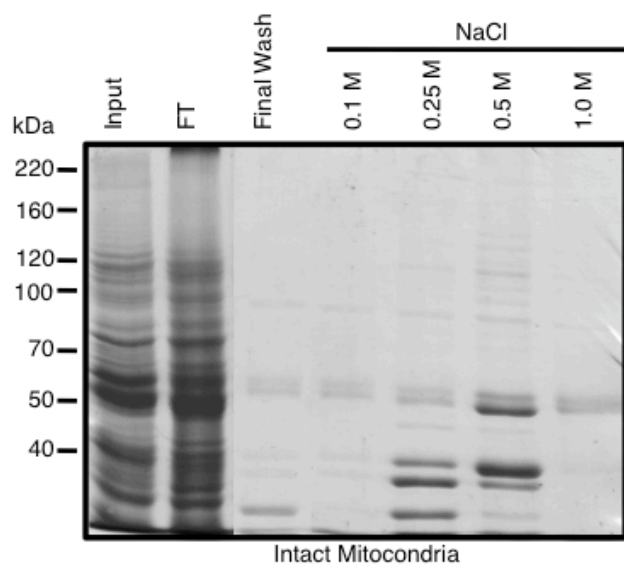
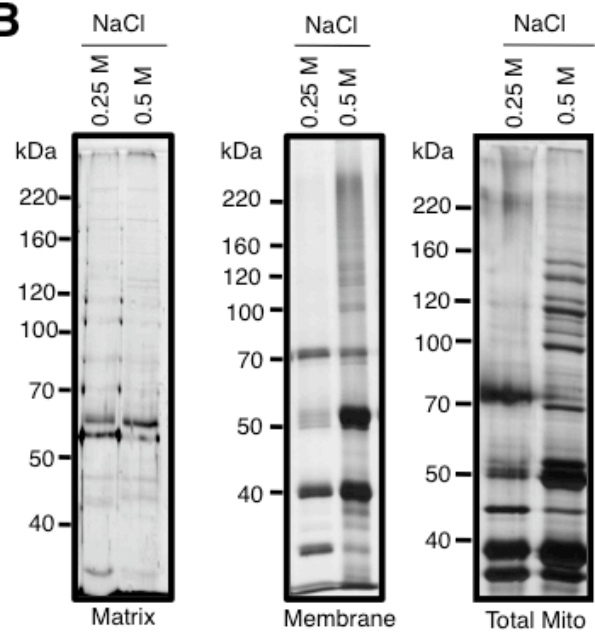
A**B**

FIGURE 2.2. Affinity purification of tRNA binding complexes from *T. brucei* mitochondria. **(A)** Isolated mitochondria were incubated with tRNA^{Leu(CAA)} affinity resin, treated with non-ionic detergent and unbound proteins collected in the flow through (FT). Bound proteins were washed extensively with low-salt buffer and a final wash was collected (Final Wash) prior to stepwise elution with buffer containing increasing concentrations of NaCl. Samples were analyzed on 10% SDS-PAGE and were stained with Coomassie Blue to visualize protein. Contaminating proteins found in all fractions were bovine serum albumin (66.8 kDa) from culture media and SUPERase-in (Ambion) from the affinity resin. **(B)** Purified mitochondria (left panel) and fractionated mitochondrial matrix (right panel) and membranes (right panel) were detergent solubilized, incubated with tRNA^{Leu(CAA)} affinity resin and associated proteins eluted by treatment with increasing concentrations of NaCl. Proteins eluting with 0.25 and 0.5 M NaCl were fractionated on 10% SDS-PAGE and visualized by Coomassie Blue staining.

In order to identify components of the complex that interacted with the biotinylated tRNA^{Leu(CAA)}, we used a subtractive mass spectrometry approach. Purified, intact mitochondria were incubated with resin lacking biotinylated tRNA^{Leu(CAA)} or resin containing biotinylated tRNA^{Leu(CAA)}, followed by solubilization of the bound mitochondria and washing away unbound protein. Both samples were treated identically and were analyzed by LC-MS/MS. Proteins found to non-specifically bind to the affinity resin were subtracted from the analysis, whereas proteins that specifically bound to the biotinylated tRNA^{Leu(CAA)} resin were evaluated further. Proteins of interest were selected based on the MS score (>95% Confidence), number of unique peptides (minimum of 2 peptides), predicted domain homology, predicted Mitochondrial Localization Signals (MLS) or transmembrane helices (TM). In addition, since tRNAs are imported into both BF and PF trypanosome mitochondria only proteins encoded by constitutively expressed genes (44), in both developmental stages, were analyzed further. From this subtractive LC-MS/MS analysis, 44 proteins, fitting these criteria, were identified that specifically interacted with the biotinylated tRNA^{Leu(CAA)}. For each of these proteins a minimum of two unique peptides were identified with a probability score of greater than 95% (Table 2.1). Of these proteins, 29 are currently annotated as hypothetical in the GeneDB and TriTrypDB databases with 9 having both predicted MLS and TM domains. It is important to note that only two of the proteins identified in this analysis (Tb09.v1.0420 and Tb927.10.4280) are homologous to components of the eleven-subunit *Leishmania* RIC. Based on the criteria described above, seven candidate proteins were selected for further analysis to directly test their role in mitochondrial tRNA import (Table 2.2).

TABLE 2.1

tRNA Specific Proteins: >95% Confidence/ Minimum 2 Unique Peptides	Protein ID	Predicted Mito & TM?
Tb927.5.2930	Hypothetical	Mito
Tb927.6.3740/Tb927.6.3750/Tb927.6.3800	Heat Shock 70kDa Mito Precurs	Mito
Tb09.v1.0420	Hypothetical	Both
Tb927.7.210	Proline Dehydrogenase	Mito
Tb11.01.4860	MRP2/gBP25	Mito
Tb11.55.0009	MRP1/gBP21	Mito
Tb10.6k15.2510	Hypothetical	Mito
Tb11.01.3370	PEX11	TM
Tb10.70.2920/Tb10.v4.0045	Prohibitin	Both
Tb927.10.15220	Hypothetical	Both
Tb10.70.0890	Hypothetical	No
Tb927.6.4280/Tb927.6.4300	GAPDH	No
Tb10.6k15.3250	Succinyl-CoA Ligase beta chain	Both
Tb09.160.2650	Hypothetical	No
Tb11.02.0970	Hypothetical	No
Tb927.2.2340	Hypothetical	No
Tb927.7.4390	Threonine Synthase	No
Tb927.5.1680	Hypothetical	No
Tb927.7.3330	Hypothetical	No
Tb10.05.0190	Hypothetical	No
Tb927.3.3460	Hypothetical	TM
Tb927.4.2640	Hypothetical	No
Tb11.02.1480	Mito Intermediate Peptidase Alpha	Both
Tb927.10.6400	Chaperone Hsp60 Mito Precursor	Mito
Tb11.01.4630	Hypothetical	TM
Tb927.7.6910	Hypothetical	No
Tb11.01.4590	Hypothetical	Both
Tb927.8.6980	Hypothetical	TM
Tb927.8.4380	Hypothetical	No
Tb927.3.1380	ATP Synthase Beta chain	Mito
Tb927.10.180	ATP Synthase Subunit Gamma	Mito
Tb927.6.4990	ATP Synthase Epsilon Chain	Mito
Tb927.4.1980	Hypothetical	TM
Tb927.7.480	Hypothetical	Both
Tb927.6.3790	Hypothetical	TM
Tb09.211.0610	Hypothetical	Mito
Tb09.160.4120	Hypothetical	TM
Tb927.8.3080	Hypothetical	TM
Tb927.6.4450	Hypothetical	Both
Tb927.5.1710	Hypothetical	TM
Tb11.02.0445	Hypothetical	Both
Tb09.211.1750	Mito Carrier Protein	Both
Tb927.10.4280	Hypothetical	Both
Tb11.02.4120	Hypothetical	Both
	Candidates Analyzed based on RNAi Growth Phenotype and Affect on tRNA Import	

TABLE 2.2

Mitochondrial Membrane Proteins Purified by tRNA Binding

tRNA Specific Proteins ¹	Protein ID	Predicted MLS & TM ²	Conserved Domains ³	PC/BS mRNA Expression ⁴
Tb09.v1.0420 ⁵	Hypothetical	Both	None	Yes
Tb11.01.4860	MRP2/gBP25	MLS	RNA Binding	Yes
Tb11.55.0009	MRP1/gBP21	MLS	RNA Binding	Yes
Tb927.10.15220	Hypothetical	Both	None	Yes
Tb11.01.4590	Hypothetical	Both	None	Yes
Tb11.02.0445	Hypothetical	Both	None	Yes
Tb927.10.4280 ⁵	Hypothetical	Both	UCR 14kD	Yes

¹ >95% Confidence / Minimum 2 Unique Peptides

² Predictions based on sequence analysis using MitoProt/TMHMM/TMPred software and Panigrahi et al., 2009.

³ Domain homology by blasting protein sequences through NCBI.

⁴ Life stage expression data from TriTrypDB/Nilsson et al., 2010.

⁵ Leishmania RIC homologue

In vivo validation of candidate protein components of the trypanosome tRNA import complex

In order to determine the role of the candidate proteins in mitochondrial tRNA import, we utilized an *in vivo* import assay which combines the RNAi knockdown of a candidate protein with the induction of a newly synthesized, Var-tRNA^{Met-i} that is imported into the mitochondria (17). Using the Var-tRNA^{Met-i} construct, we were able to show regulated expression of the Var-tRNA^{Met-i} in the presence of doxycycline (Figure 2.3A). To evaluate import of the Var-tRNA^{Met-i}, purified mitochondria were treated with micrococcal nuclease to remove contaminating cytosolic RNAs and mitochondrial RNA were isolated (Figure 2.3B). By ethidium bromide staining the mitochondrial RNA samples were enriched in the mitochondrial 9S and 12S rRNA and deficient in both large

and small cytosolic rRNAs. To verify that the mitochondrial RNA samples were devoid of cytosolic tRNA contaminants, blots were hybridized with a probe for the initiator methionyl tRNA (WT Met-i) (Figure 2.3C; Figure 2.4). The absence of both ethidium bromide stained cytosolic rRNAs and the cytosolic initiator methionyl tRNA indicated that our mitochondrial fractions were free of contaminating cytosolic RNAs. Using this fractionation method we found that the amount of tRNA imported into the mitochondria was highly reproducible over multiple experiments, with the average amount of Var-tRNA^{Met-i} imported being 10.3% (Figure 2.3B and D).

Previously, it was reported that RNAi knockdown of a cytosolic protein, eEF1a, reduced the amount of tRNA imported into the mitochondria while RNAi knockdown of eIF2, also an essential cytosolic protein, had no effect on tRNA import (17). We have verified these results by simultaneous doxycycline induction of expression of the Var-tRNA^{Met-i} and either eEF1a or eIF2 RNAi in our cell lines (Figure 2.3E and F). The import of Var-tRNA^{Met-i} was reduced 50-60% after 24 hours of RNAi induction in the eEF1a cells, whereas Var-tRNA^{Met-i} import was unaffected following induction of eIF2 RNAi. Based on the corroboration of previous results (17), we can utilize this *in vivo* tRNA import assay to evaluate the role of candidate proteins in tRNA import.

Identification of the tRNA import machinery by RNAi knockdown of candidate proteins is complicated by the fact that fully energized mitochondria are necessary for cell viability. Therefore, the loss of proteins affecting electron transport or oxidative phosphorylation in these experiments may give false positive results due to downstream effects on overall cellular viability.

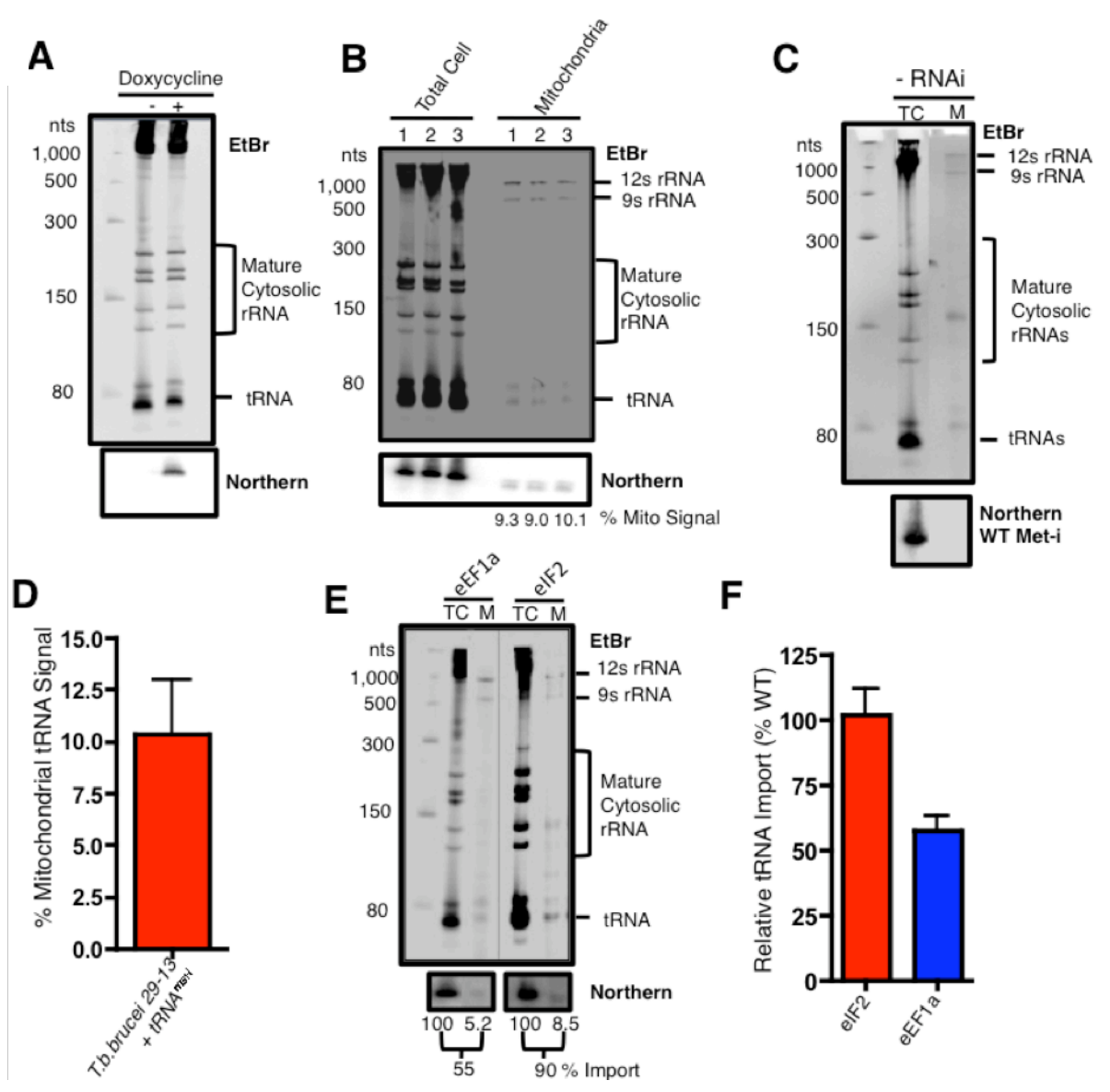


FIGURE 2.3. An *in vivo* assay for tRNA import in trypanosomes. **(A)** Doxycycline regulated expression of Var-tRNA^{Met-i} in *T. brucei*. Total cell RNA from 2×10^7 trypanosomes grown with (+) or without (-) the addition of doxycycline (1 $\mu\text{g/ml}$) for 24 hours. RNA was fractionated by non-denaturing polyacrylamide gel electrophoresis (6%)(top panel) and abundant rRNA and tRNA visualized by ethidium bromide (EtBr) staining. The Var-tRNA^{Met-i} was detected by northern blot hybridization and autoradiography (bottom panel). **(B)** Mitochondrial import of Var-tRNA^{Met-i} following doxycycline induction. Total cellular and mitochondrial RNA was isolated 24 hours post-induction with doxycycline in three independent experiments. Samples were fractionated on 6% polyacrylamide gel, stained with EtBr to visualize the mitochondrial 9S and 12S rRNAs, small mature cytosolic rRNAs and tRNAs (upper panel) and hybridized with a probe for Var-tRNA^{Met-i} (lower panel). **(C)** Total cellular and mitochondrial RNA from Control (PC 29-13) was isolated and was fractionated by non-denaturing polyacrylamide gel electrophoresis (8%)(top panel) and abundant rRNAs and tRNAs visualized by ethidium bromide (EtBr) staining. The cytosolic specific WT tRNA^{Met-i} was detected by northern blot hybridization and autoradiography. **(D)** Quantitative analysis of Var-tRNA^{Met-i} import at 24 hours post induction with doxycycline (n = 8). The percentage of tRNA import into the mitochondrion was calculated based on the hybridization of the Var-tRNA^{Met-i} probe to total cellular RNA and RNA extracted from purified mitochondria. **(E)** Analysis of tRNA import following RNAi knockdown of eEF1a or eIF2. Samples were fractionated on 6% polyacrylamide gels, stained with EtBr (upper panel) total cellular (TC) and mitochondrial (M) RNA were analyzed by northern blot (lower panel) for amount of Var-tRNA^{Met-i} imported into mitochondria. **(F)** Quantitative analysis of tRNA import for eEF1a (n = 6) and eIF2 (n = 3) was expressed relative to the average level of Var-tRNA^{Met-i} import in wild type cells (10.3%, n = 8).

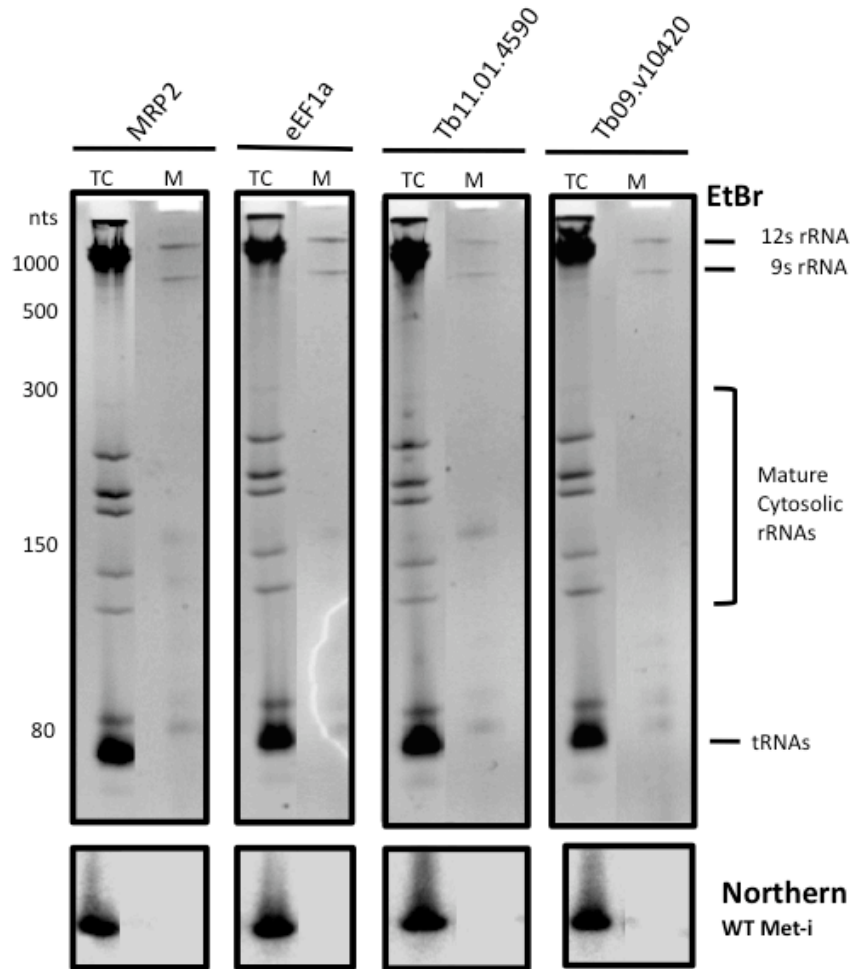


FIGURE 2.4. Validation of the purity of mitochondrial RNA preparations. Total cellular and mitochondrial RNA from MRP2, eEF1a, Tb11.01.4590, Tb09.v1.0420 RNAi cell lines 24 hours after inducing RNAi and the expression of the variant tRNA^{Met-i} of with 1mg/mL doxycycline was isolated and was fractionated by non-denaturing polyacrylamide gel electrophoresis (8%)(top panel) and abundant rRNAs and tRNAs visualized by ethidium bromide (EtBr) staining. The cytosolic specific WT tRNA^{Met-i} detected by northern blot hybridization and autoradiography.

To ensure that we were monitoring tRNA import in viable cells, cell growth and the mitochondrial membrane potential were measured for each cell line following RNAi induction (Figure 2.5; Figure 2.6). Cell lines that displayed a RNAi induced reduction in membrane potential at our 24 hr assay time point were not further characterized, as any reduction in tRNA import could represent a downstream effect due to the overall health of the cell.

RNAi knockdowns of Tb927.10.15220, Tb11.02.0445 and gBP21 had minimal affect on cell growth and tRNA import (Figure 2.5A). RNAi knockdown of the Tb927.10.4280, a member of the putative *Leishmania* RIC, slowed cell growth and reduced both the mitochondrial membrane potential and RNA import after 24 hours of induction (Figure 2.5A and B). Since Tb927.10.4280 displays homology to components of complex III of the electron transport chain, the reduction in cell growth and the decrease in mitochondrial membrane potential were expected. The loss of mitochondrial membrane potential made it impossible to evaluate the role of Tb927.10.4280 in tRNA import. In addition, this protein was not identified in subsequent PTP-pull downs with other putative tRNA translocon proteins suggesting it was a contaminant in the initial MS analysis.

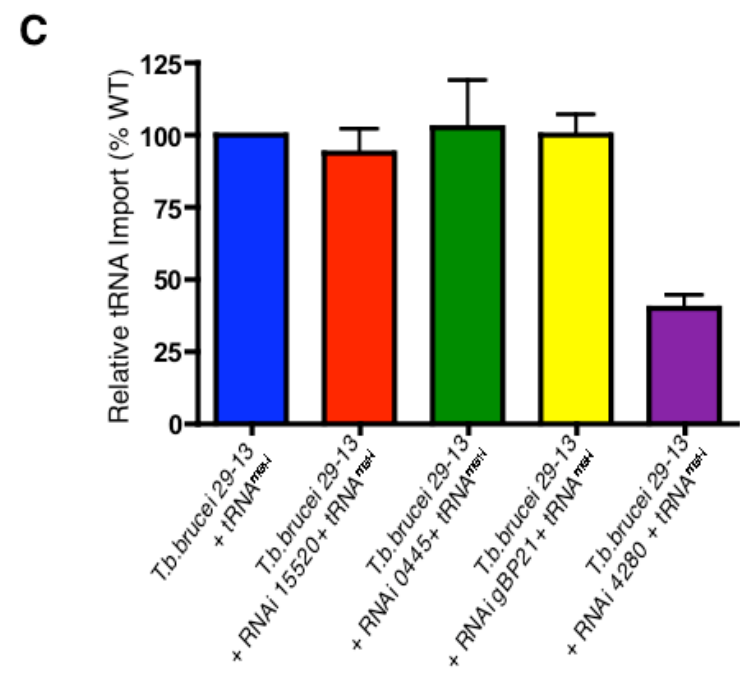
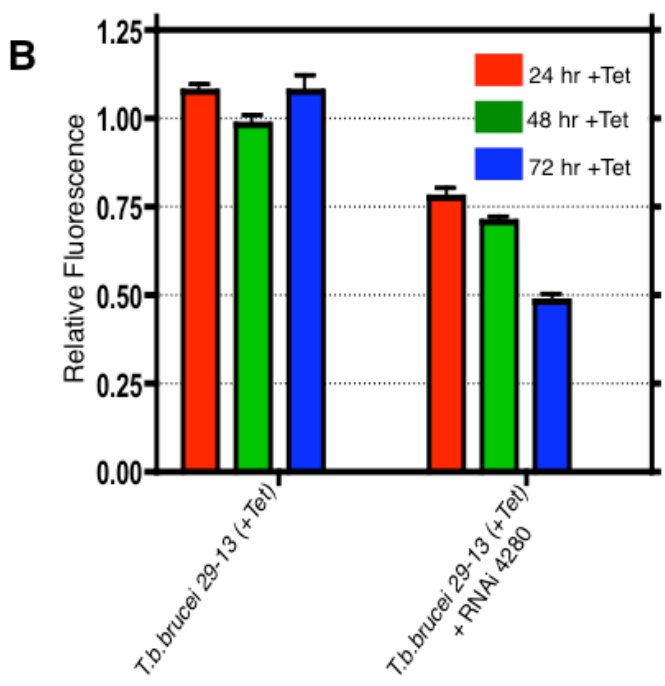
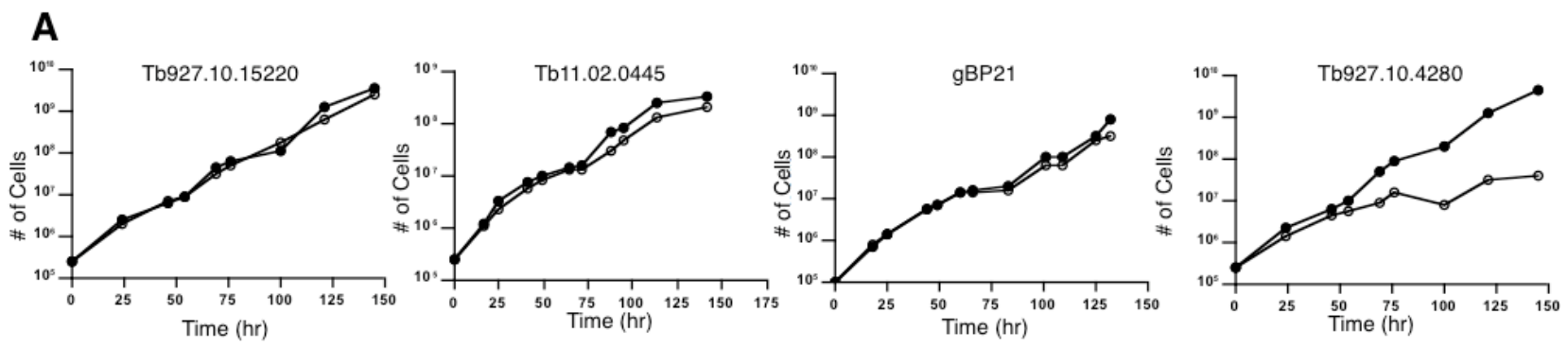
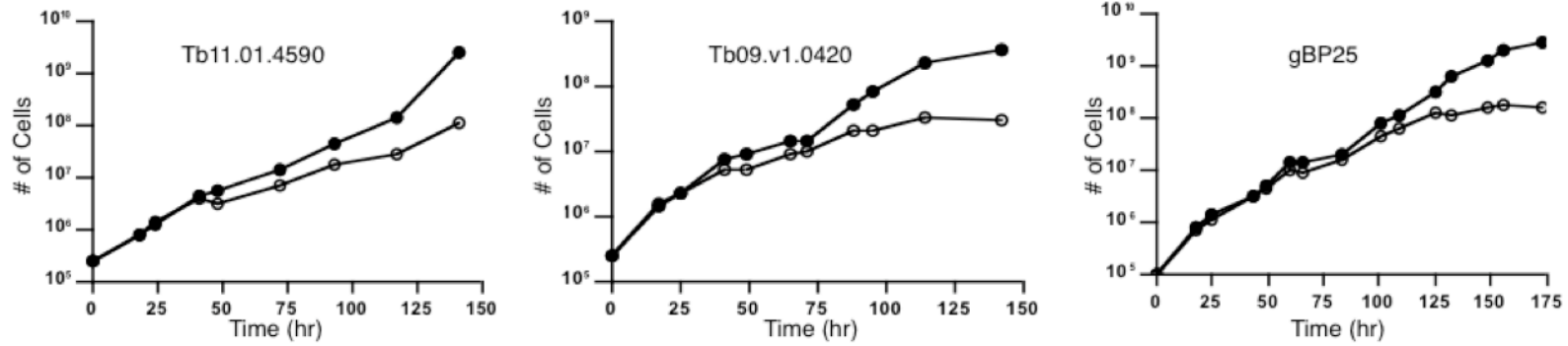


FIGURE 2.5. RNAi analysis of candidate proteins identified by LC-MS/MS. **(A)** Effects of RNAi on candidate tRNA import complex proteins on the cell growth. Untreated (closed circles) and doxycycline induced (1 μ g/ml)(open circles) cultures of Tb11.02.0445, Tb927.10.4280, Tb927.10.15220 and gBP21 were monitored for growth every 24 hours. **(B)** Mitochondrial membrane potential for wild type *T. brucei* 29-13 and RNAi cell line Tb927.10.4280. Cultures were inoculated with doxycycline (1 μ g/ml) and every 24 hours, an aliquot of cells (1×10^7 total cells) was collected, TMRM was added to a final concentration of 20 nM and mitochondrial fluorescence intensity of 2×10^6 total cells was measured (n = 3). **(C)** Quantitative tRNA import analysis for RNAi cell lines Tb927.10.15220, Tb11.02.0445, gBP21 and Tb927.10.4280. Relative import for Tb927.10.15220 (94.1% \pm 14.3) (N=8); Tb11.03.0445 (102.6% \pm 23.6) (N=4); gBP21 (99.9% \pm 12.4) (N=3); Tb927.10.4280 (40.1% \pm 9.7) (N=13) was determined 24 hours following doxycycline induction. tRNA import was expressed relative to level of Var-tRNA^{Met-i} import in wild type cells (10.3%, n = 8).

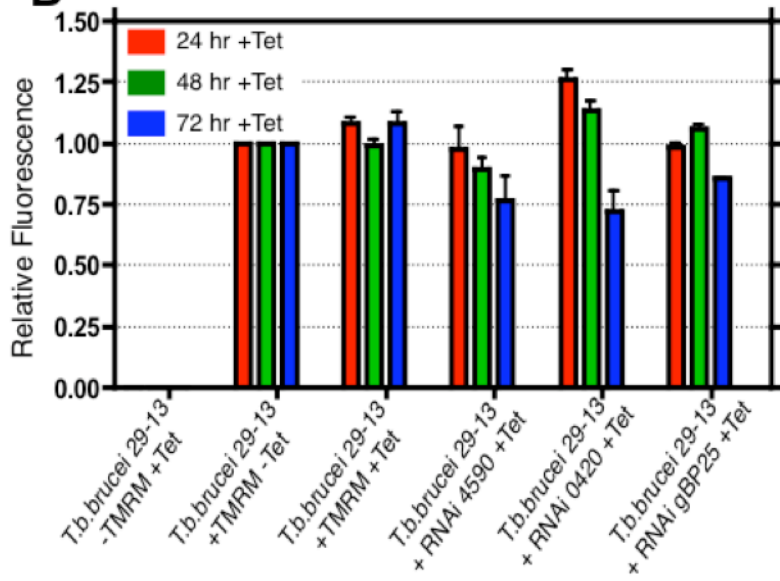
RNAi knockdown of the other three candidate proteins, Tb11.01.4590, Tb09.v1.0420 and gBP25, had no effect on cell growth or mitochondrial membrane potential in the first 24 hours of induction. However, prolonged induction resulted (48-72 hours) resulted in a significant loss of mitochondrial membrane potential and arrest of cell growth suggesting that these proteins are essential for trypanosome viability (Figure 2.6A and B). For each cell line, RNAi induction resulted in at least a two-fold reduction of mRNA for each candidate protein following 24 hours of induction (Figure 2.6C).

To determine the role of each of these proteins in mitochondrial tRNA import, we used RNAi and monitored the movement of newly synthesized, Var-tRNA^{Met-i} across the mitochondrial membrane (Figure 2.7A). RNAi of gBP25 mRNA had no effect on the import of Var-tRNA^{Met-i} (Figure 2.7A and B). Thus, while essential for mitochondrial biogenesis, gBP25 is not required for tRNA import and was likely identified by pull down analysis because of the known RNA binding properties of gBP25 (29). We next examined the effect of RNAi of Tb11.01.4590 and Tb09.v1.0420 expression on Var-tRNA^{Met-i} import and found that the amount of Var-tRNA^{Met-i} imported into the mitochondria was significantly reduced after 24 hours of induction when compared to uninduced controls (Figure 2.7A and B). RNAi knockdown of Tb11.01.4590 and Tb09.v1.0420 expression reduced the amount of Var-tRNA^{Met-i} imported into the mitochondria to 33% and 56% of wildtype levels respectively (Figure 2.7B). Further, to examine the purity of mitochondrial RNA in these preparations, it was noted that cytosolic specific rRNAs were not detected by ethidium bromide staining (Figure 2.7A).

A



B



C

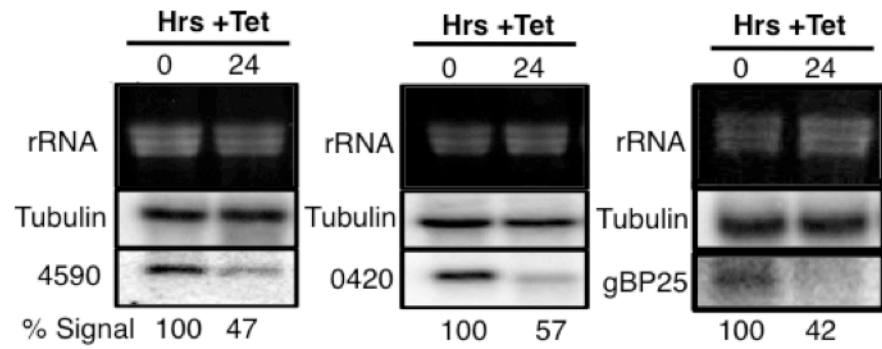


FIGURE 2.6. RNAi analysis of candidate tRNA import complex proteins. **(A)** Growth of Tb11.01.4590, Tb09.v1.0420 and gBP25 RNAi cell lines in the presence (open circles) or absence of doxycycline (1 μ g/ml)(closed circles). **(B)** Analysis of mitochondrial membrane potential analysis in wild type *T. brucei* 29-13 and RNAi cell lines Tb11.01.4590, Tb09.v1.0420 and gBP25. Cell cultures were treated with doxycycline (1 μ g/ml) and the mitochondrial membrane potential determined by relative fluorescence intensity of 2×10^6 total cells following the addition of TMRM (20 nM). Samples were tested in triplicate 24, 48 and 72 hours post induction. **(C)** Northern blot analysis of the effects of RNAi on mRNA level for Tb11.01.4590, Tb09.v1.0420 and gBP25. Total cellular RNA was isolated 0 and 24 hours post induction with 1 μ g/ml doxycycline and the percentage mRNA signal for each candidate was normalized to b-tubulin.

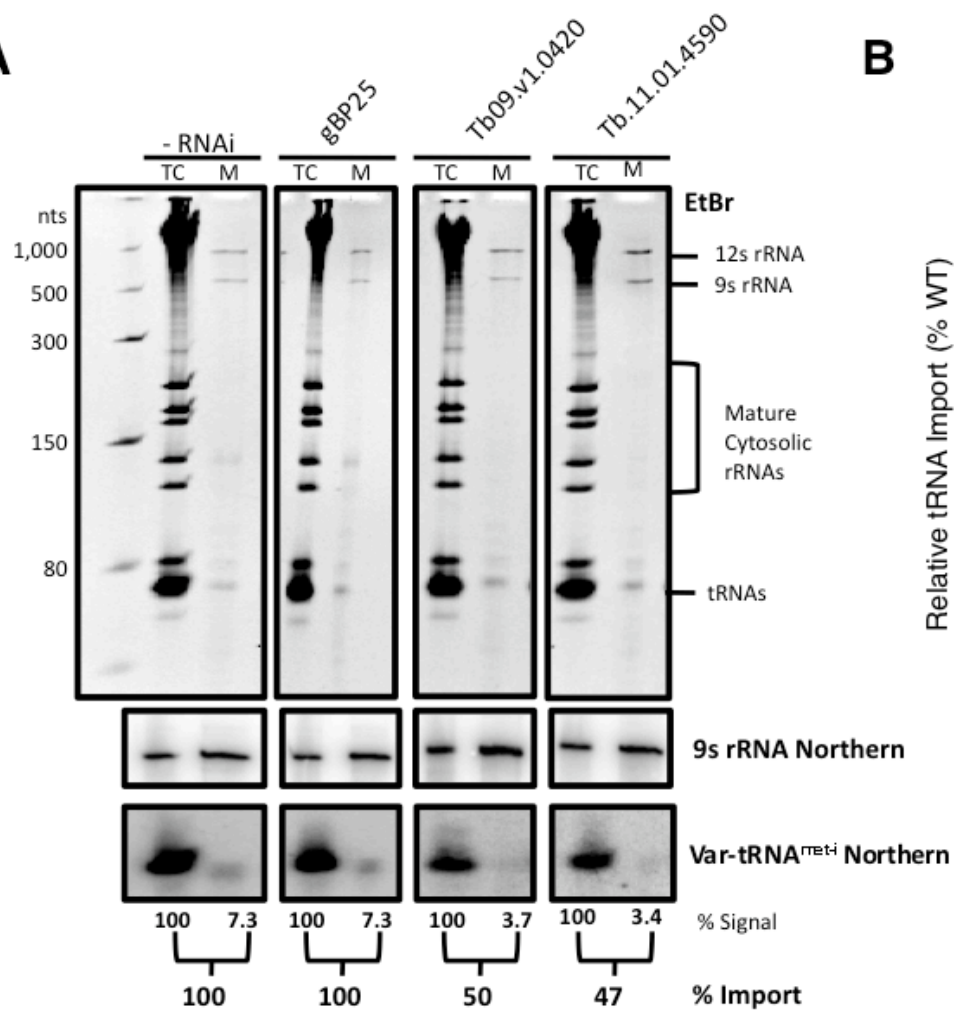
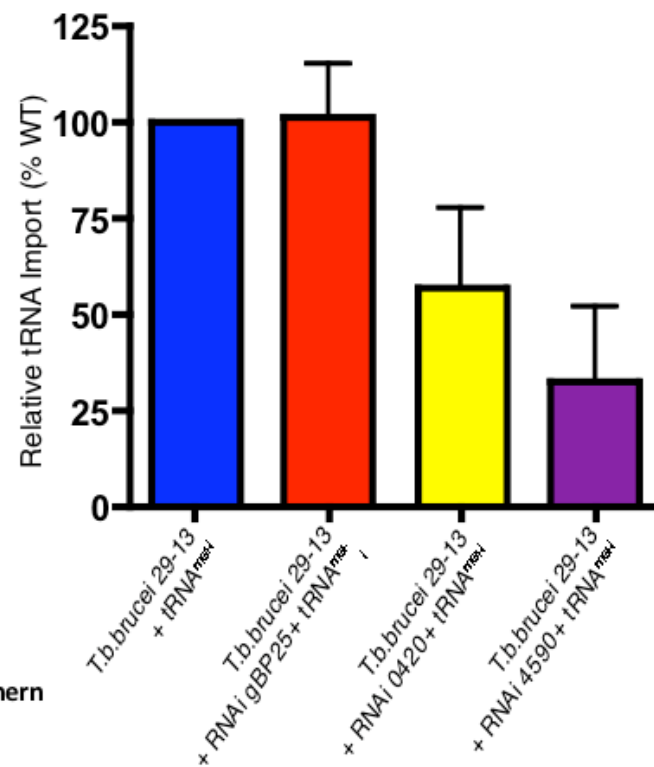
A**B**

FIGURE 2.7. Identification of two *T. brucei* mitochondrial membrane proteins required for tRNA import. **(A)** Analysis of tRNA import into gBP25, Tb09.v1.0420 and Tb11.01.4590 RNAi cell lines. Total cell (TC) RNA and mitochondrial (M) RNA was isolated 24 hours post-induction with doxycycline (1µg/ml) and was fractionated by non-denaturing polyacrylamide gel electrophoresis (8%)(top panel) and abundant rRNAs and tRNAs visualized by ethidium bromide (EtBr) staining. The Var-tRNA^{Met-i} was detected by northern blot hybridization and autoradiography (bottom panel). Percent import was expressed as the relative hybridization of mitochondrial to total cell Var-tRNA^{Met-i}. 9S rRNA used as a loading control (middle panel). **(B)** Quantitative analysis of relative tRNA import into the mitochondria for gBP25 (n = 5), Tb09.v1.0420 (n = 6) and Tb11.01.4590 (n = 8). tRNA import was expressed relative to level of Var-tRNA^{Met-i} import in wild type cells (10.3%, n = 8).

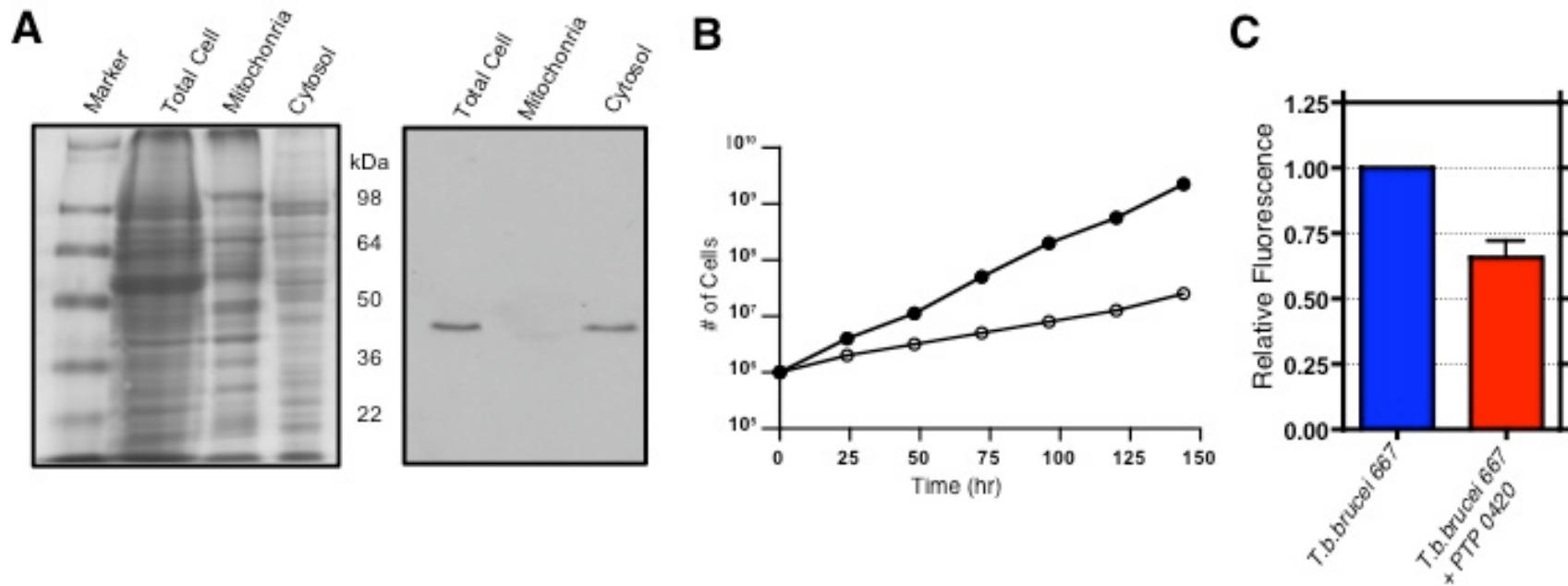


FIGURE 2.8. Analysis of PTP-tagged Tb09.v1.0420 *T.brucei* cell lines (A) Subcellular fractionation of PTP-tagged Tb09.v1.0420. Samples from total cell protein, mitochondrial and cytosolic fractions were stained with Coomassie Blue to visualize proteins (Left Panel). Western blot analysis of the gel probed with anti-Protein C. PTP-tagged Tb09.v1.0420 was effectively localized to the cytosolic fraction (Right Panel). (B) The growth rates of the PTP-tagged Tb09.v1.0420 (open circles) and wild type *T. brucei* (closed circles) were determined. (C) Measurement of mitochondrial membrane potential for wild type *T. brucei* (blue bar) and PTP-tagged Tb09.v1.0420 (Red Bar).

Mitochondrial RNA purity was also examined by probing for the cytosolic specific WT tRNA^{Met-I} (Figure 2.4). It has previously been shown that WT tRNA^{Met-i} is not imported into the mitochondria of *T. brucei* (30). By using a probe specific for WT tRNA^{Met-i}, we were able to show that our mitochondrial RNA preparations are devoid of cytosolic tRNA contamination (Figure 2.4). These studies suggest that both Tb09.v1.0420 and Tb11.01.4590 are components of the mitochondrial tRNA import complex of *T. brucei*. Interestingly Tb09.v1.0420 is the first protein found in both the trypanosome and *Leishmania* tRNA import complexes.

Identification of associated components tRNA import complex by tandem affinity purification

To further characterize the tRNA translocon of *T. brucei*, we used tandem affinity purification to identify proteins that interact with Tb11.01.4590 and Tb09.v1.0420 (31). Each protein was C-terminally PTP-tagged in order to avoid interference with a N-terminal MLS signal. However, the single allele knock in of PTP-tagged Tb09.v1.0420 resulted in a slow growth phenotype as well as a significant reduction in mitochondrial membrane potential. Subcellular fractionation analysis indicated that PTP tagged Tb09.v1.0420 was localized to the cytosol (Figure 2.8) although this protein has previously been shown to be a component of the mitochondria (32). Because the PTP-tagged Tb09.v1.0420 affected growth and mitochondrial activities it could not be analyzed further.

The PTP-tagged Tb11.01.4590 gene was expressed at levels comparable to the wildtype Tb11.01.4590 and cells exhibited normal growth and mitochondrial membrane potential (Figure 2.9A, B and C). Cell fractionation studies showed that the PTP-tagged protein of expected molecular weight (57.3 kDa) localized to the mitochondrion (Figure 2.9E). Tandem affinity purification resulted in the elution of a highly purified complex composed of several polypeptides, one the expected size (41.6 kDa) for the purified Tb11.01.4590 (Figure 2.9D). The presence of Tb11.01.4590 in the tandem affinity purified proteins was verified by western blot (Figure 2.9E). Further it was found that Tb11.01.4590 localizes to both the mitochondrial membrane and matrix fractions (Figure 2.9F). Proteins from the PTP-tagged Tb11.01.4590 purification were fractionated on SDS-PAGE and stained bands excised and analyzed by LC-MS/MS. From this analysis, nine proteins associated with Tb11.01.4590 were identified (Table 2.3). Of these 9 proteins, 7 were specifically pulled down in our tRNA binding analysis. While some of these proteins did not satisfy all of the criteria we used for further analysis, the sequence scores were highly significant (Minimum of 2 peptides with at least 1 peptide matched at >95% Confidence). Four of the proteins identified (Tim17, mHsp70, mHsp60 and mHsp20) are known to function in mitochondrial protein import or as protein chaperones in other organisms and trypanosomes (26, 33-35). Of these proteins, mHsp70, mHsp60 and mHsp20 were also purified in the initial tRNA affinity purifications and were identified by mass spectrometry (Table 2.1). Four hypothetical proteins, Tb927.6.1680, Tb09.211.2530, Tb927.8.1740 and Tb11.03.0475 were also identified by PTP-tagged Tb11.01.4590 pull downs and by tRNA affinity purification. Based on BLASTp analysis, the hypothetical protein Tb927.6.1680 contains a C2H2 zinc finger motif, however, neither Tb927.8.1740 nor Tb11.03.0475 display domain homology by BLASTp. Consistent with the requirement for tRNA import throughout the life cycle of

T. brucei, all proteins identified by the PTP-pull down of Tb11.01.4590 are constitutively expressed in both the BF and PF trypanosome life stages (36).

Finally, the identification of Tb11.01.4590 as potential component of the tRNA translocon allowed us to address the specificity of this complex for imported tRNAs. Biotinylated tRNA^{Leu(CAA)} and cytosol specific tRNA^{Met-i} were coupled to resin and used as affinity ligands for mitochondrial protein preparations (Figure 2.10). Binding to the putative tRNA translocon was evaluated using our standard purification assay monitoring the elution of PTP-tagged Tb11.01.4590 under stringent wash conditions by western blot with anti-Protein A. Both full-length tRNAs bound the PTP-tagged Tb11.01.4590 containing complex. However, binding was not non-specific. We found that intact tRNAs were needed for high affinity binding since truncated products, either the D-loop of tRNA^{Leu(CAA)} or the V-loop/T-stem of tRNA^{Leu(CAA)} were unable to bind the PTP-tagged Tb11.01.4590 containing complex (Figure 2.10).

Discussion

Here we report the identification of two mitochondrial membrane proteins (Tb11.01.4590 and Tb09.v1.0420), which are functionally involved in tRNA import. Tb09.v1.0420 is the only shared component of the RNA import machinery identified in trypanosomes and *Leishmania*. Using Tb11.01.4590 in tandem affinity purification, nine associated proteins were identified including multiple subunits of the mitochondrial protein import machinery. Seven of these proteins were also identified in LC-MS/MS

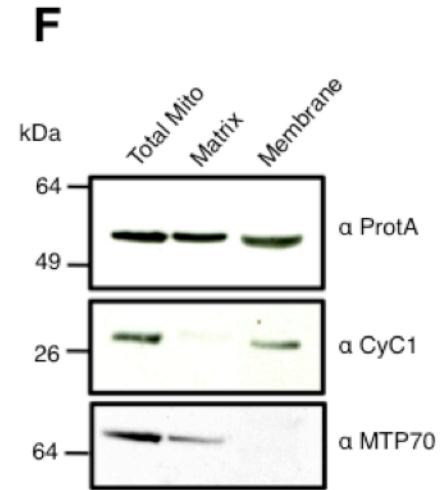
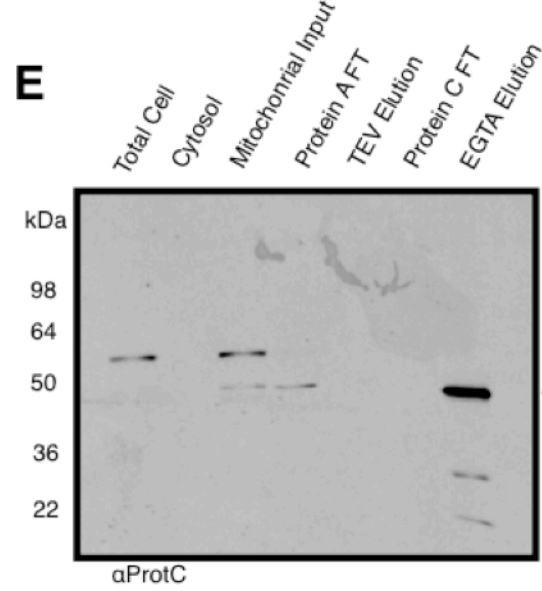
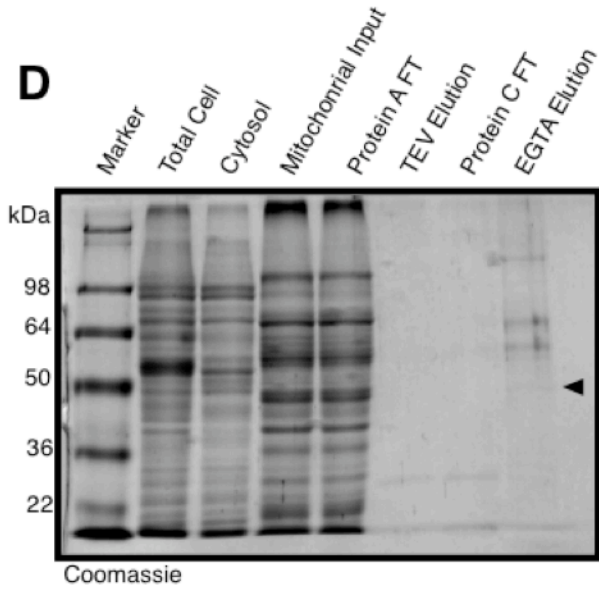
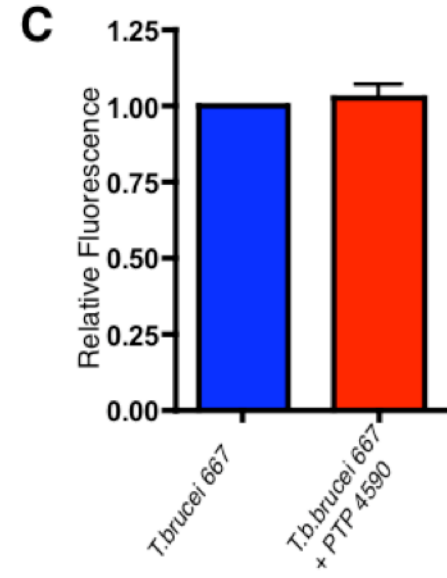
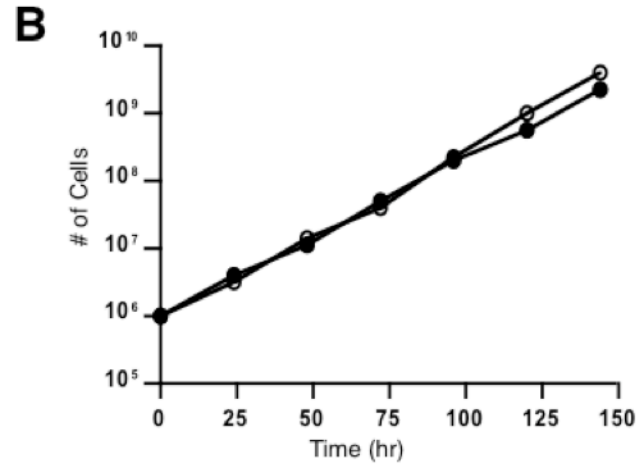
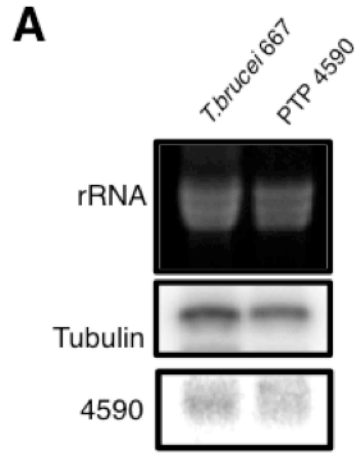


FIGURE 2.9. Purification of tRNA translocon by PTP-tagged Tb11.01.4590. **(A)** Northern blot analysis of total cellular RNA in PTP-tagged Tb11.01.4590 cell lines. The level of expression of the PTP-tagged Tb11.01.4590 mRNA was compared to the level of Tb11.01.4590 in wild type *T. brucei* (667). **(B)** The growth rates of the PTP-tagged Tb11.01.4590 (open circles) and wild type *T. brucei* (closed circles) were determined. **(C)** Measurement of mitochondrial membrane potential for wild type *T. brucei* (blue bar) and PTP-tagged Tb11.01.4590 (red bar). **(D)** Subcellular fractionation and affinity purification of PTP-tagged Tb11.01.4590 and associated proteins. Samples from total cell protein, mitochondrial and cytosolic fractions and from tandem affinity purification were stained with Coomassie Blue to visualize proteins. The flow-through (FT) and TEV protease eluate (TEV elution) from the Protein A affinity purification and the flow-through (Protein C FT) and EGTA eluate (EGTA Elution) from the Protein C affinity purification are shown. **(E)** Western blot analysis of the gel in Panel E probed with anti-Protein C. The PTP-tagged Tb11.01.4590 from the mitochondrial fraction is 57.3 kDa while the final purified protein following TEV cleavage is 41.6 kDa. **(F)** Mitochondrial fractionation and western blots of PTP-tagged Tb11.01.4590 localizes to both the matrix and membrane of the mitochondria. Cytochrome C1 (CyC1) and mitochondrial HSP70 (MTP70) were used as membrane and matrix markers respectively.

analysis of tRNA binding complexes (Table 2.1) further supporting the role for components of the protein import machinery in tRNA import.

TABLE 2.3

LC-MS/MS Identification of Proteins Purified by Tb11.01.4590 PTP Pulldowns

Gene ID	Protein ID	Pulled Down by tRNA?	Predicted MLS or TM ¹	Domain Homology ²	PC/BS mRNA Expression ³
Tb927.8.1740	Hypothetical	Yes ⁴	Mito	None	Yes
Tb11.01.4870	Tim17	No	Both	Protein Import	Yes
Tb927.3.3330	HSP20	Yes ⁴	Mito	Chaperone	Yes
Tb927.6.1680	Hypothetical	Yes ⁴	Mito	C2H2-Zinc Finger	Yes
Tb927.10.6400	HSP60	Yes	Mito	Protein Import	Yes
Tb11.03.0475	Hypothetical	Yes ⁴	Mito	None	Yes
Tb927.6.3740	HSP70	Yes	Mito	Protein Import	Yes
Tb11.02.3310	Hypothetical	Yes ⁴	Both	None	Yes
Tb09.211.2530	Hypothetical	No	Both	None	Yes

¹Predictions based on sequence analysis using MitoProt/TMHMM/TMPred software and Panigrahi et al., 2009.

²Domain homology by BLASTp analysis through NCBI/CDD.

³Life stage expression data from Nilsson et al. 2010.

⁴At least one peptide >95% confidence identified by tRNA pull down MS/MS analysis

Previous characterization of *L. tropica* led to the identification of an eleven-subunit protein complex, called the RIC, involved in the translocation of tRNAs into the mitochondrion. However, multiple protein components of the *Leishmania* RIC are subunits of mitochondrial oxidative phosphorylation complexes, including subunits IV and VI of cytochrome c oxidase and the reiske iron sulphur protein from cytochrome bc1 reductase (22). If these proteins have dual functions in mitochondrial ATP production and tRNA import in *Leishmania*, it is likely that African trypanosomes use a distinctly different tRNA translocon. Also, because of their proposed dual function in mitochondrial bioenergetics and tRNA import, care must be taken to distinguish a

primary role in tRNA translocation and simply compromising mitochondrial metabolic function.

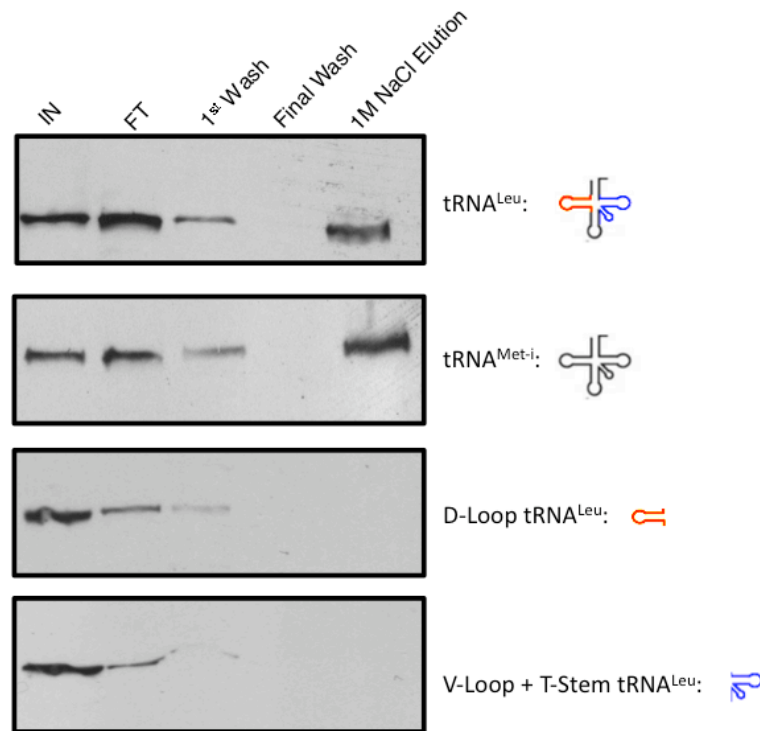


FIGURE 2.10. Binding of PTP-tagged Tb11.01.4590 containing complexes to tRNAs. Isolated, intact mitochondria from cells constitutively expressing the PTP-tagged Tb11.01.4590 protein were incubated with a resin that either contained full length tRNA^{Leu(CAA)}, the D-Loop from tRNA^{Leu(CAA)} (Red), the Variable Loop/T-stem from tRNA^{Leu(CAA)} (Blue), or the cytosolic specific WT tRNA^{Met-i}. Unbound mitochondria were washed away (IN) and the bound mitochondrial fraction was solubilized with 1% Triton (FT). After solubilization, bound protein was washed extensively using a high salt wash buffer (1st and Final Wash). Bound protein was eluted using a single 1M NaCl salt elution. PTP-tagged Tb11.01.4590 was detected by western blot analysis of the gels with anti-Protein A.

African trypanosomes have complex life cycles with extensive metabolic changes associated with transition of the parasites from the digestive tract of the insect vector to the bloodstream of the mammalian host. Whereas, the mitochondrion of the PF of the parasite is fully active, generating much of its ATP by electron transport coupled oxidative phosphorylation, the BF of *T. brucei* contains no detectable cytochromes, lacks oxidative phosphorylation and produces ATP solely by glycolysis (37-39). Transcript and proteomic analysis have verified that many of the nuclear encoded subunits of the cytochrome c oxidase and cytochrome c reductase are absent in BF *T. brucei* (38-41). Since tRNAs are imported into both PF and BF *T. brucei* mitochondria to the same extent, developmentally regulated subunits of the electron transport chain cannot be part of the constitutive tRNA translocon (25). In addition, recent studies have brought into question the role of the Reiske iron sulfur protein and the a-subunit of the F₁F₀-ATP synthase in *Leishmania* tRNA import complex (12, 23). In this study, we were able to identify only two subunits of the *Leishmania* RIC (Tb09.v1.0420 and Tb927.10.4280) by tRNA affinity purification from *T. brucei* mitochondria (Table 2.2 and Figure 2.2). These results suggest that while closely related organisms may share some components of the tRNA import machinery differences may have evolved as a consequence of distinct metabolic differences between the two. Alternatively, the presence of subunits of the mitochondrial respiratory complexes may simply reflect contamination of the *Leishmania* RIC with abundant mitochondrial membrane proteins (23). Additional studies are needed to resolve this issue.

Although much is known about the physical properties of tRNA import into trypanosome mitochondria only two mitochondrial proteins, Tim17 and mHsp70, have

been shown to be directly involved in translocation (12). Thus far, the only other protein that has been identified as having a function in mitochondrial tRNA import is the cytoplasmic eEF1a (17). Our studies fully support a role for eEF1a in tRNA import (Figure 2.3F). We also show two other trypanosome mitochondrial proteins (Tb11.01.4590 and Tb09.v1.0420) are necessary for tRNA import (Figure 2.7). Further, Tb11.01.4590 was pulled down by mitochondrial (tRNA^{Leu(CAA)}) and cytosolic (WT tRNA^{Met-i}) localized tRNAs (Figure 2.10). However, when the Variable Loop/T-Stem or the D-Loop from tRNA^{Leu(CAA)} were used as bait, Tb11.01.4590 was not pulled down. This suggests that the import complex recognizes full-length tRNA substrates and that the specificity for delivery of tRNA into mitochondria may lie within the previously characterized chaperone protein eEF1a (17), which binds specifically to the T-Stem of mitochondrially targeted tRNA and not to the cytosolic WT tRNA^{Met-i}. In addition, we identified a nine-subunit mitochondrial protein complex that interacts with Tb11.01.4590 and is composed of five hypothetical proteins and three proteins (Tim17, mHsp70 and mHsp60) that function as protein chaperones in mitochondrial protein import (Table 2.3) (26, 33, 34). The other protein identified, mHsp20, has been shown to interact with mHsp70 to refold denatured protein following import into mitochondria (35). All of the proteins identified as interacting with Tb11.01.4590 by tandem affinity purification have previously been shown to localize to the mitochondria (32). Based on the known subcellular localization of many of the identified proteins in the purified tRNA import complex (Table 2.3), it is likely that this complex represents an IMM tRNA translocon in *T. brucei*. Further, the identification of proteins known to function in mitochondrial

protein import strongly suggests that there is either a shared mechanism for mitochondrial protein and tRNA import or shared components between translocons.

The identification of components of the mitochondrial protein import machinery, as components of the *T. brucei* tRNA translocon, is consistent with mitochondrial RNA import in other systems. In yeast, it has been shown that a single nuclear encoded mitochondrial tRNA is co-imported into the mitochondrion with its corresponding pre-tRNA synthetase and requires specific interactions with components of the TIM and TOM proteins import complexes (4). Similarly, in plants, tRNA import requires interactions with Tom20 and Tom40 prior to mitochondrial translocation (5). Though specific mitochondrial components have not been identified, characterization of the mechanism of 5S rRNA import into mammalian mitochondria showed a dependence on protein import machinery and co-transport with the cytoplasmic chaperone protein rhodanese (42, 43). In each case, a shared mechanism of import has been suggested for protein and RNA translocation into mitochondria.

Recent studies have shown that the *T. brucei* Tim17 and mHsp70 are necessary for both protein and tRNA import into trypanosome mitochondria (12). Here we show that Tim 17, mHsp70 and two other proteins implemented in protein import are physically associated with the tRNA translocon of *T. brucei*. These results further support the possibility of a membrane transporter capable of moving both RNA and protein into trypanosome mitochondria and suggest that yeast, plant, mammals and trypanosomes may have functionally similar translocons. Further functional analysis of the components of the tRNA translocon will need to be conducted in order to determine if

there is truly a shared mechanism between mitochondrial protein and tRNA import in these organisms.

Materials and Methods

Trypanosome cell culture and mitochondrial purification

T. brucei 667 (TREU 667) and *T. brucei* 29-13 strains were used and were maintained in a SM culture media supplemented with 10% (v/v) heat inactivated fetal bovine serum (44). For tRNA and Protein C-TEV-Protein A (PTP) affinity purification experiments, mitochondria were isolated from procyclic form (PF) *T. brucei* TREU 667 and mitochondrial membrane and matrix fractions were prepared by differential detergent extraction of hypotonically isolated mitochondria (0.5% Triton X-100 for matrix followed by 2% n-Dodecyl β -D-maltoside for membrane) as previously described (45, 46). Mitochondrial matrix and membrane localization of PTP tagged proteins were determined by probing for the protein A domain (PAP Antibody, Abcam) Transmission electron microscopy of isolated mitochondria was conducted as previously described (47).

Native tRNA binding assay

Uniformly labeled *T. brucei* tRNA^{Leu (CAA)} (α -³²P-UTP, 800 Ci/mmol) was generated by *in vitro* transcription by T7 polymerase using a DNA template with a T7 promoter according to the manufacturer's recommended procedure (Ambion, MEGashortscript High Yield Transcription Kit). To identify the tRNA import complex,

solubilized total mitochondrial (2% n-Dodecyl β -D-maltoside) extracts were prepared from 4×10^9 cells. Each sample was mixed with an equal volume of a protein binding buffer (160 mM MOPS, 310 mM sucrose, 6.25 mM MgCl_2 , 100 mM KCl, 9 mM DTT, 2 units/300 μl SUPERase-in (Ambion), 1 mg/ml BSA) and incubated for 30 minutes at 4°C with uniformly labeled $\text{tRNA}^{\text{Leu}(\text{CAA})}$. Following incubation, mitochondrial protein (30 μg) and associated tRNA were run for 18 hours at 4°C on blue native gradient gels (4-13%) (27). Following electrophoresis, gels were dried and exposed to x-ray film (Kodak, Biomax MS) to visualize tRNA-protein complexes.

tRNA affinity purification of mitochondrial membrane complexes

For tRNA affinity purification, 500 μl of paramagnetic streptavidin resin (SA) (DYNAL Magnetic Beads, Invitrogen), containing 6.7×10^8 - beads/ml, was washed three times in 500 μl of 0.5 x SSC buffer (1 x SSC; 150 mM NaCl, 15 mM tri-sodium citrate dihydrate, pH 7.2). The washed resin was resuspended in 0.5 x SSC, synthetic 5' biotinylated *T. brucei* $\text{tRNA}^{\text{Leu}(\text{CAA})}$ (Dharmacon) was added to a final concentration of 0.2 μM and incubated at 65°C for 10 minutes to form the tRNA-streptavidine affinity resin. After incubation, the tRNA bound resin was washed three times with 300 μl of 0.1 x SSC and equilibrated in 300 μl of protein-binding buffer (see above). tRNA binding complexes were affinity purified following binding to intact mitochondria or detergent extracts (1% Triton X-100) of total mitochondria or purified mitochondrial membranes. Each of the starting samples were adjusted to 6.0×10^9 cell equivalents and volumes were adjusted to 300 μl with protein binding buffer. Samples were incubated with the tRNA affinity resin for 30 minutes at 4°C , unbound protein was collected and the tRNA affinity

resin was washed six times in 500 μ l of protein binding buffer prior to elution of tRNA bound proteins using buffers containing increasing ionic concentrations of NaCl (0.1M to 1M NaCl; 20 mM MOPS, pH 7.2). Protein samples from each elution were precipitated and fractionated on 8-12% SDS-PAGE. For LC-MS/MS analysis, tRNA bound proteins were eluted with 20 mM MOPS, 1M NaCl, pH 7.2 and analyzed directly. Mitochondrial proteins were also incubated with paramagnetic streptavidin resin without bound tRNA as a control in order to identify proteins that bound to the resin nonspecifically. To evaluate the specificity of binding, synthetic 5' biotinylated *T. brucei* specific tRNA^{Leu(CAA)}, tRNA^{Met-i}, tRNA^{Leu(CAA)} D-Loop (nt 9-27) and tRNA^{Leu(CAA)} Variable Loop/T-Stem (nt 44-74) pull downs of PTP-tagged Tb11.01.4590 were conducted exactly the same, except SUPERase-in (Ambion) was removed from the binding procedure as it was found to cross react with both Protein A/PAP (Abcam) and Protein C (Delta Biolabs) polyclonal antibodies. Also, prior to a single NaCl elution (1M NaCl, 20mM MOPS, pH 7.2), the bound fraction was washed six times in 600 μ l of binding buffer in which the concentration of KCl had been increased to 300mM.

Subtractive mass spectrometry analysis

Using the tRNA affinity purification strategy, mitochondrial proteins that specifically bound tRNA and those that nonspecifically interacted with the paramagnetic resin were prepared. Both samples were analyzed by LC-MS/MS (Thermo Fisher LTQ Linear Ion Trap) and peptides identified from the resin only sample were subtracted from the indexed proteins specifically bound to the *T. brucei* tRNA^{Leu(CAA)} resin. Domain homology for each protein was determined by using the protein-protein BLAST

(BLASTp) algorithm from the National Center for Biotechnology Information (NCBI) and the Conserved Domain Database (CDD).

Inducible expression of a variant tRNA^{Met-i}

A tetracycline inducible tRNA expression construct was previously generated and characterized (17). This tRNA is a variant of *T. brucei* tRNA^{Met-i} (Var-tRNA^{Met-i}), which contains an internal sequence tag within the D-loop and the T-stem from tRNA^{Met-e} (Figure 2.11). This construct has been shown to be aminoacylated and processed upon mitochondrial import by *in vitro* and *in vivo* assays (17, 48). The original construct containing the phleomycin resistance gene was replaced with the puromycin resistance gene by use of the *MscI* restriction sites flanking the phleomycin gene. Upon *NotI* digestion, linearized vector was electroporated and transfected into *T. brucei* 29-13 cell lines.

RNAi to validate candidate tRNA import complex proteins

All RNAi constructs were prepared in the doxycycline inducible pZJM vector containing opposing T7 promoters (49). RNAi inserts for eIF2 and eEF1a were generated as previously described (17). Inserts for Tb09.v1.0420 (nucleotides 1-597), Tb927.10.4280 (nucleotides 1-606), Tb927.10.15220 (nucleotides 1-438), Tb11.01.4590 (nucleotides 1-787), Tb11.02.0445 (nucleotides 1-282), gBP21 (nucleotides 1-621), gBP25 (nucleotides 1-675) were also prepared. Constructs were linearized by cleavage with *NotI* and transfected into the clonal cell line containing the inducible Var-tRNA^{Met-i} gene. Upon induction with doxycycline each cell line simultaneously expressed the Var-tRNA^{Met-i} and double stranded RNA to knockdown expression of targeted proteins.

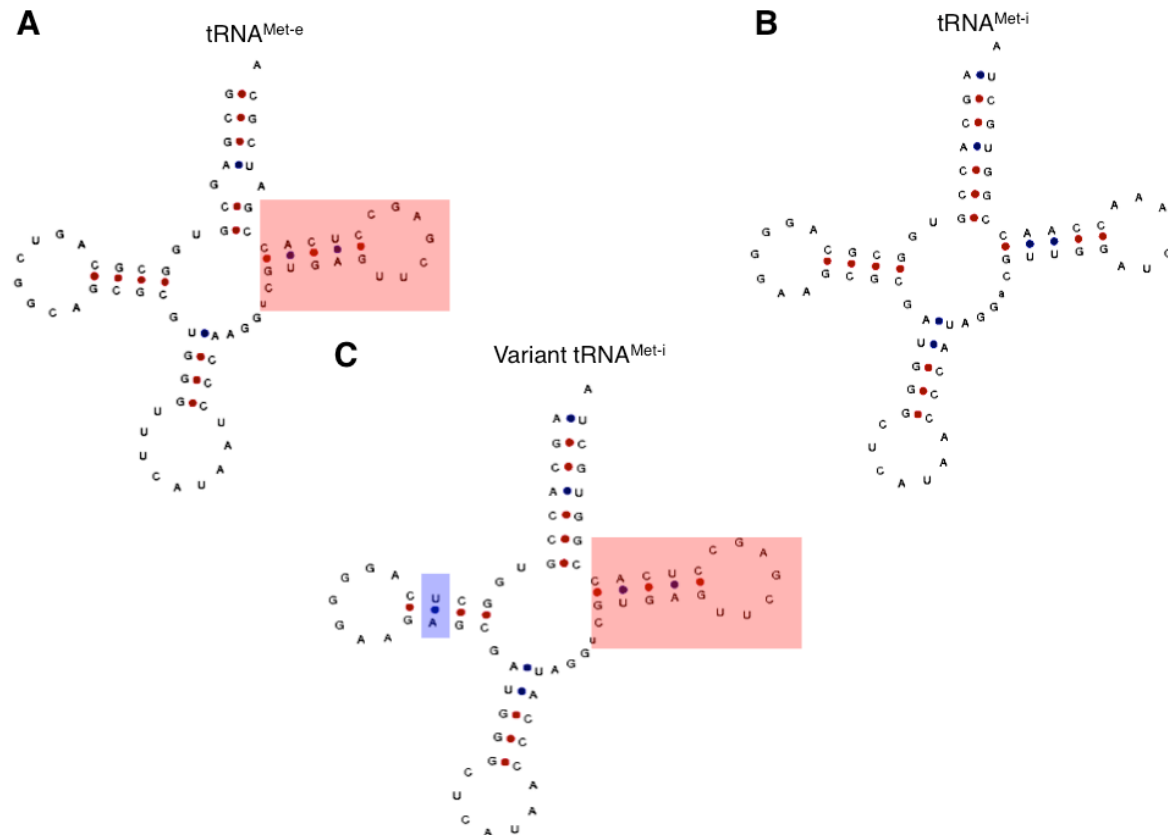


FIGURE 2.11. Predicted secondary structures of (A) WT tRNA^{Met-e} (B) WT tRNA^{Met-i} (C) Var-tRNA^{Met-i} - Blue box highlights area where the D-loop sequence was mutated to an U:A base pair (12:22), generating a unique sequence tag. Red box highlights the T-stem from WT tRNA^{Met-e}, which was used to replace the T-stem from the WT tRNA^{Met-i}. Together, the D-loop (Blue) and T-stem (Red) modifications of the WT tRNA^{Met-i} were used to generate the Var-tRNA^{Met-i}. Models generated by tRNAscan-SE software.

Measurement of mitochondrial membrane potential

RNAi cell lines for each protein were induced with doxycycline for 24, 48 or 72 hours to determine the effect knockdown of each candidate protein had on mitochondrial membrane potential. Samples from untreated and doxycycline induced (1 μ g/ml) cells were incubated with the cationic fluorescent dye tetramethyl rhodamine methyl ester (TMRM) (20 nM) for 10 minutes. TMRM rapidly accumulates in the mitochondria and has previously been used to determine the mitochondrial membrane potential by use of microfluoremetry (50). After incubation with TMRM, cells were centrifuged and the pellet resuspended in fresh SM media and allowed to equilibrate for 30 minutes followed by two washes with PBS. Washed cells were resuspended at a final concentration of 4 x 10⁶ cells/ml in SM media. Mitochondrial membrane potential was then determined by analyzing 2 x 10⁶ total cells in a microfluoremeter with an excitation of 548 nm and emission of 573 nm (Luminescence Spectrometer LS 55, Perkin Elmer).

RNA isolation and quantization of imported tRNA

Mitochondrial and total cellular RNA was prepared from cells as previously described (17). Briefly, 4 x 10⁸ cells were resuspended in 500 μ L 20 mM Tris-HCl, pH 7.5, 0.6 M sorbitol, 2 mM EDTA, pH 7.5 containing 0.05% digitonin and incubated for 5 minutes on ice. Cells were then centrifuged (8,000 x g) at 4°C for 5 minutes and the supernatant was discarded. The pellet containing the crude mitochondrial fraction was then resuspended in 500 μ L of micrococcal nuclease buffer (10 mM Tris, 1mM CaCl₂, pH 8.0, 10% glycerol) containing 300U of micrococcal nuclease (USB) and incubated at

room temperature for 30 minutes to digest contaminating cytosolic RNAs. The nuclease reactions were stopped by the addition of 10 mM EDTA. The crude mitochondrial fraction was then centrifuged (8,000 x g) at 4°C for 5 minutes and the supernatant was discarded. RNA was extracted from the mitochondrial and total cellular fractions with TriPure according to the manufacturer's recommendation (Roche). Total cellular and mitochondrial RNAs from 2×10^7 cells were fractionated on 8 M urea/8 % polyacrylamide-sequencing gels. Gels were stained with ethidium bromide to visualize RNAs prior to transfer to a positively charged nylon membrane (Amersham Hybond N+, GE Healthcare) and subsequent probing with ^{32}P end labeled probe specific for the D-loop sequence tag of inducible Var-tRNA^{Met-i} (CGCTCTTCCCCTGAGCCA), with a probe specific for the T-Stem of the WT tRNA^{Met-i} (GTTGGTTTTCGATCCAACG) or with a probe specific for the mitochondrial encoded 9S rRNA (AATTACCGCAACGGCTGGCTGGCATCCATTTCTGAC). The amount of total cellular and mitochondrial tRNA were quantified by exposure of the probed membrane to a phosphorimager plate (Storage Phosphor Screen, Molecular Dynamics) and then developed and analyzed by PhosphorImager (GE healthcare, model STORM-860).

Tandem affinity of PTP Tb11.01.4590 and associated proteins

Constructs for PTP-Tb11.01.4590 (Nucleotides 276-1047) were designed with *Apal* and *NotI* restriction sites to clone into the pC-PTP-Neo vector (31). PTP tagged Tb11.01.4590 was linearized with *BarI* (SibEnzyme) and transfected into PF *T. brucei* TREU667 targeting a single allele for the endogenous expression of the PTP-tagged construct. Isolated mitochondria from 7.2×10^{10} cells were solubilized as previously

described by resuspending (400 μ g mitochondria/40 μ L) in Solubilization Buffer A (50 mM NaCl, 50 mM Imidazole/HCl, 2 mM 6-Aminohexanoic Acid, 1 mM EDTA, pH 7.0) containing 2 % dodecyl maltoside in a rotator for 30 minutes at 4°C (27). PTP tagged protein complexes were subsequently purified as described previously (51). Samples from the tandem affinity purification were examined by 8-10% polyacrylamide gel electrophoresis and immunoblot with anti-Protein C (Delta Biolabs). Eluted proteins were excised from gels and analyzed by LC-MS/MS (Thermo Fisher LTQ Linear Ion Trap). Raw tandem mass spectra were converted to mzXML files then into mascot generic files via the Trans-Proteomic Pipeline (Seattle Proteome Center, Seattle, WA). MGF files were searched using Mascot (Matrix Scientific, Boston, MA) against target and decoy National Center for Biotechnology Information (NCBI) databases for trypanosome proteins. ProteoIQ (Nusep, Bogart, GA) was used for data analysis and a 5% protein false discovery rate was applied to confirm the presence of proteins.

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CHAPTER 3

A TIM17 CONTAINING MITOCHONDRIAL MEMBRANE COMPLEX IS
REQUIRED FOR BOTH PROTEIN AND tRNA IMPORT INTO *TRYPANOSOMA*
BRUCEI MITOCHONDRIA¹

¹ Seidman, D., Johnson, D., Orlando, R., Hajduk, S.L. To be submitted to The Journal Of Biological Chemistry

Abstract

The import of nuclear encoded proteins and RNAs into mitochondria is essential for all eukaryotes and provides a reminder of the emergence of this organelle from a symbiotic origin. In most eukaryotes, the mitochondrial protein translocon is compositionally and mechanistically conserved with homologues to both the Translocase Inner Membrane (TIM) and Translocase Outer Membrane (TOM) readily identifiable. An exception is found in the members of the Order Kinetoplastidae. A sole component of the canonical TIM/TOM complex, a highly divergent homologue of Tim17, has been identified. Recent studies have presented evidence for a novel macromolecular translocon, necessary for both protein and RNA import into trypanosome mitochondria. Here we identify a Tim17 containing mitochondrial membrane complex in *Trypanosoma brucei* required for both tRNA and protein import. This translocon was purified from mitochondrial membranes by the tandem affinity purification of Tim17 and subsequent identification of constituent proteins by LC-MS/MS. In this analysis, components of a recently characterized tRNA import complex were identified. Further, the Tim17 containing complex specifically interacts with tRNA substrates. Using *in vivo* protein and tRNA import assays, in combination with RNAi, we show that both proteins and tRNAs are imported into the mitochondria of *T. brucei* by the same translocon. This represents the first characterization of a mitochondrial import complex functional for both tRNA and protein import.

Introduction

Macromolecular import of RNA and proteins is necessary for mitochondrial inheritance and function in all eukaryotes. Despite the universal need for membrane channels to facilitate movement from the cytosol to the mitochondrial matrix, distinct translocation mechanisms have evolved. Among the most divergent is the protein import machinery in the early branching eukaryote, *Trypanosoma brucei*. In this organism, the canonical Translocase Outer Membrane (TOM) and Translocase Inner Membrane (TIM) complexes are absent and only a highly divergent homologue of Tim17 has been identified (1). Recently, a novel mitochondrial import channel of *T. brucei* containing an ortholog the bacterial Omp85 superfamily, termed the archaic translocase of the outer mitochondrial membrane (ATOM) was identified (2). In bacteria, this protein is involved in protein secretion and assembly of outer membrane complexes suggesting that trypanosomes use a vestige of an ancient protein translocase to move macromolecules into the mitochondrion (1-3).

Despite the physical differences between tRNAs and proteins, both molecules are imported into mitochondria of yeast, plants and humans by the same mitochondrial translocon, albeit by very different mechanisms (4-7). Independent studies on tRNA and protein import in *T. brucei* have shown that Tim17 is necessary for import of both molecules and that Tim17 has also been identified as a component of a tRNA binding complex localized to mitochondrial membranes (1, 8, 9). While these results suggested that Tim17 is a necessary component of both tRNA and proteins translocons, they fail to distinguish whether import occurs through a single macromolecular translocase or

whether distinct protein and tRNA import complexes, sharing Tim17 as a common component, function independently.

Consistent with a single translocon model, many of the biophysical features are shared between trypanosome mitochondrial protein and RNA import, including requirements for ATP hydrolysis and mitochondrial membrane proteins. A potentially important difference in mitochondrial protein and tRNA import in trypanosomes is the requirement for an electrochemical gradient across the inner mitochondrial membrane however, this remains unresolved since contradictory findings have been reported (1, 8, 10-14).

Here we show that the mitochondrial tRNA and protein translocon in *T. brucei* share the same proteins and has similar energetic requirements. We have established a physical and functional linkage network showing that Tim17, mitochondrial HSP60 (mHSP60), mitochondrial HSP20 (mHSP20), mitochondrial HSP70 (mHSP70) and three hypothetical proteins (Tb927.8.1740, Tb09.v1.0420 and Tb11.01.4590) function both in tRNA and protein import within the same essential mitochondrial membrane translocon. In addition, dissipation of the mitochondrial membrane potential resulted in identical inhibition kinetics for both protein and tRNA import further supporting a shared translocon model.

Results

A role for Tim17 in tRNA Import

Previously, we identified a mitochondrial membrane complex that specifically interacts with tRNAs and showed that two of the protein components, Tb09.v1.0420 and Tb11.01.4590, were necessary for tRNA import into the *T. brucei* mitochondrion (9). To identify other components of this membrane complex, Tb11.01.4590 was tagged with Protein C-TEV-Protein A (PTP) for tandem affinity purification (9). Proteins associated with PTP-Tb11.01.4590 were analyzed by LC-MS/MS and eight proteins were identified with high confidence. Two of these proteins, Tim17 and Tb927.8.1740, were analyzed for their role in tRNA import using inducible tRNA expression/RNAi cell lines to determine the gene specific effect of RNAi knockdown on tRNA translocation (Figure 3.1). RNAi induction showed that both Tb927.8.1740 and Tim17 were essential for sustained growth, with arrest occurring between 72-96 hours post induction. However, no affect on cell growth was observed during the initial 24 hours of induction (Figure 3.1A). During this initial 24 hour period, the levels of both Tb927.8.1740 and Tim17 mRNA were reduced relative to uninduced cells (24% and 37% of uninduced respectively) without a change in the levels of tubulin mRNA (Figure 3.1B). To ensure that overall function of the mitochondrion in RNAi cells was unimpaired we measured the mitochondrial membrane potential, as an indicator of electron transport generated proton gradient. No decrease in the membrane potential was detected up to 48-72 hours post induction (Figure 3.1C). Based on the growth and mitochondrial membrane potential, tRNA and later protein import was measured after 24 hours of RNAi induction.

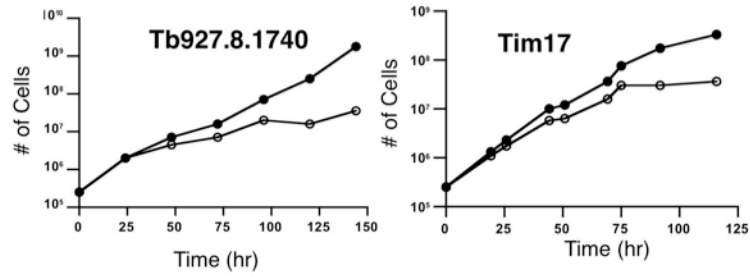
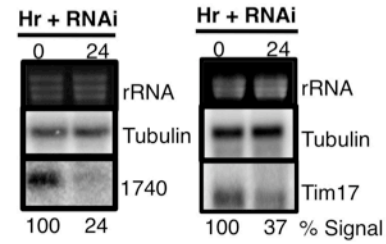
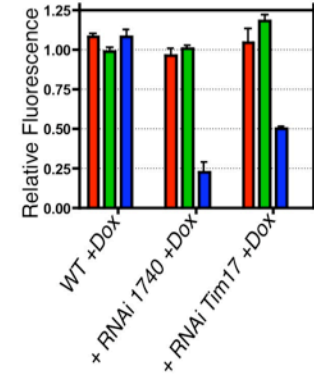
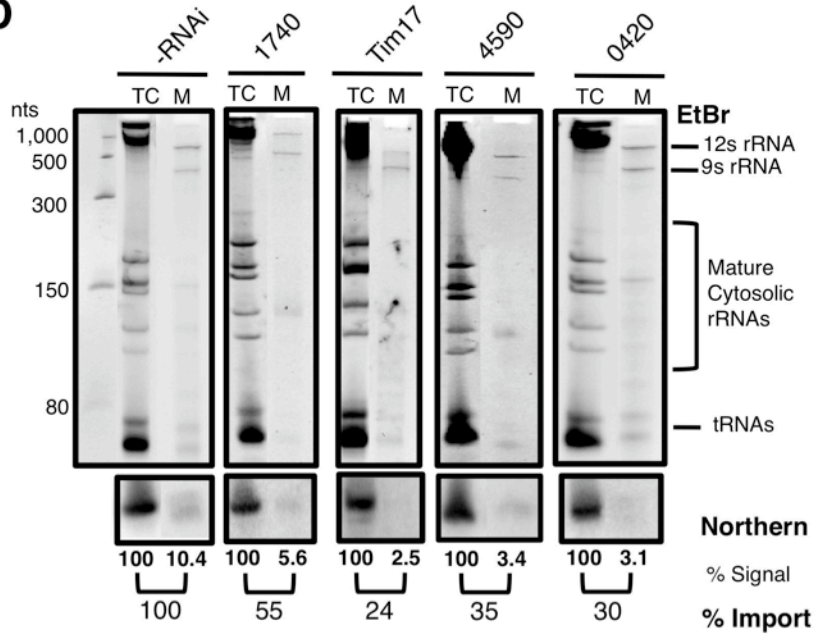
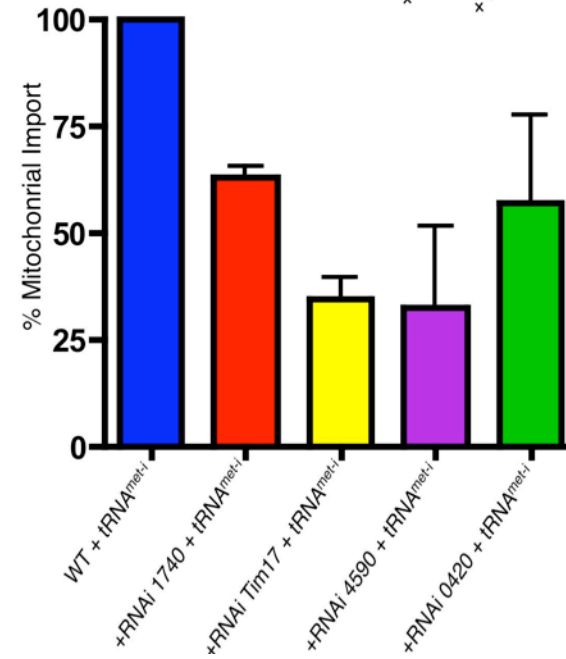
A**B****C****D****E**

FIGURE 3.1. RNAi analysis of tRNA import complex components. **(A)** Growth of Tb927.8.1740 and Tim17 in the presence (open circle) or absence (closed circle) of doxycycline (1 $\mu\text{g/ml}$). **(B)** Northern blot analysis of the effects of RNAi on mRNA levels for Tb927.8.1740 and Tim17. Total RNA was collected from cells at 0 and 24 hours post RNA induction with 1 $\mu\text{g/ml}$ doxycycline and the percentage mRNA signal for Tb927.8.1740 and Tim17 at each time point was normalized to β -tubulin. **(C)** Analysis of mitochondrial membrane potential in wild type *T. brucei* 29-13 and RNAi cell lines for Tb927.8.1740 and Tim17 in the presence of 1 $\mu\text{g/mL}$ doxycycline. Samples were collected in triplicate at 24 (Red Bar), 48 (Green Bar) and 72 (Blue Bar) hours and the mitochondrial membrane potential of 2×10^6 cells was determined by the relative fluorescence of 20 nM TMRM mitochondrial accumulation. **(D)** *In vivo* tRNA import analysis from RNAi Tb927.8.1740, Tim17, Tb11.01.4590 and Tb09.v1.0420 cell lines. Total cellular (TC) and mitochondrial (M) RNA was isolated 24 hours post induction with 1 $\mu\text{g/ml}$ doxycycline and fractionated by non-denaturing polyacrylamide gel electrophoresis (8%) (Top Panel) and abundant rRNA and tRNA were visualized by ethidium bromide (EtBr) staining. The Var-tRNA^{Met-i} was detected by northern blot hybridization and autoradiography (bottom panel). Percent import was expressed as the relative hybridization of mitochondrial to total cell Var-tRNA^{Met-i}. **(E)** Quantitative analysis of relative tRNA import for Tb927.8.1740 (n = 9), Tim17 (n = 8), Tb11.01.4590 (n = 8) and Tb09.v1.0420 (n = 6). tRNA import was expressed relative to Var-tRNA^{Met-i} import in wild type cells (10.3%, n = 8).

Stable trypanosome lines were prepared which allowed for the simultaneous induction of a sequence tagged tRNA (Var-tRNA^{Met-i}), allowing measurement of import of a newly synthesized tRNA, and RNAi induction of Tb927.8.1740 and Tim17. At 24 hours post-induction the level of mitochondrial tRNA import was reduced for both the Tb927.8.1740 and Tim17 RNAi cell lines to 55% and 24% of wild type levels respectively (Figure 3.1D & E). Control RNAi studies, with the previously characterized Tb09.v1.0420 and Tb11.01.4590 cell lines, showed similar inhibition of tRNA import after 24 hours of induction (35% and 30% of wild type levels) (Figure 3.1D & E). Further, RNAi knock down of the essential mitochondrial RNA binding protein gBP25 did not have an effect on tRNA import (9). These findings are consistent with a previous characterization of Tim17, which showed an involvement in tRNA import (8).

Identification of Tim17 Associated Proteins

To determine the composition of the Tim17 complex, the protein was modified for tandem affinity purification by the addition of a C-terminal PTP-tag. The PTP-Tim17 gene was linearized and cloned into the endogenous Tim17 locus of *T. brucei*. Cells expressing the PTP-tagged Tim17 gene at wild type levels, grew normally and did not show altered mitochondrial activity as measured by membrane potential (Figure 3.2 A, B & C). Sub-cellular fractionation showed that the PTP-tagged Tim17 was mitochondrial and largely localized to membrane fractions (Figure 3.2D, E & F). Tim17 associated proteins were recovered by tandem affinity purification, fractionated on SDS-PAGE and analyzed by LC-MS/MS. The recovered Tim17 complex contained seven proteins (Table 3.1) of which five were previously identified as part of a tRNA binding complex in the trypanosome mitochondrial membrane. The sixth protein, Tim17, will be shown later to

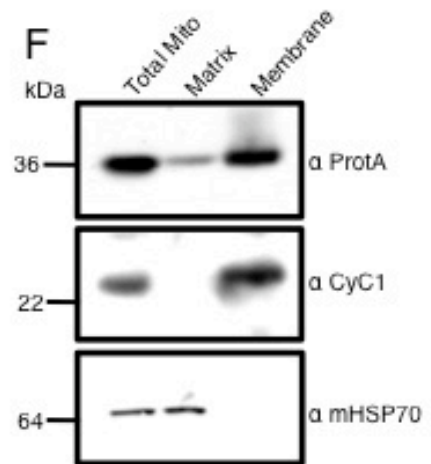
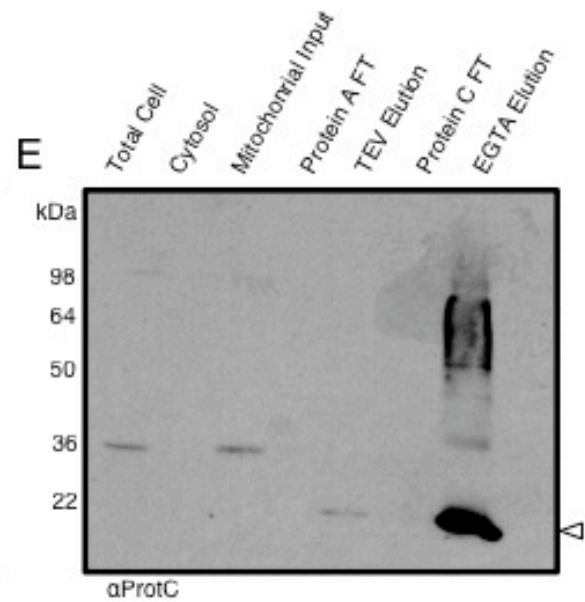
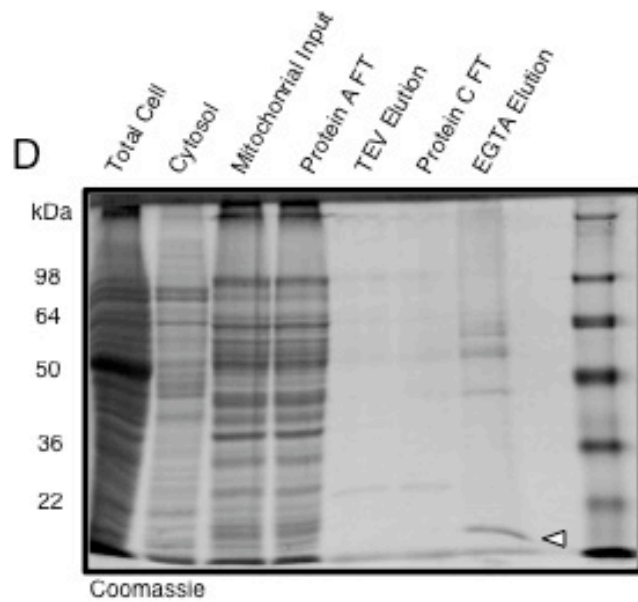
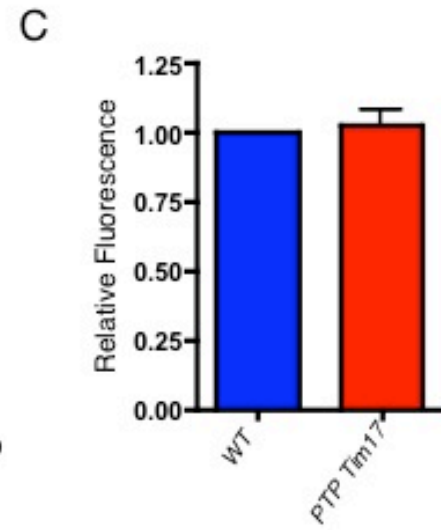
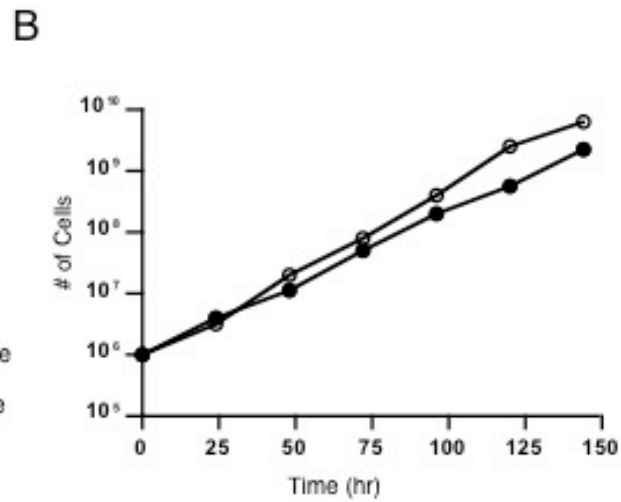
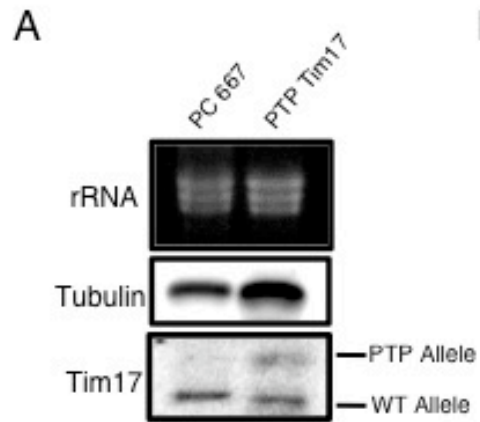


FIGURE 3.2. Purification and localization of PTP-Tim17 associated proteins. **(A)** Northern blot analysis of total cellular RNA in PTP-Tim17 cell lines. The level of expression of the Tim17 PTP-Allele and WT Allele mRNA were compared to wild type *T. brucei* (TREU667) Tim17 mRNA levels. **(B)** The growth rates of PTP-Tim17 (open circles) and wild type *T. brucei* (closed circles) were determined. **(C)** Mitochondrial membrane potential of 2×10^6 cells was determined for wild type *T. brucei* (blue bar) and PTP-Tim17 (red bar) ($n = 3$) **(D)** Subcellular localization and tandem affinity purifications of proteins associated with PTP-Tim17. Protein samples from total cell, cytosolic, mitochondrial and tandem affinity purification fractions were visualized with Coomassie Blue staining. The flow through (Protein A FT) and TEV protease elution (TEV elution) from Protein A affinity purification and the flow through (Protein C FT) and EGTA eluate (EGTA Elution) from the Protein C affinity purification are shown. White hatch mark indicates Protein C-Tim7 elution product. **(E)** Western blot analysis of gel from Panel D, probed with Anti-Protein C. PTP-Tim17 from the mitochondrial fraction is 35.6 kDa while the final purification product after TEV cleavage is 20.1 kDa. White hatch mark indicates Protein C-Tim7 elution product. **(F)** Mitochondrial fractionation and western blots of PTP-Tim17 localizes mainly to the membrane fraction. Cytochrome c1 (CyC1) and mitochondrial HSP70 (mHSP70) were used as markers for the membrane and matrix fractions respectively.

TABLE 3.1

LC-MS/MS Identification of Proteins Purified by Tim17 PTP Pulldowns

Gene ID	Protein ID	PTP Purification ID ¹	Pulled Down by tRNA? ²	Predicted MLS or TM ³	Domain Homology ⁴	PC/BS mRNA Expression ⁵
Tb927.8.1740	Hypothetical	PTP 4590 & PTP Tim17	Yes	Mito	None	Yes
Tb11.01.4870	Tim17	PTP 4590 & PTP Tim17	Yes ⁶	Both	Protein Import	Yes
Tb927.3.3330	HSP20	PTP 4590 & PTP Tim17	Yes	Mito	Chaperone	Yes
Tb927.10.6400	HSP60	PTP 4590 & PTP Tim17	Yes	Mito	Protein Import	Yes
Tb927.6.3740	HSP70	PTP 4590 & PTP Tim17	Yes	Mito	Protein Import	Yes
Tb11.02.3310	Hypothetical	PTP 4590 & PTP Tim17	Yes	Both	None	Yes
Tb09.211.2530	Hypothetical	PTP 4590 & PTP Tim17	No	Both	None	Yes

¹ Identification of PTP 4590 binding partners from Seidman et al. 2012.

² Data from Seidman et al., 2012.

³ Predictions based on sequence analysis using MitoProt/TMHMM/TMPred software and Panigrahi et al., 2009.

⁴ Domain homology by BLASTp analysis through NCBI/CDD.

⁵ Life stage expression data from Nilsson et al. 2010.

⁶ Tim17 specifically pulled down by tRNA (Figure 3).

be part of a tRNA binding complex (Figure 3.3) (9). Furthermore, all seven proteins that were pulled down by PTP-tagged Tim17 were also co-purified by PTP-tagged Tb11.01.4590, a component of the putative tRNA translocon of *T. brucei* (9).

The finding that mitochondrial membrane complexes purified by PTP-Tb11.01.4590 or PTP-Tim17 share protein components suggest that protein and tRNAs are imported into trypanosome mitochondria by the same machinery. If this is the case, Tim17 should be in a complex that binds tRNA. To address this question, purified *T. brucei* mitochondria were incubated with synthetic, biotinylated *T. brucei* tRNA substrates, tRNA^{Leu} (CAA) and tRNA^{Met-i} or a mitochondrially encoded small RNA (guide RNA A6ES1) coupled to paramagnetic streptavidin resin. Binding to these RNAs was monitored by western blot analysis with anti-Protein A to detect the PTP-Tim17 (Figure

3.3A). The Tim17 complexes bound both tRNA substrates but not bind the mitochondrially encoded guide RNA. Identical binding specificity was observed for the previously characterized PTP- Tb11.01.4590 (Figure 3.3B) (9).

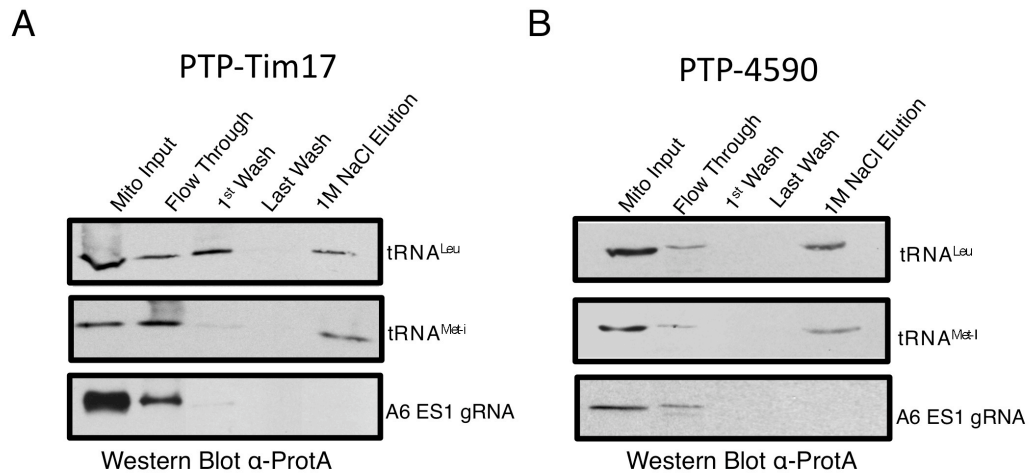


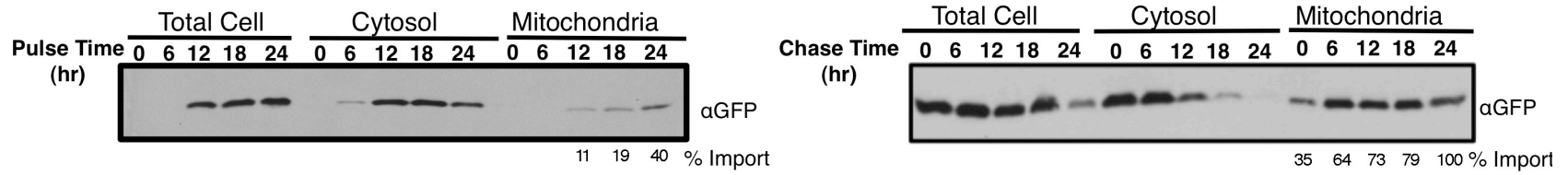
FIGURE 3.3. Binding of PTP-Tim17 containing complexes to RNA. **(A)** Isolated, intact mitochondria from cells constitutively expressing the PTP-Tim17 protein were incubated with a resin that either contained full length tRNA^{Leu(CAA)}, the cytosolic specific wild type tRNA^{Met-i} or A6 ES1 gRNA. Starting mitochondrial protein (input), unbound protein (flow through), samples from the first (1st wash) and last (last wash) high salt washes and the bound proteins released with 1 M NaCl (1 M NaCl Elution). PTP-Tim17 was detected by western blot analysis of 10% SDS-PAGE with anti-Protein A. **(B)** As described above for PTP-Tim17, isolated, intact mitochondria from cells constitutively expressing the PTP-4590 were incubated with a resin that contained either full length tRNA^{Leu(CAA)}, the cytosolic specific wild type tRNA^{Met-i} or A6 ES1 gRNA. PTP-4590 was detected by western blot analysis of 10% SDS-PAGE with anti-Protein A.

An In Vivo Assay for Mitochondrial Protein Import

To evaluate the role of the Tim17 complex in protein import, doxycycline regulated cell lines were developed that allowed inducible expression of the Green Fluorescence Protein with an N-terminal mitochondrial localization sequence (MLS-GFP) as well as simultaneous expression of an RNAi cassette to knockdown mRNA levels for a specific gene. The regulated expression and mitochondrial trafficking of MLS-GFP were evaluated by cell fractionation in pulse-chase experiments (Figure 3.4). In the absence of doxycycline ($T = 0$), there was no detectable MLS-GFP expression, showing that induction of the construct is tightly regulated (Figure 3.4A). Expression of MLS-GFP, in total *T. brucei* extracts and in cytosolic and mitochondrial fractions was followed for 24 hours post induction with doxycycline by western blotting with anti-GFP (Figure 3.4A). After 24 hours of induction (pulse samples), the levels of MLS-GFP were roughly equal in the cytosolic and mitochondrial samples (60 and 40% respectively) and the cytosolic MLS-GFP was efficiently translocated into the mitochondria when doxycycline was removed (chase samples) (Figure 3.4 A & B). These results show that MLS-GFP expression and localization can be used to measure mitochondrial protein import *in vivo*.

MLS-GFP lines were co-transfected with RNAi cassettes and the *in vivo* import assay was used to evaluate the role of Tim17 complex proteins in mitochondrial import. Having found that multiple components of the tRNA translocon are known components of the mitochondrial protein import machinery and that the tandem affinity purification of Tim17 yielded a similar protein complex, we wanted to determine whether this complex also functioned in mitochondrial protein import. RNAi knockdown of Tim17 has been

A



B

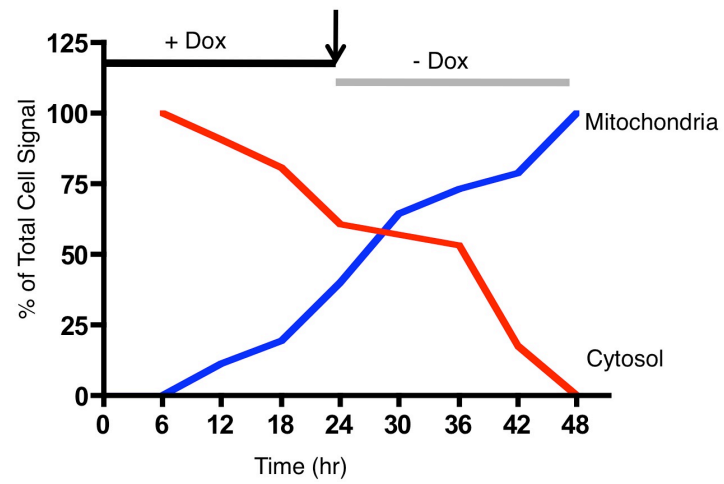


FIGURE 3.4. An *in vivo* assay for protein import in *T. brucei*. **(A)** Time course of expression and localization of mitochondria targeted MLS-GFP. *T. brucei* containing the inducible MLS-GFP construct were treated (Pulse) with 1 $\mu\text{g/ml}$ doxycycline and samples were collected every 6 hours out to 24 hours post induction. The distribution of MLS-GFP in total cell, cytosol (cyto) and mitochondria (mito) was determined by cell fractionation followed by western blot with anti-GFP. **(B)** After 24 hours doxycycline was removed and MLS-GFP localization was followed for another 24 hours (Chase). The percent mitochondrial signal was determined relative to total cellular MLS-GFP for each time point. **(C)** Analysis of MLS-GFP import from the cytosol to the mitochondria during the pulse-chase induction. Cells were treated with 1 $\mu\text{g/ml}$ doxycycline for 24 hours (black bar) prior to washing the doxycycline out of the culture (arrow) and analyzing import for another 24 hours in the absence of doxycycline (grey bar). MLS-GFP distribution in the cytosol (red line) and mitochondria (blue line).

shown to significantly impair mitochondrial protein import *in vitro* (1). RNAi knockdown of Tim17 in cells expressing the MLS-GFP protein reduced import to 20% of wild type levels (Figure 3.5A & B). In addition to Tim17, three other proteins, Tb927.8.1740, Tb09.v1.0420 and Tb11.01.4590, have been shown to be necessary for efficient tRNA import (Figure 3.1D & E) (9). To determine whether these proteins are also required for protein import, cell lines containing both the doxycycline inducible MLS-GFP and RNAi constructs for Tim17, Tb11.01.4590, Tb09.v1.0420 and Tb927.8.1740 were prepared. After 24 hours of RNAi induction, the mitochondrial levels of MLS-GFP were determined in the Tim17, Tb11.01.4590, Tb09.v1.0420 and Tb927.8.1740 RNAi cell lines. Protein import was reduced to 20, 38, 42 and 28% of wild type levels respectively (Figure 3.5A & B). Control RNAi knockdown of the essential mitochondrial RNA binding protein gBP25, which is not associated with the Tim17 complex, resulted in little or no reduction in protein import (Figure 3.5 A & B). These findings show that components of the identified mitochondrial membrane import complex are functional for the mitochondrial translocation of both protein and tRNA in *T. brucei*.

Both tRNA and Protein Import Require Mitochondrial Membrane Potential

The protein compositions of the mitochondrial tRNA and protein translocons are largely shared and RNAi knockdowns of these putative translocon proteins, revealed a functional relationship between tRNA and protein import. We reasoned that if the import machinery was shared, then biophysical requirements such as membrane potential should be similar. To investigate this possibility, we tested the requirement for a membrane potential in tRNA and protein import (Figures 3.6 & 3.7).

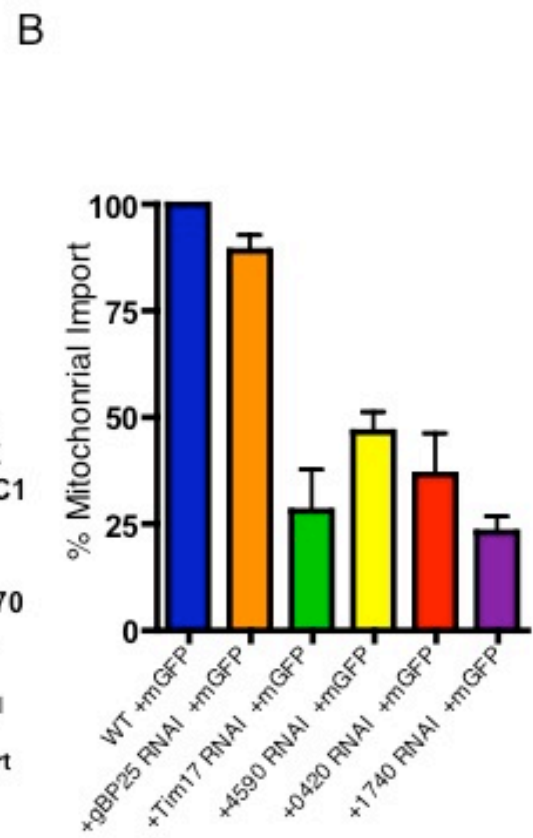
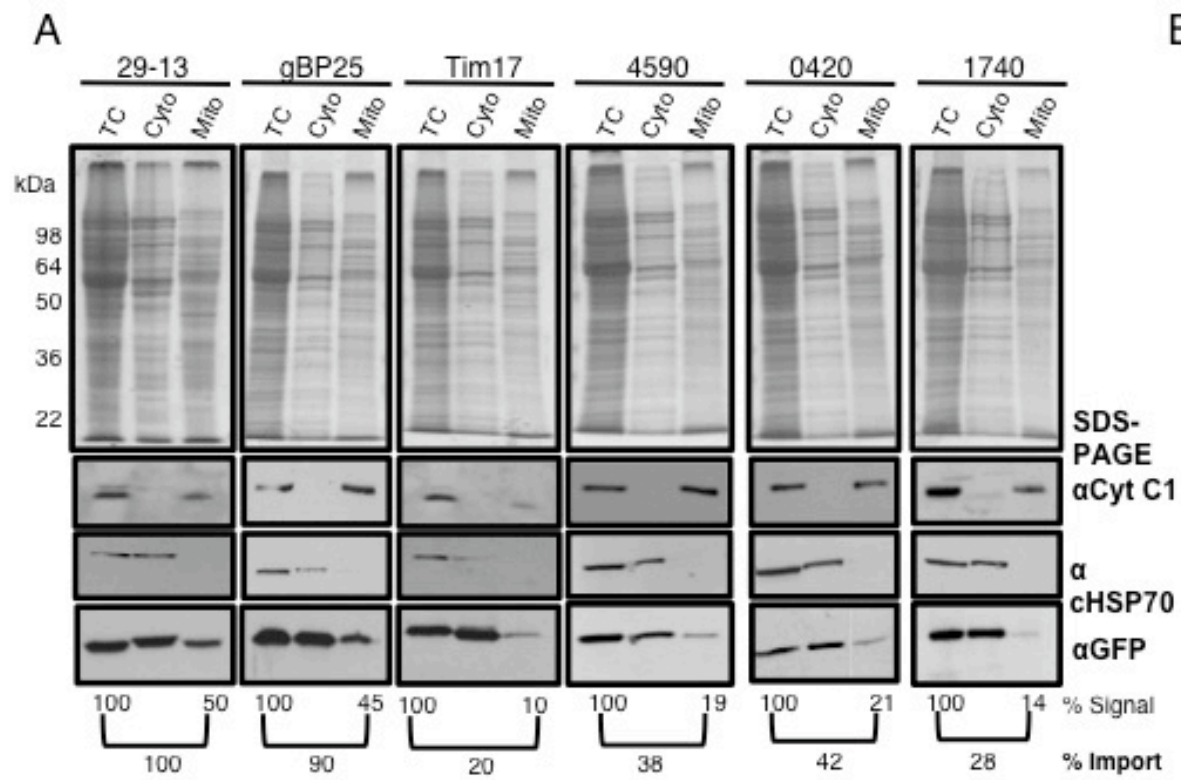


FIGURE 3.5. Identification of mitochondrial membrane proteins required for mitochondrial protein import. **(A)** The *in vivo* MLS-GFP import analysis was used to examine *T. brucei* RNAi lines for gBP25, Tim17, Tb11.01.4590, Tb09.v1.0420 and Tb927.8.1740. Total cellular (TC), cytosolic (Cyto) and mitochondrial (Mito) protein samples were isolated 24 hours post induction with 1 μ g/mL doxycycline and fractionated by SDS-PAGE (10%) gel electrophoresis (top Panel). Protein was visualized by colloidal Coomassie Blue staining. MLS-GFP was detected by anti-GFP western blot (bottom panel). Cytochrome c1 (Cyt C1) and cytosolic HSP70 (cHSP70) were used as mitochondrial and cytosolic markers respectively (middle panels). Percent import was determined by comparing the relative MLS-GFP signals from mitochondrial fractions to total cell signal. **(B)** Quantitative analysis of relative MLS-GFP import for gBP25, Tim17 (n = 6), Tb11.01.4590 (n = 6), Tb09.v1.0420 (n = 6) and Tb927.8.1740 (n = 6). MLS-GFP import was expressed in relationship to WT import (45.6%, n = 6).

Mitochondrial membrane potential is produced during electron transport in procyclic *T. brucei*. Inhibition of electron transport either by drug treatment or loss of critical protein components of the inner mitochondrial membrane electron transport complexes results in decreased membrane potential. Initially, to determine whether electron transport and subsequently a proton gradient across the inner mitochondrial membrane is necessary for both protein and RNA import, an RNAi cell line for the Rieske iron sulfur protein (ISP) was prepared. Cell growth was largely unaffected for the first 24 hours following RNAi induction whereas mitochondrial membrane potential rapidly declined to approximately 50% of wild type levels and remained at this level for up to 72 hours (Figure 3.6 A & B). Both protein and tRNA import also decrease during the initial 24 hours, which is consistent with the decrease in membrane potential resulting in inhibition of translocation of both substrates (Figure 3.6 C, D & E).

To directly test the role of membrane potential in mitochondrial import, inducible tRNA and MLS-GFP cell lines were simultaneously treated with the uncoupling ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Figure 3.7 A-C). The effect of CCCP on mitochondrial membrane potential was determined using the cationic fluorescent dye TMRM, which is rapidly sequestered within active mitochondria (9, 15). Treatment with 5 μ M CCCP for 24 hours depleted membrane potential by greater than 90% (CCCP-pulse) (Figure 3.7A). Cells were viable after CCCP treatment and membrane potential returned to near wild type levels 24 hours after removal (CCCP-chase) (Figure 3.7A). Simultaneous treatment of cell lines with CCCP and doxycycline to induce expression of the Var-tRNA^{Met-i} resulted in a 60% decrease in Var-tRNA^{Met-i} import relative to untreated cells (Figure 3.7 B & C).

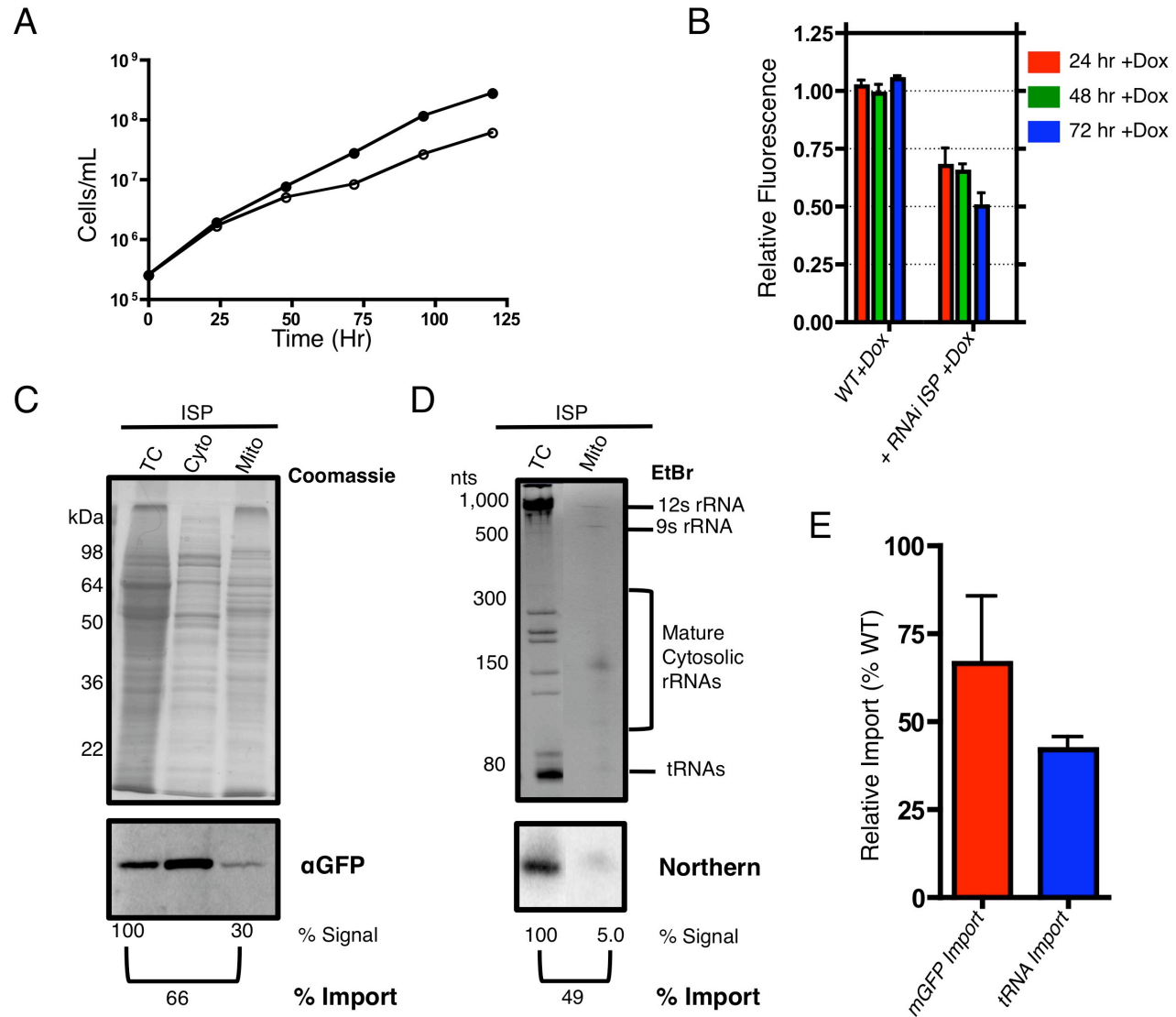


FIGURE 3.6. Loss of mitochondrial membrane potential and tRNA and protein import following RNAi knockdown of the Rieske ISP. **(A)** Growth of *T. brucei* 29-13 RNAi Rieske ISP in the presence (open circle) or absence (closed circle) of doxycycline (1 $\mu\text{g/ml}$). **(B)** Analysis of mitochondrial membrane potential in wild type *T. brucei* 29-13 and the RNAi cell line for Rieske ISP in the presence of 1 $\mu\text{g/ml}$ doxycycline. Samples were collected in triplicate at 24, 48 and 72 hours and the mitochondrial membrane potential of 2×10^6 cells was determined by the relative fluorescence of 20 nM TMRM mitochondrial accumulation. **(C)** The *in vivo* MLS-GFP import analysis was used to examine the Rieske ISP RNAi lines. Total cellular (TC), cytosolic (Cyto) and mitochondrial (Mito) protein samples were isolated 24 hours post induction with 1 $\mu\text{g/ml}$ doxycycline and fractionated by SDS-PAGE (10%) gel electrophoresis (top panel). Protein was visualized by colloidal Coomassie Blue staining. MLS-GFP was detected by anti-GFP western blot (bottom panel). Percent import was determined by comparing the relative MLS-GFP signals from mitochondrial fractions to total cell signal. **(D)** *In vivo* tRNA import analysis from RNAi Rieske ISP cell line. Total cellular (TC) and mitochondrial (Mito) RNA was isolated 24 hours post induction with 1 $\mu\text{g/ml}$ doxycycline and fractionated by non-denaturing polyacrylamide gel electrophoresis (8%) (Top Panel) and abundant rRNA and tRNA were visualized by ethidium bromide (EtBr) staining. The Var-tRNA^{Met-i} was detected by northern blot hybridization and autoradiography (bottom panel). Percent import was expressed as the relative hybridization of mitochondrial to total cell Var-tRNA^{Met-i}. **(E)** Quantitation of mitochondrial mGFP (red bar) and Var-tRNA^{Met-i} (blue bar) import in comparison to WT protein (45.6%) and tRNA (10.3%) import levels (n = 6).

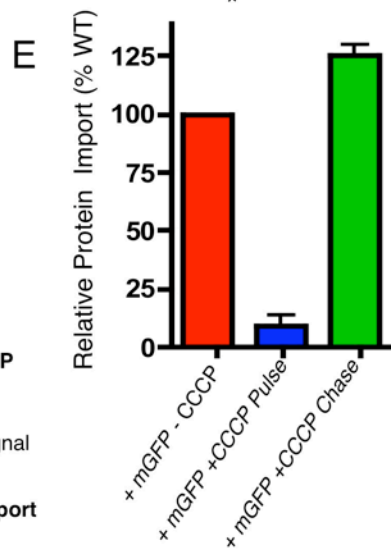
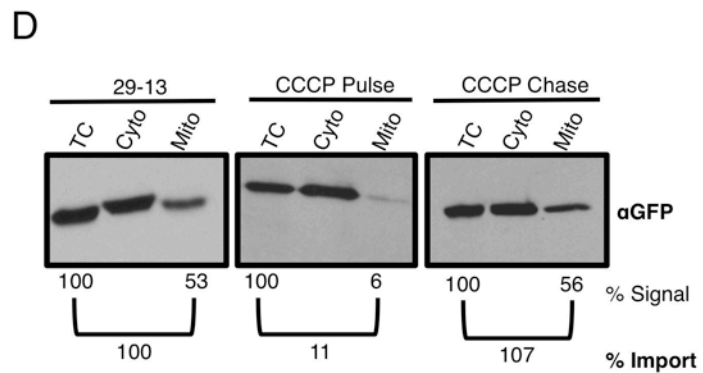
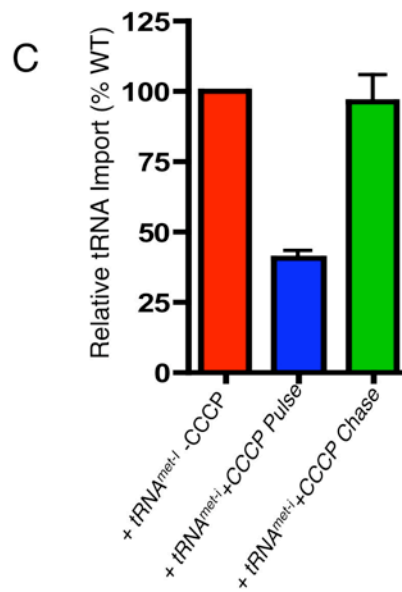
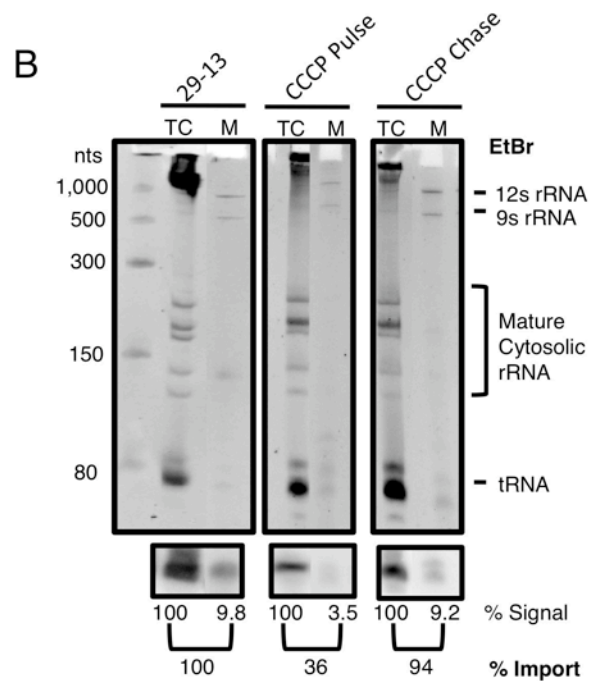
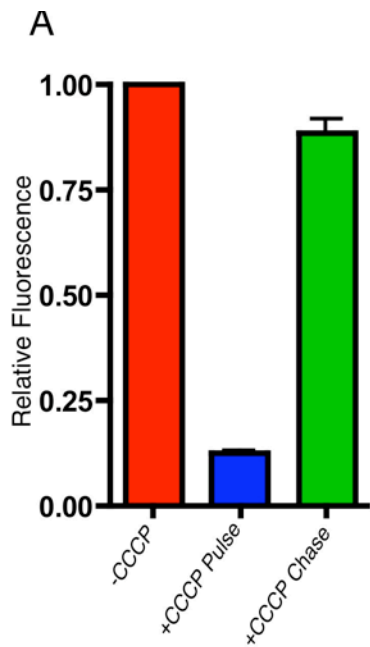


FIGURE 3.7. An active membrane potential is required for both mitochondrial tRNA and protein import. **(A)** Measurement of mitochondrial membrane potential from 2×10^6 cells by relative TMRM fluorescence (20 nM). WT *T.brucei* 29-13 cell lines containing the Var-tRNA^{Met-i} expression construct were either treated with 5 μ M of the membrane potential uncoupler CCCP (+CCCP) or untreated (-CCCP) and membrane potential was monitored 24 hours post treatment (-CCCP and +CCCP Pulse). After 24 hours, CCCP was washed out of the culture (+CCCP Pulse) and cells were allowed to recover for another 24 hours followed by the measurement of membrane potential (+CCCP Chase). **(B)** *in vivo* tRNA import analysis from - CCCP (29-13) and + CCCP (CCCP Pulse and CCCP Chase) treated cell lines. Total cellular (TC) and mitochondrial (M) RNA was isolated 24 hours post induction with 1 μ g/ml doxycycline and fractionated by non-denaturing polyacrylamide gel electrophoresis (8%) (top panel) and abundant rRNA and tRNA were visualized by ethidium bromide (EtBr) staining. The Var-tRNA^{Met-i} was detected by northern blot hybridization and autoradiography (bottom panel). Percent import was expressed as the relative hybridization of mitochondrial to total cell Var-tRNA^{Met-i}. **(C)** Quantitative analysis of relative tRNA import for - CCCP (n = 3), + CCCP Pulse (n = 3) and +CCCP chase (n = 3). tRNA import was expressed relative to Var-tRNA^{Met-i} import in wild type cells (10.3%, n = 8). **(D)** Protein fractions from uninduced (left panel) or CCCP (5 μ M) treated (right panel) MLS-GFP cell lines. MLS-GFP expression and localization detected by anti-GFP. **(E)** Quantitative analysis of MLS-GFP mitochondrial import 24 hours post induction with 1 μ g/ml doxycycline (n = 6). The percentage of MLS-GFP import was determined by anti-GFP western blot signal of MLS-GFP from total cellular and mitochondrial protein fractions and compared to WT import levels (45.6%).

The inhibition of Var-tRNA^{Met-i} import was restored to near wild type levels when CCCP was removed (Figure 3.7 B & C). Similar studies were done with *T. brucei* lines containing the doxycycline regulated MLS-GFP gene. Treatment of these cells, with 5mM CCCP also resulted in a reversible inhibition of mitochondrial protein import (Figure 3.7 D & E). These studies show that mitochondrial import of both tRNA and protein in *T. brucei* requires functional mitochondria and that membrane potential is required for both mitochondrial tRNA and protein import. These findings lend additional biophysical evidence for a shared transporter of tRNAs and proteins in *T. brucei* mitochondria

Discussion

In this study we show that trypanosome mitochondria have a single transporter for the import of both proteins and tRNAs. This conclusion was reached based on the shared protein composition of independently purified protein and tRNA import complexes, the diminished import of both tRNA and protein in RNAi knockdowns of these proteins, and a requirement for mitochondrial membrane potential for tRNA and protein import.

Characterization of the mitochondrial tRNA import complex led to the identification of several protein components, including Tim17 and mHSP70, known to be necessary for mitochondrial protein import in *T. brucei* (1, 8). These results raised the possibility of a common translocon for both proteins and tRNAs. An analogous situation exists in other diverse organisms where tRNA and protein imported into the mitochondria appear to share the same import machinery (4-7). Tandem affinity purification of the *T.*

brucei Tim17 homologue yields a complex composed of seven proteins (Table 1). Several of the Tim17 associated proteins, Tb927.8.1740, Tb11.02.3310 and Tb09.211.2530, are currently annotated as hypothetical while the other four, Tim17, mHSP70, mHSP60 and mHSP20, are associated with mitochondrial protein import in trypanosomes and other organisms. Consistent with a shared translocon for both proteins and tRNAs, all of the subunits of this complex were previously identified as components of a *T. brucei* mitochondrial membrane tRNA binding complex (9).

Import of tRNAs and proteins into the mitochondria, by a single translocon, should also share biophysical requirements. Consistent with this possibility, both tRNA and protein import into *T. brucei* mitochondria requires internal and external pools of ATP and hydrolysis (11, 12). However studies evaluating the need for a membrane potential in trypanosome, yeast, plant and human tRNA import have yielded diverse and controversial results (4, 6, 7, 11-14, 16, 17). Paris *et al.* reported that RNAi depletion of the Rieske ISP in *T. brucei*, an essential redox component of the trypanosome electron transport Complex III, had no effect on either the steady state levels of mitochondrial tRNAs or the *in vitro* import of tRNAs into mitochondria isolated from the RNAi cells (14). Based on these findings, the authors suggested that there were two independent translocons for mitochondrial protein and RNA import in trypanosomes. However, these studies are difficult to evaluate in terms of tRNA import requirements. First, mitochondrial membrane potential was not measured following Rieske ISP RNAi knockdown (14). Second, controls to ensure that protein import was inhibited were lacking in Rieske ISP RNAi knockdowns, although the authors previously postulated that Complex I, IV and residual components of complex III may be able to maintain

membrane potential at a level that allows for protein import (18). Third, the specificity for tRNA import *in vitro* was not evaluated as no attempt was made to examine whether or not tRNAs retained within the cytosol (tRNA^{Met-i} and tRNA^{Sec}) or non specific RNA substrates were imported into the mitochondria. Furthermore, tRNAs are the most highly expressed RNAs in the cell and are known to have very long half lives, ranging from hours to five days (19-21). Without knowing the stability of tRNA in *T. brucei*, it is impossible to determine if mitochondrial import is affected simply by the examination of steady state tRNA levels. The authors also argue that others have also reported that a membrane potential was not required for tRNA import in *T. brucei* and reference a study from Schneider and coworkers (12). However, Schneider has recently shown that a membrane potential is needed for tRNA import *in vivo* and that the earlier results were likely a consequence of artifacts associated with the *in vitro* import assay used (12, 22). Further, it has been postulated that the ability to isolate intact, active mitochondria could explain the discrepancies found by use of *in vitro* import assays in trypanosomes, yeast and human (23). The recent development of a reproducible *in vivo* tRNA import system by the Schneider group provides a more reliable measure of tRNA import (13). Using this *in vivo* tRNA import system, our initial studies showed that the formation of the mitochondrial proton gradient was be inhibited by RNAi mediated depletion of the Rieske ISP and that this led to decreased protein and tRNA import (Figures 6). We also found that depolarization of the mitochondrial membrane potential by the use of the uncoupler CCCP, reduced mitochondrial tRNA import to 40% of wild type levels. The effects of CCCP were fully reversible since removal of CCCP resulted in restoration of membrane potential and wild type tRNA import levels (Figure 7 B & C). Together these

results show that an active mitochondrial membrane potential is a biophysical requirement both protein and tRNA import into the mitochondrion of *T. brucei*.

We previously purified a *T. brucei* mitochondrial membrane complex by tRNA affinity chromatography that was necessary for mitochondrial tRNA import (9). In the characterization of this complex, several subunits were identified that are known to function in mitochondrial protein import. Among these mitochondrial proteins was Tim17 which had previously been shown to play a role in tRNA import (8). To directly address whether Tim17 containing mitochondrial membrane complexes bind tRNAs we conducted pulldown experiments with both tRNA and non-tRNA substrates under stringent binding conditions (Figure 3.3). We found that the protein complex containing Tim17 has strong, specific interactions with tRNA, further supporting the hypothesis that the mitochondrial protein import machinery may be required for tRNA translocation. Since these studies were conducted with isolated mitochondria with intact inner and outer membranes we infer that the *T. brucei* Tim17 associated complex traverses both mitochondrial membranes. It will be interesting to determine whether this complex can also directly bind preproteins or whether other outer membrane proteins are involved in preprotein recruitment and binding (2).

Previous studies in yeast, plant and mammalian organisms have all implicated the function of components of mitochondrial protein import machinery in the import of RNA (4-7). In yeast it has been shown that the nuclear encoded tRNA^{Lys}(CUU) is co-imported with its corresponding precursor aminoacyl tRNA synthetase through interactions with Tom20 and Tim44 and that import was dependent upon the mitochondrial membrane potential (4, 24). Further, the import of tRNA in the plant, *S. tuberosum*, has been shown

to require a mitochondrial membrane potential and involvement with Tom20 and Tom40 (5). In mammals, the mitochondrial import of tRNA, *RNase P* RNA and 5S rRNA is inhibited when the membrane potential is depleted or when the protein import channel is blocked (6). Consistent with a single translocon, we found that Tim17, associated proteins and membrane potential are required for both tRNA and protein import into *T. brucei* mitochondria. The identification of canonical protein import machinery in *T. brucei* as having a function in mitochondrial tRNA import further supports a universal mechanism for RNA import in eukaryotic organisms.

Materials and Methods

Trypanosome cell culture and mitochondrial purification - Cell cultures were maintained in SM media supplemented with 10 % (v/v) heat inactivated fetal bovine serum (25). For this study, *T.b. brucei* 667 (TREU 667) and *T.b. brucei* 29-13 strains were used. Mitochondria used in tRNA and PTP affinity purification experiments were isolated and membrane and matrix fractions were prepared as previously described (26, 27).

tRNA affinity purification of mitochondrial membrane protein complexes - Pulldown of PTP-Tim17 by tRNA was performed as previously described (9). Trypanosoma specific tRNA^{Leu(CAA)}, tRNA^{Met-i}, and A6 Editing Site 1 (A6 ES1) gRNA were synthetically generated (Dharmacon) with a 3' biotin tag.

Doxycycline inducible expression of a variant tRNA^{Met-i} - Previously, an inducible variant of tRNA^{Met-i} was generated which has been shown to be imported, aminoacylated

and processed upon mitochondrial translocation *in vitro* and *in vivo* (13, 28). The phleomycin resistance cassette was replaced with a puromycin resistance cassette by use of the flanking *MscI* restriction sites.

Generation of RNAi cell lines to validate protein components function tRNA and protein import - All RNAi constructs were prepared in the doxycycline inducible pZJM vector containing opposing T7 promoters (29). RNAi inserts for gBP25, Tb11.01.4590 and Tb09.v1.0420 were generated as previously described (9). Inserts for Tim17 (nucleotides 1 - 459) and Tb927.8.1740 (nucleotides 841 - 1441) were also prepared. Inserts for the RNAi knockdown of the Rieske ISP were generated as previously described (18). Constructs were linearized by cleavage with *NotI* and transfected into the clonal cell line containing the inducible Var-tRNA^{Met-i} gene. Upon induction with doxycycline each cell line simultaneously expressed the Var-tRNA^{Met-i} and double stranded RNA to knockdown expression of targeted proteins. Mitochondrial membrane potential measurements upon induction of RNAi were conducted as previously described (9).

Inducible expression of a mitochondria targeted protein – To generate a doxycycline regulatable protein substrate, we utilized the previously characterized pLew100 vector, which contains a T7 regulatable PARP promoter and phleomycin resistance cassette (30). As a first step in the generation of the expression construct, the phleomycin resistance cassette was replaced with a puromycin resistance cassette by use of the *MscI* restriction sites flanking the resistance cassette. We generated primers specific for the coding sequences of Green Fluorescent Protein (GFP), which contained a mitochondrial localization signal (MLS) from *T. brucei* dihydrolipoyl dehydrogenase (Tb11.01.8470,

nucleotides 1-42). (Primer description: Lowercase nucleotides are overhang, Bolded nucleotides are *HindIII* or *XhoI* restriction sites, Italicized nucleotides represent the MLS Signal, Uppercase non-bold nucleotides represent GFP coding sequence. Sense: gatc**AAGCTT***TATGTTCCGTCGCTGCTTCCGATCTTTA***ACCCCTACGATGTCATGAGTAAA** GGAGAAGA**ACTTTTC**. Antisense: gatc**GGATCCTT***ATTTGTATAGTTCATCCATGCC*).

After PCR amplification and cloning into pLew100 by use of the *HindIII* and *XhoI* restriction sites, we had generated a doxycycline regulatable MLS-GFP fusion protein. Upon *NotI* digestion, linearized vector was electroporated and transfected into *T. brucei* 29-13 cell lines and clones were selected with puromycin. Clonal cell lines could then be induced with 1µg/mL doxycycline for the expression of MLS-GFP. RNAi constructs for Tim17, Tb927.8.1740, Tb09.v1.0420, Tb11.01.4590, gBP25 and Rieske ISP were stably transfected into clonal MLS-GFP cells.

Cell fractionation, RNA and protein import assays - Total cellular and mitochondrial RNA fractions were collected as previously described (9, 13). Protein fractions from total cell, cytosolic and mitochondrial fractions were collected by a similar method. 4 x 10⁸ cells were resuspended in 500µL of 20 mM Tris-HCl, pH 7.5, 0.6 M sorbitol, 2 mM EDTA, pH 7.5 containing 0.05% digitonin and incubated for 5 minutes on ice. The cells were then centrifuged (8,000 x g) at 4°C for 5 minutes and the supernatant containing the non-mitochondrial fraction (cytosolic) was retained. The pellet was then resuspended in 500 µL of SME-20 (0.25 M sucrose, 20 mM MOPS/KOH at pH 7.2 and 2 mM EDTA) containing 5 mM CaCl₂, 100 mM KCl. Proteinase K was then added to a final concentration of 30 µg/mL and incubated at room temperature for 10 minutes. The reaction was then terminated by adding one volume of cold SME-20 containing 4mM

PMSF and the sample was centrifuged (8,000 x g) at 4°C for 5 minutes and the supernatant was discarded. Total cellular, cytosolic and mitochondrial protein were extracted from equal cell equivalents by the addition of equal volumes of SDS cracking buffer. Protein fractions from 5 x 10⁶ cellular equivalents were resolved by 10% SDS-PAGE electrophoresis. GFP was detected by anti-GFP (Invitrogen) immunoblot, cytosolic and mitochondrial fractions were detected by anti-HSP70 and anti-CytC1 respectively.

Tandem affinity of PTP Tim17 and associated proteins - Constructs for PTP-Tim17 (Nucleotides 6-459) were designed with *Apal* and *NotI* restriction sites to clone into the pC-PTP-Neo vector (31). PTP tagged Tim17 was linearized with *AvaI* (NEB) and transfected into PF *T. brucei* TREU667 targeting a single allele for the endogenous expression of the PTP-tagged construct. Isolated mitochondria from 6 x 10¹⁰ cells were solubilized as previously described by resuspending (400 µg mitochondria/40 µL) in Solubilization Buffer A (50 mM NaCl, 50 mM Imidazole/HCl, 2 mM 6-Aminohexanoic Acid, 1 mM EDTA, pH 7.0) containing 2 % dodecyl maltoside in a rotator for 30 minutes at 4°C (32). PTP tagged protein complexes were subsequently purified as described previously (33). Samples from the tandem affinity purification were examined by 8-10% polyacrylamide gel electrophoresis and immunoblot with anti-Protein C (Delta Biolabs). Eluted proteins were excised from gels and analyzed by LC-MS/MS (Thermo Fisher LTQ Linear Ion Trap). Raw tandem mass spectra were converted to mzXML files then into mascot generic files via the Trans-Proteomic Pipeline (Seattle Proteome Center, Seattle, WA). MGF files were searched using Mascot (Matrix Scientific, Boston, MA) against target and decoy National Center for Biotechnology Information (NCBI)

databases for trypanosome proteins. ProteoIQ (Nusep, Bogart, GA) was used for data analysis and a 5% protein false discovery rate was applied to confirm the presence of proteins.

CCCP Pulse-Chase Analysis to Monitor tRNA and Protein Import – Carbonyl cyanide m-chlorophenylhydrazone (CCCP) is an ionophore that rapidly uncouples mitochondrial membrane potential gradients. Cells were either untreated or treated (Pulse) with 5 μ M CCCP (Sigma) for 24 hours. After 24 hours of inhibition with CCCP, a fraction of cells were collected and the mitochondrial membrane potential of 2×10^6 total cells was determined by use of microfluorimetry as previously described (9). The treated fraction of cells was then allowed to recover for 24 hours (Chase) in the absence of CCCP, after which the mitochondrial membrane potential of 2×10^6 total cells was again monitored. This process was conducted using clonal cell lines containing either the inducible tRNA import construct (Var-tRNA^{Met-i}) or the mitochondrial targeted protein import construct (MLS-GFP). At the indicated time points, subcellular fractions were collected from 4×10^8 Untreated, Pulsed and Chased cells which had been induced (1 μ g/ml doxycycline - Sigma) for either the expression of mitochondrially targeted tRNA (Var-tRNA^{Met-i}) or protein (MLS-GFP) as earlier described. Total cellular and mitochondrial RNAs from 2×10^7 cellular equivalents were resolved on 8 M Urea/8 % polyacrylamide-sequencing gels and the amount of Var-tRNA^{Met-i} imported into the mitochondria was quantitated as previously described (9). Protein fractions 5×10^6 cellular equivalents were resolved by 10% SDS-PAGE electrophoresis and the levels of mitochondrially imported MLS-GFP were detected and quantitated as described earlier.

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CHAPTER 4

CONCLUSIONS AND DISCUSSION

The eukaryotic mitochondrial genome encodes for a minimal number of protein and RNA genes. In order to maintain mitochondrial function, all eukaryotic organisms must import nuclear encoded proteins and RNA into their mitochondria. The mechanisms and mitochondrial membrane protein complexes responsible for protein translocation have been well characterized and defined in numerous eukaryotes (1-6). The recent identification of mitochondrial protein complexes that function in mitochondrial RNA import suggests that all eukaryotes may import RNA through the protein import channels (7-11). The mitochondrial genome of *Trypanosoma brucei* is unique in that there are no genes encoding for tRNA, instead all of the tRNAs contained within the mitochondria are nuclear encoded and imported from the cytosol (12-14).

Though much is known about the biophysical requirements for mitochondrial tRNA import, very little is known about the mitochondrial proteins that are required for translocation. In the closely related kinetoplastid *Leishmania tropica*, an eleven-subunit inner mitochondrial membrane (IMM) RNA import complex (RIC) was identified as functioning in tRNA import (15). Upon closer analysis though, it was found that numerous subunits of the complex are not required for mitochondrial tRNA import in *T. brucei* (11, 16). Further, concerns about the validity of the RIC identification have been raised (17). To determine if the RIC was shared between *L. tropica* and *T. brucei*, we initially aimed to investigate what the components make up the tRNA import complex in

T. brucei (Chapter 2). From this study, only one component (Tb09.v1.0420) of the previously characterized RIC was identified as being required for mitochondrial tRNA import, though it should be noted that this protein component was not subsequently found in our characterized *T. brucei* tRNA import complex (Chapter 2, Table 2.3). By tandem affinity purification of a candidate protein (Tb11.01.4590), which we identified as binding to tRNA as well as affecting mitochondrial import, a unique protein complex was identified, distinct from the *Leishmania* RIC (18). This complex was found to both localize to the mitochondrial membrane and also bound specifically to tRNA substrates (Chapter 2, Figure 2.10 and Chapter 3, Figure 3.3). Of interest, it was found that multiple subunits of this complex are also known components of the mitochondrial protein import machinery (Tim17, mHSP70, mHSP60 and mHSP20) in *T. brucei*. This raised the possibility that tRNA may use components of the mitochondrial protein import machinery, or that tRNA and protein are imported through the same complex in *T. brucei*. To determine if components of the protein import complex were shared with those of the tRNA import complex, we identified proteins that co-immunoprecipitated (CoIP) with Tim17, a known component of the TIM complex (Chapter 3, Table 3.1) (19). We found that all of the proteins that CoIP'd with Tim17 were also components of our characterized tRNA import complex. This result further indicated that the identified complex may function in both mitochondrial tRNA and protein import.

The mitochondrial protein import complex of *T. brucei* has also yet to be identified and the few components of the canonical translocase outer (TOM) and translocase inner membrane (TIM) that have been identified, share very low homology to other eukaryotes (20, 21). Further, recent studies have found that components of the

protein import complex of *T. brucei* are of bacterial origin and may represent an ancient relic of macromolecular transport in early divergent eukaryotes (22, 23). Not knowing the identity of the components of the mitochondrial protein import machinery, as well as the identification of Tim17 in our characterized tRNA import complex led to the hypothesis that the identified tRNA import complex of *T. brucei* may also function in mitochondrial protein import. To test this, we developed an *in vivo* protein import assay in which we could monitor the mitochondrial import of newly synthesized protein at the same time as we targeted components of the tRNA import complex for RNAi knockdown (Chapter 3) (19). We found that each protein (Tim17, Tb11.01.4590, Tb09.v1.0420 and Tb927.8.1740), which was found to affect mitochondrial tRNA import, also impaired mitochondrial protein import (Chapter 3, Figure 3.5). These functional and physical results show that tRNA and protein are imported into the mitochondria by use of the same import complex (Figure 4.1).

Previously it had been postulated that tRNA and protein are imported into the mitochondria of *T. brucei* by separate mechanisms as their import was dependent on different biophysical energy requirements; specifically it was found that the membrane potential was not required for tRNA import (16, 24). However, these findings were a result of a flawed *in vitro* tRNA import assay, which was found to nonspecifically import small RNAs and cytosolic specific RNAs (25, 26). In this study, we have been able to determine that mitochondrial tRNA import requires an active membrane potential *in vivo* (Chapter 3, Figure 3.7). These findings corroborate previous *in vivo* tRNA import studies in *T. brucei*, which also showed a dependency on the membrane potential for import (26).

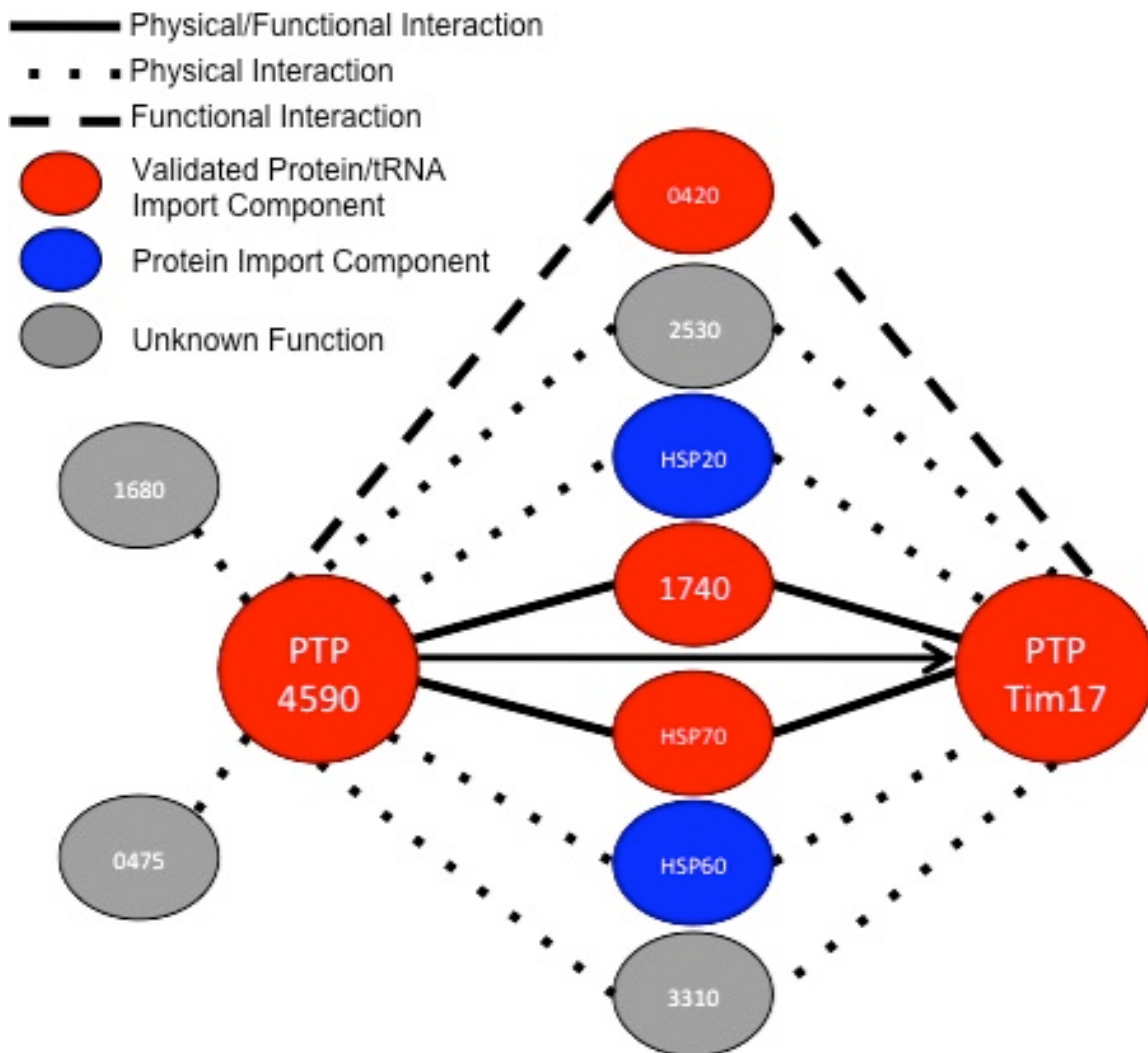


FIGURE 4.1 The mitochondrial tRNA/protein import complex of *T. brucei*. Proteins are listed by their known identity or last 4 digits of their gene ID (Full gene ID in Tables 2.2, 2.3 and 3.1). Of the proteins characterized as components of they physically associated complex, 6 are known to function in mitochondrial protein import (4590, Tim17, HSP70, 1740, HSP20 and HSP60) and 4 function in both protein and tRNA import (4590, Tim17, HSP70 and 1740). Tb09.v1.0420 (0420) was not coimmunoprecipitated by either 4590 or Tim17, but was found to function in both tRNA and protein import by *in vivo* import analysis.

Further, through the *in vivo* mitochondrial protein import assay, it was also found that protein import requires the mitochondrial membrane potential (Chapter 3, Figure 3.7 D, E). Our findings are supported by previous *in vitro* studies showing that the membrane potential is required for mitochondrial protein import (19, 27). Together, the results of this study in *T. brucei* show that both mitochondrial tRNA and protein import require the membrane potential *in vivo* (Chapter 3, Figure 3.6 and 3.7).

Based on the findings of this thesis, as well as previous studies, we have proposed a model for how tRNA and protein are imported into the mitochondria of *T. brucei* (Figure 4.2). This represents the first mitochondrial membrane complex that has been characterized as functioning in both tRNA and protein import. Nuclear encoded tRNA and protein are both transported from the cytosol through the identified mitochondrial membrane protein complex in an energy dependent manner, requiring ATP hydrolysis and an active mitochondrial membrane potential (Chapter 2, Table 2.3. Chapter 3, Table 3.1 and Figure 3.7). The import complex was isolated from intact mitochondria, suggesting that the identified components of the complex functionally span both the OMM and the IMM. For tRNA import, the tRNAs are first aminoacylated and bound by eEF1a where they are then delivered to the mitochondria (26). At this point, eEF1a disassociates from the tRNA where it is either directly transferred to the import complex or possibly bound by another protein cofactor where further transport to the mitochondria or co-import may occur. This model supports a universal mechanism for mitochondrial RNA and protein import as all other eukaryotic organisms studied to date utilize components of the protein import machinery for both mitochondrial RNA and protein import (7-10).

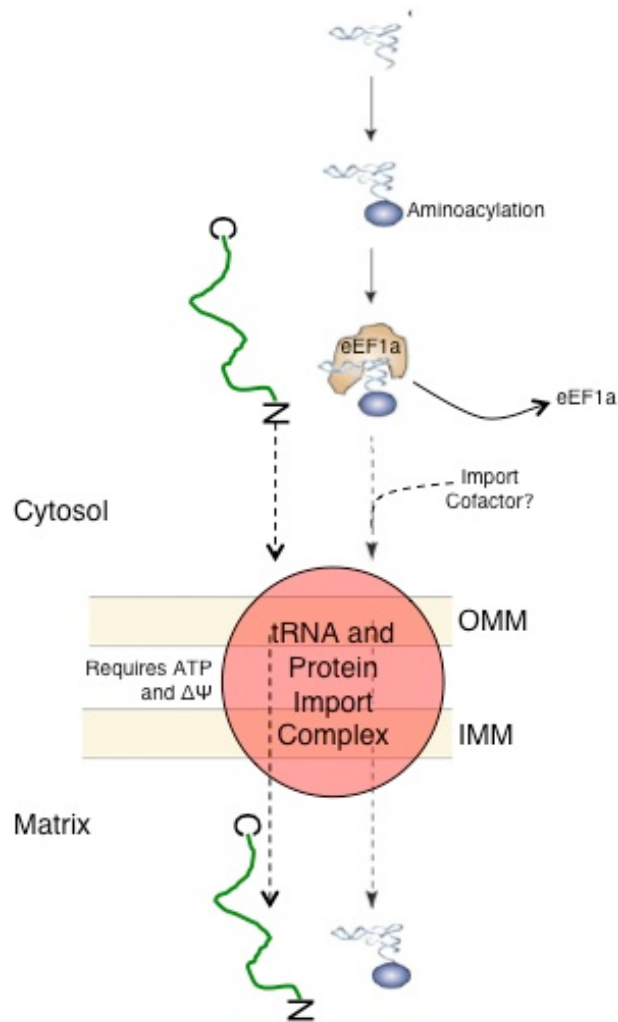


FIGURE 4.2 Model for mitochondrial tRNA and Protein import in *Trypanosoma brucei*. Elongator tRNAs (except for tRNA^{Sec}) are aminoacylated prior to binding to eEF1a (26). eEF1a then delivers tRNA to the mitochondria where it is then either bound by the receptor of the import channel or another cytosolic cofactor. Protein and tRNA are transported through the same mitochondrial membrane protein complex. This translocation of both protein and tRNA requires ATP hydrolysis as well as an active membrane potential. (Adapted from Salinas *et al.*, Trends in Biomedical Science 2008) (28).

The role of Tb09.v1.0420 in mitochondrial protein and tRNA import proves to be interesting in two aspects: 1) It is the only component of the *Leishmania* RIC to be identified in *T. brucei* and 2) C-terminal tagging causes the protein to mislocalize to the cytosol, resulting in severely impaired mitochondria (Chapter 2, Figure 2.8). Tb09.v1.0420 is a known mitochondrial component and most mitochondrial proteins contain an N-terminal mitochondrial localization signal (MLS) (29). However, proteins targeted for assembly into the OMM can utilize an alternative MLS located within the C-terminus of the protein (30). Therefore, the mislocalization of Tb09.v1.0420 to the cytosol could be due to interference with a C-terminal MLS. It would be of interest to determine if this is the case, possibly by using the N-terminal version of the PTP-Tag, as Tb09.v1.0420 could provide the functional linkage to the OMM for protein and tRNA import.

To fully address whether or not protein and tRNA import are imported into the mitochondria by the same import channel, a combination of *in vivo* and *in vitro* import assays could be used. Previously, a fusion protein consisting of the N-terminal domain of the mitochondrially targeted dihydrolipoamide dehydrogenase and mouse dihydrofolate reductase (LDH-DHFR) has been used to identify components of the TOM protein import complex *in vivo* (22, 31). In *T. brucei*, treating cells with methotrexate (MTX) results in a MTX and LDH-DHFR complex where LDH-DHFR is retained in a folded state. In this state, the N-terminus of LDH-DHFR is able to bind to the TOM and TIM complexes, however, due to the folded state of the DHFR domain, it cannot be imported into the mitochondria, thus blocking the import channel (22). Similar to the previously

described *in vivo* import assays, generating inducible *T. brucei* cell lines which express LDH-DHFR as well as either a mitochondrially targeted tRNA or protein could be used to examine mitochondrial import *in vivo*. Upon treatment of cells with MTX, the mitochondrial protein import complex would be sterically blocked by LDH-DHFR. If tRNA is imported through the same complex as protein, then mitochondrial tRNA should be inhibited by MTX treated LDH-DHFR.

Inhibition of mitochondrial protein import would also impair the import of protein components of macromolecular import complexes and in turn, inhibit RNA import. tRNA and protein substrates are first detected within the mitochondria 12-18 hours post induction *in vivo*. During this time span, it is possible that inhibition of protein import by LDH-DHFR could have downstream effects that result in impaired tRNA import. An alternative to this method would be to monitor import into isolated, wild type mitochondria. This would provide the ability to directly inhibit the protein import channels *in vitro* without affecting homeostatic mitochondrial protein levels (i.e. complexes that are required for macromolecular import that would also need to be imported *in vivo*). The specificity for the *in vitro* import of mitochondrially-targeted substrates must first be established in order to obtain reliable results.

Previously, *in vitro* analysis examining shared mechanisms for protein and tRNA import have been achieved by three different experimental techniques: antisera, competition and steric inhibition of the protein import complex. Firstly, studies in *S. cerevisiae* have been able to use isolated mitochondria to examine the effects of antisera on mitochondrial tRNA import (32). Deletion of the Tom20 gene, one of the TOM complex receptors, resulted in impaired tRNA import *in vitro*, but these findings

could simply be a result of impaired protein import (7). To address this, the researchers isolated wild type mitochondria and incubated them with antisera directed towards Tom20. Afterwards, tRNA import was analyzed *in vitro* and it was found that import was completely inhibited (32). Two putative components of the OMM protein import complex of *T. brucei* have been identified, ATOM and Sam55 respectively. By raising antibodies towards these proteins, we could examine whether ATOM and Sam55 also affect tRNA import (21, 22). Similar analysis in the plant *S. tuberosum* found that Tom20 and Tom40 are also required for mitochondrial tRNA import. However, it was shown that although components of the protein import complex are required for tRNA import, the tRNA is not actually transported through the same complex as protein (8, 33). This was achieved by competition analysis in which unlabeled tRNA and protein substrates were used as competitors for the import of labeled tRNA substrate. In this assay, it was found that as the concentration of competitor increased, only the unlabeled tRNA competed for import, whereas protein had no effect (8, 33). By utilizing antisera directed towards components of the protein import machinery in *T. brucei* as well as import competitors, we could further examine whether or not tRNA and protein are imported through the same channel.

These two experimental techniques would provide more insight into whether or not our characterized *T. brucei* mitochondrial import complex is functional in both the import of tRNA and protein substrates. In human mitochondria, it has been shown that 5s rRNA is imported through the outer membrane preprotein import channel (9). This was achieved by providing isolated mitochondria with an unfoldable preprotein substrate analogue, similar to LDH-DHFR, which is able to initiate import, but cannot be fully

imported into the mitochondria. This construct was shown to effectively block the protein import complex and inhibit both protein and RNA import (9). By employing a combination of the above *in vitro* assays and by ensuring specificity for import, it could be definitively determined if protein and tRNA are indeed imported through our characterized import complex in *T. brucei*.

The identification of a shared mechanism for RNA and protein import could be applied to identify trypanosomal drug targets as well as to further study mitochondrial biogenesis in trypanosomes. The diversity of the trypanosomal genome from that of other eukaryotic organisms, including the human host, provides a vast array of unique druggable targets. In particular, the mitochondrial import complexes appear to be highly divergent from most other eukaryotic organisms. By developing potent inhibitors of the mitochondrial import complex, mitochondrial macromolecular import could be shut down in *T. brucei*, which in turn would kill the parasite. Inhibitors of the mitochondrial import complex would be stronger as it may be more difficult for the parasite to generate resistance to the inhibition of both protein and tRNA import. However, delivery of the inhibitor to the mitochondria may be difficult, though a recent liposome system has been developed for the delivery of functional cargo and potential therapeutics to the mitochondria of living cells (34, 35). It is possible that such a system could be employed, which would deliver inhibitors to the mitochondria of *T. brucei*.

Further, understanding the delivery mechanism of tRNA substrates to the mitochondria could be used to study mitochondrial biogenesis. The mitochondrial transcripts of *T. brucei* are extensively edited in order to generate appropriate open reading frames (ORF). Additionally during the editing process one mitochondrial

transcript can be alternatively edited to create multiple different ORFs and thus generating distinct proteins. The quest to understand the function of these alternatively edited proteins has only recently begun (36). Genetic systems to identify the function of unknown mitochondrial transcripts are limited. Thus further characterization of our mitochondrial import complex may prove to be beneficial to characterize the function of unknown mitochondrial transcripts in *T. brucei*. It is possible that by coupling a mitochondrially import tRNA with an antisense RNA or Ribozyme, we could specifically target alternatively edited mitochondrial mRNA transcripts for degradation. In turn, we would be able to determine the function of and alternatively edited protein within the mitochondria. tRNAs have previously been used in coupled RNA transport through nuclear pores, so it is entirely possible that this type of coupling would be able to translocate across the mitochondrial membranes as well (37).

The import of RNA and protein into the mitochondria is essential for mitochondrial biogenesis in all eukaryotic organisms. In this study we have identified a mitochondrial membrane protein complex that specifically binds to tRNA and is functional for both tRNA and protein import in *T. brucei*. Like other characterized eukaryotic RNA import systems, the identified *T. brucei* protein complex contains numerous subunits of the canonical mitochondrial protein import machinery, suggesting that RNA and protein may utilize the same import machinery. Further analysis of components of this complex showed that the identified complex is required for both mitochondrial tRNA and protein import. Based on these findings, we conclude that the import of RNA and protein by use of a shared mitochondrial membrane complex is a universal trait among all eukaryotic organisms.

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