Published in final edited form as:
Wiley Interdiscip Rev RNA. 2011 ; 2(6): 802–817. doi:10.1002/wrna.93.

tRNA travels from the cytoplasm to organelles

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Abstract

Transfer RNAs (tRNAs) encoded by the nuclear genome are surprisingly dynamic. Although tRNAs function in protein synthesis occurring on cytoplasmic ribosomes, tRNAs can transit from the cytoplasm to the nucleus and then again return to the cytoplasm by a process known as the tRNA retrograde process. Subsets of the cytoplasmic tRNAs are also imported into mitochondria and function in mitochondrial protein synthesis. The numbers of tRNA species that are imported into mitchondria differ among organisms, ranging from just a few to the entire set needed to decode mitochondrially encoded mRNAs. For some tRNAs, import is dependent on the mitochondrial protein import machinery, whereas the majority of tRNA mitochondrial import is independent of this machinery. Although cytoplasmic proteins and proteins located on the mitochondrial surface participating in the tRNA import process have been described for several organisms, the identity of these proteins differ among organisms. Likewise, the tRNA determinants required for mitochondrial import differ among tRNA species and organisms. Here, we present an overview and discuss the current state of knowledge regarding the mechanisms involved in the tRNA retrograde process and continue with an overview of tRNA import into mitochondria. Finally, we highlight areas of future research to understand the function and regulation of movement of tRNAs between the cytoplasm and organelles.

Keywords

nuclear tRNA import; nuclear tRNA export; mitochondrial tRNA import; nucleus; mitochondria

tRNAs encoded by nuclear genomes are transcribed in the nucleus and are then exported to the cytoplasm where they perform their essential function of delivering amino acids to growing polypeptide chains as specified by mRNA codons. Since protein synthesis occurs in the cytoplasm, it was a surprising discovery that cytoplasmic tRNAs can travel in a reverse direction from the cytoplasm to the nucleus via the tRNA retrograde process – a process that is conserved in yeast and vertebrate cells [1–4]. Here we discuss the possible functions of the tRNA retrograde process, describe its regulation, and explore the mechanism(s) of tRNA nuclear import. Protein synthesis also occurs in organelles-mitochondria and chloroplasts. Mitochondrial genomes of some organisms encode all the tRNAs necessary for organellar protein synthesis; mitochondrial genomes of other organisms do not encode a complete set of tRNAs or, in some cases, completely lack tRNA genes, thereby requiring import of tRNAs encoded by the nucleus in order for protein synthesis to occur. It appears that all organisms are able to import tRNAs from the cytoplasm to mitochondria, regardless of whether a complete set of tRNAs is encoded by the organellar genome [5]. In addition to the systems to import cytoplasmic tRNAs into mitochondria, in at least one organism, *S. cerevisiae*, removal of introns from pre-tRNAs occurs on the mitochondrial outer surface [6, 7] and thus pre-tRNAs exported from the nucleus must be located to the mitochondrial surface.

Here, we also discuss the different mechanisms by which tRNAs are imported from the cytoplasm to and into mitochondria. Finally, we highlight areas of future research in tRNA subcellular dynamics.

tRNA MOVEMENT BETWEEN THE NUCLEUS AND THE CYTOPLASM

In contrast to previous dogma that tRNA movement is unidirectional, nucleus to cytoplasm, it is now known that tRNAs move bi-directionally between the nucleus and the cytoplasm and from the cytoplasm to and into organelles (Fig. 1). tRNA bi-directional movement between the nucleus and the cytoplasm occurs in both yeast and vertebrate cells [1–4]. The movement of tRNA between the nucleus and the cytoplasm consist of three steps. The first step, initial tRNA export, delivers newly transcribed and partially or fully processed tRNAs to the cytoplasm. The second step, tRNA retrograde nuclear import, is the movement of cytoplasmic tRNAs into the nucleus. Finally, tRNAs that had been imported into the nucleus from the cytoplasm are able to return to the cytoplasm by the step know as tRNA re-export [8].

tRNA nuclear export

The Ran-GTP binding β-importin family which functions in most macromolecular trafficking between the nucleus and the cytoplasm plays a key role in tRNA nuclear/cytoplasmic dynamics as at least two family members participate in tRNA nuclear export: (1) yeast Los1/vertebrate Exportin-t/plant PAUSED and (2) yeast Msn5/vertebrate Exportin-5, (Figure 1). For yeast it is easy to distinguish the roles of proteins in the tRNA initial vs. re-export steps because the removal of introns from pre-tRNAs occurs in the cytoplasm on the outer mitochondrial surface [6, 7] (Figure 1). Thus, tRNAs experiencing the initial tRNA nuclear export step contain introns (if the tRNAs are encoded by intron-containing genes), whereas tRNAs re-exiting the nucleus do not contain introns as they were spliced in the cytoplasm before being imported into the nucleus. By this type of analysis Los1 was shown to participate in both the initial and the re-export steps, whereas Msn5 participates only in the tRNA re-export step, at least for tRNAs encoded by intron-containing genes [9].

Los1/Exportin-t/PAUSED is the primary tRNA exporter in yeast, vertebrate, and plant cells [10–13]; however, insects lack a Los1/Exportin-t ortholog [14]. Substrate binding studies demonstrated that vertebrate Exportin-t preferentially interacts with tRNAs with mature 5′ and 3′ termini with appropriate tertiary structure [15, 16]. These results were recently verified by a 3.2 Å resolution structural study of *S. pombe* Los1/Exportin-t, Xpo-t, in complex with Ran-GTP and tRNA which documented that Xpo-t contacts the tRNA acceptor arm and the TΨC and D loops, leaving the anticodon loop exposed [17].

Msn5/Exportin-5/HASTY serves multiple functions. In addition to its role in tRNA re-export in yeast [2, 9, 18] and tRNA export in insects [14], in yeast it also serves as a nuclear exporter for several transcription regulatory proteins, dependent upon their appropriate phosphorylation [19]. In plants and vertebrate cells Exportin-5/HASTY serves primarily in the nuclear export of pre-microRNAs [20–22], though it has also been implicated as a minor exporter of mature tRNAs in vertebrate cells [23, 24]. Although there are structural studies for the interaction of exportin-5 with microRNAs, tRNA-exportin-5 structures have not been reported [25].

Los1 and Msn5 and their orthologues are unessential in budding yeast and the other model organisms in which they have been deleted [2, 26–29]; even yeast lacking both Los1 and Msn5 are healthy [2]. Thus, not all the gene products that function in tRNA nuclear export have been identified.

tRNA nuclear import

Why import tRNAs into the nucleus?—Retrograde movement of tRNA from the cytoplasm is conserved in yeast and vertebrate cells, indicating that this dynamic behavior serves an important function(s). Moreover, the Fassati group proposed that HIV usurped the tRNA retrograde pathway as one mechanism to move reverse transcribed complexes through nuclear pores to the genome of nondividing neuronal cells [3]. Hints as to the cellular function of tRNA nuclear import come from the yeast studies in which nuclear accumulation of previously cytoplasmic tRNA occurs under particular conditions: (1) tRNAs missing 3′ CCA nucleotides [2, 30]; (2) defects in tRNA aminoacylation [31–34]; (3) nutrient deprivation of amino acids [1], glucose [8], or phosphate [35]. Nuclear accumulation of previously cytoplasmic tRNA was also reported for rat hepatoma cells upon amino acid deprivation [4]. The results support two different possible roles for the tRNA retrograde process – tRNA quality control and regulation of protein synthesis in response to nutrient status.

The need for tRNA quality control might result from the fact that tRNAs are long-lived and may suffer damage in the cytoplasm, such as loss of the 3′-CCA extension. Indeed tRNAs with damaged CCA termini are imported into both yeast and HeLa cell nuclei [2, 3, 30], in contrast to unstructured tRNAs which are not imported into nuclei in permeabilized HeLa cells [3]. Importing such damaged tRNAs into the nucleus would remove them from the pool of proteins that interact with tRNAs for protein synthesis. However, since the CCA adding enzyme is both cytoplasmic and nuclear [36], it is difficult to understand why such damaged tRNAs would not be repaired in the cytoplasm. Another role in tRNA quality control might result because many tRNA cleavages and modifications occur solely in the nucleus [37] and there may be competition between completing tRNA processing and tRNA nuclear export, sometimes erroneously resulting in export of end-extended and/or hypomodified tRNAs. Retrograde nuclear import of such putative tRNAs would remove them from the translation machinery and could provide a 2nd opportunity for completion of processing. If the retrograde import step serves a 2nd opportunity role, it is also likely to be in competition with the two known tRNA turnover pathways - the nuclear and the cytoplasmic 5′>3′ tRNA exonucleolytic rapid tRNA decay, RTD, pathway that degrades mature tRNAs with “loose” tertiary structure due to hypomodification [29, 38] and the nuclear polyadenylation-TRAMP 3′>5′ nuclear exosome Rex1 pathway that degrades pre-tRNA$^{Met}_{i}$ lacking m^{1}A and other misfolded or unprocessed RNA [39–41]. These ideas regarding roles for the tRNA retrograde pathway in tRNA quality control await testing.

Accumulation of previously cytoplasmic tRNA in the nucleus when cells are deprived of nutrients or when tRNAs are uncharged is consistent with the idea that tRNAs might accumulate in the nucleus under conditions that protein synthesis should be down-regulated, as has been proposed for the movement of mRNAs to P-bodies upon glucose deprivation [42]. Because tRNAs re-enter the cytoplasm upon re-feeding and mRNAs can move back to polysomes upon addition of glucose [8, 42], translation could be quickly restored when nutrients are replete, providing an inexpensive and quick process to control the rate of protein synthesis or the constellation of proteins translated. It should be noted that tRNA charging, though necessary, is not sufficient for tRNA re-export [8]. To date, tests of the hypothesis that the tRNA retrograde nuclear import serves as a novel mechanism to regulate protein synthesis in response to nutrient deprivation has not been reported.

There are other possibilities for the function of tRNA nuclear import. For example, a previous study claimed that protein synthesis can occur in the nucleus [43] which would require the presence of mature charged nuclear tRNAs; however, this study has been called into question [44]. Since tRNAs serve roles in addition to their essential function in protein synthesis such as targeting proteins for degradation via the N-end rule pathway [45], signaling in the general amino acid control pathway [46], regulation of apoptosis by binding cytochrome

C [47], and as reverse transcription primers and for strand transfer during retroviral replication [48, 49], it is also possible that tRNAs serve a yet to be discovered function in nuclei.

Mechanism of tRNA nuclear import—tRNA nuclear import is an active process as import is energy-requiring and temperature-dependent, *in vivo* in yeast [2] and in vertebrate cells [4], and in nuclear import assays using permeabilized HeLa cells [3]. There is conflicting data regarding the role of the Ran pathway in the tRNA import process. Studies in yeast employed a temperature-sensitive mutation of the RanGAP, *rna1-1*; at the nonpermissive temperature, the *rna1-1* mutation causes defects in macromolecular nuclear/cytoplasmic trafficking for all Ran-dependent trafficking [50]. One group reported that cytoplasmic tRNAs could be imported into nuclei of haploid *rna1-1* cells at the nonpermissive temperature [2], suggesting that the Ran pathway is not required for tRNA nuclear import. A second group employing yeast heterokaryon studies and the *rna1-1* mutation reported that tRNA accumulation was markedly reduced in the nucleus not encoding the tRNA [1], suggesting that the Ran pathway is required for tRNA nuclear import. Ran-dependency is supported by the fact that retrograde tRNA nuclear accumulation is dependent upon the β-importin family member, Mtr10 (vertebrate TNPO3/TRN-SR2 or Transportin-3) [1, 35] (Figure 1). It is difficult to reconcile the different results, but perhaps there are both Ran-independent and Ran-dependent tRNA nuclear import pathways.

How Mtr10 affects nuclear accumulation of cytoplasmic tRNAs is unknown. A likely way would be for Mtr10 to serve as a tRNA importer by binding tRNA, either directly or via an adaptor. As Mtr10 is known to import other RNA binding proteins such as Npl3 [51], this is an attractive idea, but it is unknown whether Mtr10 binds tRNA directly or interacts with tRNA via an adaptor. It is also feasible that in yeast Mtr10 acts as a positive effector of the retrograde import machinery or that it imports a protein that is responsible for nuclear retention of imported tRNA; this latter possibility seems unlikely because when *MTR10* is deleted in combination with deletion of either of the tRNA exporters, Los1 or Msn5, there is no nuclear tRNA accumulation [9].

Although it was reported that Ran components are not required for tRNA-mediated nuclear import of HIV retrotranscribed complexes (RTC) in HeLa cells [3], TNPO3/TRN-SR2 has also been implicated in HIV RTC nuclear accumulation, but appears to act by binding tRNA in the nucleus and thereby disassembling the tRNA-RTC complex prior to viral genomic integration (A. Fassati, personal comm.).

Regulation of tRNA nuclear import and/or re-export—Cytoplasmic tRNAs accumulate in nuclei under various conditions: when tRNAs are damaged or uncharged or when cells are deprived of nutrients. One explanation as to why cytoplasmic tRNAs accumulate in nuclei under these conditions is that tRNAs might be imported into nuclei only under stress conditions. However, two types of studies showed that tRNA nuclear import is constitutive. First, in heterokaryons in fed conditions, tRNAs encoded by one nucleus accumulate in the second nucleus not encoding the tRNA when tRNA nuclear export was inhibited by deletion of *LOS1*[1]. The data show that tRNAs enter the second nucleus under fed conditions, rather than only under stress conditions. Second, studies employing a nuclear tethered tRNA modification enzyme that modifies only spliced tRNAs (i.e., previously resided in the cytoplasm) showed that tRNAs enter the nucleus both in replete and nutrient deprived states [52]. Constitutive tRNA nuclear import likely also occurs in vertebrate cells [3].

Since tRNAs accumulate in the nucleus when cells are nutrient deprived or when tRNAs are damaged and tRNA nuclear import is constitutive, the distribution of these tRNAs between

the nucleus and the cytoplasm could be the result of up-regulation of tRNA nuclear import, down-regulation of tRNA re-export, or the combination of both, although down-regulation of re-export is favored[9, 18]. Redistribution of tRNA between the nucleus and cytoplasm dependent on nutrient availability is fast, occurring within ~15 min. of removal or introduction of particular nutrients, implicating a posttranscriptional process. Signaling is independent of the general amino acid control pathway as *gcn2Δ* cells are not blocked for starvation-induced tRNA nuclear accumulation [8]. The TOR amino acid sensitive regulatory pathway has an undefined role in tRNA nuclear-cytoplasmic dynamics as rapamycin treatment failed to cause the expected tRNA nuclear accumulation for this pseudo-starvation condition [8]. Cells defective in the protein kinase A (PKA) pathway or the glucose derepression pathways were blocked for starvation-induced tRNA nuclear accumulation [8]. Since these strains are not only defective for PKA signaling and glucose repression, but exist in a constitutively stressed state, the lack of tRNA nuclear accumulation may be due to the inability to experience acute starvation signaling [8]. Thus, at present, it is unknown which nutrient-dependent signaling pathway(s) is involved in the tRNA retrograde pathway.

Recent studies have shown that there is coordinate regulation between mRNA cytoplasmic dynamics and tRNA nuclear/cytoplasmic trafficking. When cells are deprived of nutrients, P-bodies co-assemble with mRNAs released from polysomes and the mRNAs can be recruited back to ribosomes upon re-feeding [42]. Mutation or over-expression of genes involved in P-body formation - Pat1 and Dhh1 - affect the tRNA retrograde pathway in a parallel fashion[53]. Thus, co-deletion of *PAT1* and *DHH1* that prevents P-body formation and translational repression upon glucose deprivation [54] also prevents accumulation of cytoplasmic tRNAs in the nucleus upon nutrient deprivation; likewise, over-expression of *PAT1* or *DHH1* causes P-body formation and tRNA nuclear accumulation, even in nutrient replete conditions [53, 54]. However, coordinate regulation of P-body formation/translation repression and tRNA nuclear accumulation is restricted to the early part of the P-body pathway as gene products functioning in late steps of the pathway, such as the decapping enzyme and the Xrn1 5′>3′ endonuclease, do not affect tRNA nuclear/cytoplasmic dynamics[53]. Coordination of early steps of P-body formation with tRNA nuclear/cytoplasmic dynamics may provide cells with effective survival in response to adverse conditions. There are other indications that tRNA nuclear/cytoplasmic dynamics are coordinated with stress responses. One of the yeast major heat shock proteins, Ssa2 binds tRNA in an ATP-sensitive manner under nutrient deprivation conditions. Deletion of *SSA2* prevents tRNA nuclear accumulation upon nutrient stress but not when cells are provided with appropriate nutrients (T. Yoshihisa, person comm.). This chaperone-dependent mechanism may define another tRNA nuclear import process that functions in parallel with the Mtr10 Ran-dependent process.

Remaining questions

There are many questions regarding the movement of tRNA from the cytoplasm to the nucleus. The function of this process still must be deciphered and indeed it is quite possible that tRNA retrograde nuclear accumulation serves more than a single biological role. tRNA nuclear import is constitutive but could also be up-regulated upon nutrient deprivation and it seems quite likely that import involves both Ran-dependent and Ran-independent mechanisms. The carriers for neither mechanism have been established, although Mtr10 and Ssa2 are likely candidates. Finally, how tRNA retrograde nuclear import is connected to nutrient-sensitive signaling processes and P-body formation need to be delineated.

tRNA MOVEMENT FROM THE CYTOPLASM TO THE MITOCHONDRIAL SURFACE

Intron-containing pre-tRNAs can easily be detected in the nucleus by fluorescence *in situ* hybridization of tRNAs in wild-type yeast cells [6, 12]. However, under normal conditions these tRNAs are not detected in the cytoplasm, even though introns are not removed until tRNAs contact the outer surface of mitochondria [6]. Cytoplasmic intron-containing tRNAs accumulate if the tRNA splicing endonuclease is defective and the tRNAs appear to be diffusely distributed throughout the cytoplasm, rather than accumulated at the mitochondrial surface [6]. The results document that tRNA splicing on mitochondria occurs very efficiently upon tRNA nuclear export. Whereas it is possible that tRNAs exiting the nucleus simply diffuse to the mitochondrial surface where tRNA splicing endonuclease is located, it seems more likely that a mechanism(s) exists to aid tRNA mitochondrial location (Figure 1). At present there is no information whether such a system does exist and, if so, what gene products are required for mitochondrial delivery.

tRNA IMPORT INTO MITOCHONDRIA

The eukaryotic cell has undergone amazing genetic circuitry rearrangements upon the endosymbiotic appearance of mitochondria from an α-proteobacterial ancestor [55–57]. During evolution, many mitochondrial genes have been either functionally transferred to the nucleus or replaced by pre-existing nuclear genes of similar function. In maintaining mitochondrial function, a vast number of mitochondrial proteins are encoded in the nucleus, synthesized in the cytoplasm, and subsequently imported into the organelle. Together, a few mitochondria-encoded subunits along with the many nucleus-encoded mitochondria-targeted proteins assemble into fully operational respiratory complexes, generating ATP through oxidative phosphorylation[58].

In early analyses of the *Tetrahymena pyriformis* mitochondrial genome, Suyama did not find two-thirds of the tRNA genes needed to complete mitochondrial protein synthesis. This led to his original proposal that cytosolic tRNAs must be imported into mitochondria [59] (Figure 1). To date, multiple occurrences of tRNA import have been described in diverse organisms: *Tetrahymena* [60–62], *Trypanosoma* and *Leishmania* [63–65], *Toxoplasma* [66], yeast [67, 68], *Triticum*[69], Paramecium [70], plants [71] marsupials [72], and most recently, placental mammals [5]. The number of imported tRNAs varies greatly from a few tRNAs in the case of yeast to a full set of tRNAs in some protists [73]. In the kinetoplastids *Leishmania* and *Trypanosoma*, no tRNA genes are encoded in the mitochondrial genome. As a consequence, these mitochondria strictly require tRNA import for the translation of the few mitochondrial proteins still encoded in the organellar genome. In other organisms, such as yeast and humans, where most tRNAs are encoded in the mitochondrial genome, the import of some nucleus-encoded tRNAs is proposed to function in mitochondrial wobble codon decoding [68], as shown by the mitochondrial import of one nucleus-encoded lysyl tRNA to decode a lysine codon under stress conditions[74]. In the present review, we provide a general overview of current knowledge regarding the diversity of import systems, examine some experimental discrepancies, and accentuate future challenges.

The disappearance of a varying number of tRNA genes from mitochondrial genomes among different organisms has perhaps driven the independent evolution of systems that permit import of tRNAs from the cytoplasm [73]. Common to all systems is the fate of the given tRNA in traversing an outer and an inner lipid-bilayer membrane before reaching the mitochondrial matrix, the site for mitochondrial translation. However, there are various systems residing in the cytosol commissioned to the delivery of tRNAs to the mitochondrion. The protein(s) involved in these mitochondrial delivery systems all have had

prior assigned functions, such as glycolysis, translation, and amino acid activation [73]. As a consequence, these proteins currently implicated in mitochondrial delivery of tRNA have now become classified as multi-functional proteins. Although there are various systems proposed to recognize a given tRNA in the cytosol and deliver it to the mitochondrion, the possibility that a given tRNA may reach the mitochondrion by the direct recognition or recruitment of a component of the outer membrane still remains, as suggested by existing *in vitro* import systems.

Given the integral association with components of the translation machinery, tRNAs also need to be recycled during protein synthesis. Therefore, one conundrum is how tRNAs are freed from the cytoplasmic translation machinery to reach the mitochondrion. One proposal suggests that a fraction of the nucleus-encoded tRNAs escapes the cytosolic translation machinery by interacting with protein factors that will consequently be directed to the mitochondrion. In *Trypanosoma brucei*, the cytosolic translation elongation factor 1a (eEF1a) is thought to play this role as a specificity determinant for a small subset of imported tRNAs[75] (Figure 2). The major role of EF1a is to deliver the aminoacylated tRNA (aa-tRNA) to the ribosome in the form of an EF1a-aa-tRNA-GTP ternary complex[76]. However, in *T. brucei*, the knockdown of cytosolic eEF1a, but not of initiation factor 2, inhibits mitochondrial import of newly synthesized tRNAs before translation or growth is affected[75]. tRNA import requires both eEF1a and aminoacylation of the tRNA[75]. How this complex avoids interaction with the ribosome to enter the mitochondrial import pathway has yet to be explained.

Mitochondrial tRNA import systems

In refraining from the relaxed use of the term “mechanism”, we separate the mitochondria import systems into two broadly defined types (Figure 2). Type A tRNA import utilizes the known protein import pathway and has strict requirements for the presence of a membrane potential and ATP hydrolysis. In systems that fall into the type B category, tRNA import itself occurs independently of the canonical protein import pathway[77, 78]. Type A import is limited to the single known example from yeast where direct participation of the canonical and well-characterized protein import machinery helps drive the tRNA across the mitochondrial double membrane [79]. The protein(s) that reside in the mitochondrial membranes to comprise the tRNA import machinery themselves have to be translocated from the cytoplasm and/or inserted into the mitochondrial membranes. Therefore, there exists a delicate nuance in the study of tRNA transport systems in carefully teasing the secondary effects of protein import from that of the downstream function tRNA import.

Type A mitochondrial tRNA import in yeast—In *S. cerevisiae*, the molecular mechanism of cytosolic $tRNA^{Lys}$(CUU), tRK1, mitochondrial targeting involves interaction of tRNA with cytosolic factors, enabling the tRNA to escape from the cycle of cytosolic protein synthesis. A portion of aminoacylated cytosolic $tRNA^{Lys}$(CUU) can be specifically recognized by one of the two isoforms of the glycolytic enzyme enolase, Eno2p[80]. Eno2p is then targeted to a large glycolytic macromolecular complex known to be tightly associated with the outer mitochondrial surface. [81] (Figure 2).

The aminoacylated cytosolic $tRNA^{Lys}$(CUU) tRK1 is taken up by the precursor mitochondrial lysyl-tRNA synthetase, pre-mito LysRS (preMSK1p), which is responsible for the aminoacylation of its true substrate, the mitochondria-encoded $tRNA^{Lys}$ counterpart in the mitochondrial matrix (Figure 2). Therefore, pre-mito LysRS is a multi-functional cytosolic protein that also serves as the carrier for cytosolic $tRNA^{Lys}$(CUU) translocation into the mitochondrial matrix [82]. Further genetic and biochemical assays have also isolated three components of the ubiquitin/26S proteasome system, Rpn13, Rpn8, and Doa1, that

interact with the tRNA and the pre-mito LysRS [81]. These proteins plays a dual regulatory role, since the overall inhibition of cellular proteasome activity reduces tRNA import, while specific depletion of Rpn13 or Doa1 increases import of $tRNA^{Lys}(CUU)$[81]. It is clear that unfolding is a key step in protein import into mitochondria; thus, how a structured tRNA-precursor synthetase complex enters and traverses the mitochondrial membranes remains unclear [79].

Type B tRNA import in yeast—*S. cerevisiae* is a unique case in which the two disparate systems, Type A and Type B import, co-exist. Type B import in yeast results in the mitochondrial delivery of two cytosolic tRNAs, $tRNA^{Gln}(CUG)$ and $tRNA^{Gln}(UUG)$[68]. Independence from the protein import pathway for $tRNA^{Gln}$ was demonstrated by *in vitro* experiments using isolated mitochondria where the membrane potential was inhibited, no cytosolic factors were present, but the tRNA was efficiently imported. The localization of both the cytoplasmic $tRNA^{Gln}$ and the cytosolic glutaminyl sythetase (GlnRS) in highly purified mitochondria was also clearly demonstrated by *in vivo* immunofluorescence. The successful rescue of a mitochondrial *S. cerevisiae* in-frame amber stop codon mutant demonstrated the functional tie of an amber suppressor imported $tRNA^{Gln}$ and the cytoplasmic GlnRS in the organelle in generating mitochondrial pools of $Gln\text{-}tRNA^{Gln}$. However, it is possible that in mitochondria the transamidation pathway also generates $Gln\text{-}tRNA^{Gln}$ by a tRNA-dependent glutamate modification of $Gln\text{-}tRNA^{Glu}$, found in bacteria, archaea, and chloroplasts [83–86]. One study uncovered mitochondrial import of the cytosolic glutamate synthetase that is capable of mischarging the mitochondria-encoded $tRNA^{Gln}$, forming $Glu\text{-}tRNA^{Gln}$, that is subsequently fed into the transamidaton pathway [87]. The same study was unable to detect the presence of any cytosolic $tRNA^{Gln}$ species in yeast mitochondria [87]. The The failure of detection of cytosolic $tRNA^{Gln}$ in this mitochondrial study remains a discrepancy. There may still be the interesting possibility that two distinct events contribute to the generation of $Gln\text{-}tRNA^{Gln}$ species in *S. cerevisiae* mitochondria where these events may well be subject to differences in strain and/or culture growth conditions.

Type B tRNA import into mammalian mitochondria—The Type B tRNA import system also exists in mammalian mitochondria [5]. Using clean subcellular RNA fractions from rat liver and human cells, nucleus-encoded $tRNA^{Gln}(CUG)$ and $tRNA^{Gln}(UUG)$ species have been shown to localize to mitochondria in vivo[5]. Import of *in vitro* transcribed tRNAs, but not of heterologous RNAs, into isolated mitochondria also demonstrates that this process is tRNA-specific and does not require cytosolic factors. Although this *in vitro* system requires ATP, it is resistant to inhibitors of the mitochondrial electrochemical gradient, a key component of protein import. Therefore, $tRNA^{Gln}$ import into mammalian mitochondria proceeds by a mechanism distinct from protein import. Evidence shows that both $tRNA^{Gln}$ species and a bacterial $pre\text{-}tRNA^{Asp}$ can be imported *in vitro* into mitochondria isolated from myoclonic epilepsy with ragged-red fiber cells, given that sufficient ATP (2 mM) is provided[5]. This work suggests that tRNA import is more widespread than previously thought and may be a universal trait of mitochondria.

Type B tRNA import machinery in the trypanosomatids—Among the most studied tRNA import pathways are those of kinetoplastids. To date, the RNA import complex (RIC) of *Leishmania tropica* is by far the system with the most proteins proposed to import tRNAs into mitochondria [88–90]. Mass spectrometry analyses of the multi-protein ~580 kDa *L. tropica* RIC revealed a total of 122 nuclear-encoded ORFs [88]. Further Western blot and RNAi analyses led to the final composition of eleven major subunits, RIC1, 2, 3, 4A, 4B, 5–7, 8A, 8B and 9[88–90]. The functional characterization of the RIC showed import of tRNAs into phospholipid vesicles in an ATP-dependent manner [90, 91].

Recent studies have explored whether the described *L. tropica* RIC components have functional conservation with another related kinetoplastid, *Trypanosoma brucei*. The involvement of the Rieske subunit of complex III (RIC6) is essential for both maintenance of membrane potential and respiration in *T. brucei* [92]. However, down-regulation of the Rieske protein levels by RNAi had no effect on mitochondrial tRNA import *in vivo* and *in vitro* in *T. brucei* [92]. These findings demonstrate that the Rieske protein does not play a major role in mitochondrial tRNA localization in *T. brucei* and corroborates previous observations [93, 94]. In addition, despite relying heavily on glycolysis instead of respiration, the bloodstream stage mitochondria still import tRNAs[95]. Therefore, subunits of respiratory complexes are not required for both protein and tRNA import[95, 96].

Remarkably, the ATP requirement and lack of a requirement for membrane potential is a conserved feature of the *T. brucei* and *L. tarentolae* pathways [93, 94], but again differs from what has been shown in *L. tropica* [97]. Highlighting the *L. tarentolae in vitro* import system is the observation that the system reaches saturation at micromolar tRNA concentration, which strongly implies that the *in vitro* assay exhibits dynamics akin to receptor-mediated systems, is consistent with the levels of *in vivo* localization of these tRNAs providing a valid mimic of the in vivo situation [94, 98, 99]. Currently, the true nature of the *L. tropica* import complex remains questionable and is under further scrutiny[100]. Taken together, these data highlight the numerous incongruities among the various tRNA import systems analyzed, not only between diverse organisms, but also among closely related ones. More information regarding the transporters is required before establishing commonalities among different factors involved in tRNA transport.

Type B tRNA import machinery in plant mitochondria—In plants, import of nucleus-encoded cytosolic tRNAs is essential for mitochondrial biogenesis. tRNA import is also ATP-dependent and does not require any added cytosolic factors [101]. Plant mitochondrial tRNA import can be inhibited *in vitro* by valinomycin or oligomycin, suggesting the requirement for both a membrane potential and a functional respiratory chain, which would make it a candidate for Type A import. Indeed, two proteins from the classical protein import pathway were found to be important for tRNA import. Specifically, the translocase of the outer mitochondrial membrane complex, TOM20 and TOM40, are important in binding tRNA. However, the translocases of the inner membrane (TIMs) from the protein import pathway are apparently not involved in tRNA import to plant mitochondria [102], as they are in Type A import of yeast lysyl tRNA, where TOM and TIM protens are needed to import the precursor lysyl synthetase protein with tRNA as its cargo[103]. Therefore, plant mitochondrial import of tRNAs seems to follow the Type B import pathway.

The plant mitochondrial voltage-dependent anion channel (VDAC) interacts with tRNA *in vitro* (Figure 2) and is proposed to be a major component of the channel involved in the tRNA translocation step through the plant mitochondrial outer membrane[102]. Playing an essential role in metabolite transport, the VDAC provides a permeability pathway through the mitochondrial outer membrane by forming voltage-gated channels with an open pore radius of 3 nm to 1.8 nm in the closed state[104]. Remarkably, the VDAC is capable of selecting among small molecules, ions, and negatively charged metabolites such as succinate, malate, or ATP with a much smaller effective radius, 0.4±0.5 nm. How the VDAC performs another function in the transport of tRNAs into plant mitochondria in its open state remains a puzzle, since the tRNA tertiary L-shaped structure is large. This raises the further question of whether tRNAs must unfold prior to translocation through the VDAC.

In contrast to plants, in *T. brucei* there is no identifiable homolog of TOM40 in the genomic database, despite maintaining a tRNA import mechanism. Additionally, import of tRNAs occurs efficiently in *T. brucei* VDAC knockout cells. Thus, unlike tRNA import in plant mitochondria, the VDAC is not required for mitochondrial tRNA import in *T. brucei* [105].

Sequence determinants for tRNA mitochondrial import

Structural folding of a tRNA molecule is controlled by base-pairing of the primary sequence into secondary structure, the cloverleaf structure, followed by further stabilization by co-axial stacking of the helices and higher order tertiary interactions. Despite the existence of numerous tRNAs in the cell, in general, most naturally fold into one canonical L-shape tertiary structure. However, within the L-shaped structure, tRNAs have extraordinary structural versatility that fine-tunes local structure. While it seems likely that specific cis-acting sequences may act as determinants or anti-determinants for tRNA mitochondrial import, as summarized below, many studies conclude that the global tertiary fold of the tRNA molecule that seems to direct import.

In some organisms, the anticodon is an important determinant for mitochondrial tRNA import. For example, in the protozoa *Tetrahymena*, only one of the three isoacceptors, $tRNA^{Gln}$(UUG), travels into mitochondria and substitution of a single anticodon nucleotide (UUA-->UUG) is both necessary and sufficient for tRNA import and confers import on a normally non-imported glutamine tRNA [62](Figure 3A).

In higher plants, import into tobacco cell mitochondria involve essential determinants in the anticodon, D-arm for $tRNA^{Gly}$(UCC) and the anticodon, D-arm, and TΨC-arm for $tRNA^{Val}$(AAC) [101, 106107] (Figure 3B and 3C). However, in *Phaseolus vulgaris* and the lower plant *Marchantia polymorpha*, recognition of tRNAs for import appears idiosyncratic and specific to each tRNA or isoacceptor group [108, 109].

Earlier observations suggested that in *Trypanosoma brucei*, dicistronic precursors containing the $tRNA^{Ser}$ and $tRNA^{Leu}$ transcripts with a 59-nucleotide intergenic sequence were the substrate for tRNA import *in vivo* (Figure 3D) [110]. In contrast, other experiments showed that at least in the case of some tRNA isoacceptors, import occurs regardless of the sequence context of the imported tRNA, implying that the tRNA pre-sequence does not contain sequence-specific determinants for import in *T. brucei* [111]. For some tRNAs, internal nucleotides involved in eEF1a interactions are important. A major localization determinant of *T. brucei in vivo* import was found within the TΨC-stem nucleotide base-pair at nucleotides 51 and 63 within $tRNA^{Met}$, $tRNA^{Ile}$ and $tRNA^{Lys}$ [75] (Figure 3E). However, the cytosol-specific initiator $tRNA^{Met}_i$ containing a different nucleotide pair at 51 and 63, corresponding to the main anti-determinant, was found to prevent its interaction with the cytosolic eEF1a. Being the only other cytosol-specific tRNA in *T. brucei*, $tRNA^{Sec}$ has its own elongation factor and does not bind eEF1a. However, upon introduction of a mutation of $tRNA^{Sec}$ to render it capable of binding to eEF1a, the mutant $tRNA^{Sec}$ was imported into mitochondria[75].

In *L. tarentolae*, mature tRNAs are also substrates for mitochondrial import *in vivo* [99, 109]. Localization of $tRNA^{Ile}$(UAU) is mostly within the mitochondrion, while $tRNA^{Gln}$(CUG) is primarily in the cytosol [98, 112, 113]. Both *in vivo* and *in vitro* experiments further demonstrated that swapping the D-stem and loop from the mainly cytosolic $tRNA^{Gln}$ with that from the $tRNA^{Ile}$ produced increased mitochondrial localization of the tRNA[94, 112]. This D-loop exchange did not eliminate the mitochondrial localization of $tRNA^{Ile}$(D-Gln) *in vivo* and *in vitro*. The role of tertiary tRNA structure or additional sequence elements were proposed to contribute an essential role in import (Figure 3F) [94, 112], since proper folding of

the chimeric $tRNA^{Gln}$(D-Ile) was confirmed by successful aminoacylation by the cytosolic glutaminyl tRNA synthetase [114].

In *Leishmania tropica* mitochondria, imported tRNAs have one of two signature consensus sequences: Class I tRNAs or Class II [115, 116]. Class I tRNAs contain a conserved sequence motif in the D arm that positively stimulates import of type II molecules into the mitochondrial matrix. In contrast, class II tRNAs, with motifs within the variable region and the TΨC domain, are poorly imported and inhibit import of class I tRNAs [115, 116].

In contrast to the studies designed to identify tRNA determinants within the context of the L-structure, other studies have shown that structures deviating from the canonical tRNA tertiary model may be substrates for mitochondrial import. One approach addresses minimal substrates sufficient for mitochondrial import. The D-arm of $tRNA^{Tyr}$, AUGGCAGAG, was used to isolate the *L. tropica* RIC^{88} (Figure 3G). However, in another study, more sequences were extensively surveyed, no obvious consensus sequence correlated with tRNA localization, calling into question its *in vivo* importance [117]. In *L. tarentolae* mitochondria, smaller RNAs including five different 16 to 17-nucleotide mini-helix RNAs, some of non-tRNA origin, and one unstructured could be efficiently imported *in vitro*; however, unstructured RNAs of greater sizes (up to 33 nucleotides in length) failed to support import. [94]. Therefore, while shorter RNA molecules may be efficiently imported *in vitro*, the import of short RNA substrates strikingly leads to the loss of discrimination by the mitochondria [94].

In yeast, an alternative tRNA structure may be imported into mitochondria. Although for $tRNA^{Lys}$(CUU), studies mapped the acceptor stem base-pair, the discriminator nucleotide, and the wobble position C_{34} of the anticodon, to be important for mitochondrial import [118–120] (Figure 3H), an alternative conformation of $tRNA^{Lys}$(CUU) into an F-structure[121] (Figure 3I). In the F-structure, there is a proposed formation of a helix between base pairs $C_{74}G_{57}$ to $G_{69}C_{62}$, such that the 3′-end of the acceptor stem forms a helix with the 3′ portion of the TψC stem, therefore re-structuring the anticodon nucleotides into a long hairpin with the 5′-end of the TψC stem[121]. Enolase is thought to modulate the tRNA structure and play a role in mitochondrial import selectivity[121]. How the lysyl tRNA synthetases recognize the F-structure remains an open question.

Remaining questions

Intracellular trafficking of tRNA molecules may not be limited only to the nucleus and mitochondria, but may also expand other organelles. Inspection of chloroplast genomes shows that most tRNAs needed for translation are encoded by the organelle [122]. However, the data do not eliminate the possible import of tRNAs into the chloroplast. The plastid genome of a non-photosynthetic parasitic flowering plant *Epifagus virginiana* lacks all genes for photosynthesis and respiration found in chloroplast genomes of green plants [123]. This highly reduced, compact plastid genome of *E. virginiana* maintains functionality while missing six ribosomal proteins and 13 tRNA genes which are found in the chloroplast DNAs of its photosynthetic flowering plant host [123]. To compensate for these losses, import of nucleus-encoded gene products including tRNAs may occur [124]. tRNA import into chloroplasts and plastids remains an unexplored area.

A daunting number of mutations located within tRNA genes account for numerous human mitochondrial neuromuscular and neurodegenerative disorders [125, 126]. Within the current horizon, experimental designs incorporate the transplantation of a surrogate tRNA import machinery into a human cell line to deliver a given tRNA to the defective mitochondria [127–130]. Since mammalian mitochondria actively import tRNAs, Since mammalian mitochondria actively import tRNAs, the prospect of mitochondrial therapy is

possible. However, the rescue of tRNA import *in vitro* into mitochondria from MERFF patients required the addition of excess ATP. This observation raises the possibility that unless alternative ATP sources are provided *in vivo*, both native and surrogate import systems may fail as potential therapies[5]. Use of a surrogate system has been reported to rescue some level of mitochondria function. However, it is not clear how this or any *in vitro* system represents the *in vivo* situation with whole organisms. It could well be that import systems may themselves be compromised by mutations that affect certain mitochondrial functions such as the ability to synthesize enough ATP. Secondly, the proteins involved in tRNA import may require the protein import pathway for proper assembly in the membranes. Therefore, in examining defects in tRNA import, the careful teasing apart of secondary effects due to a malfunction of mitochondrial protein import seems pertinent.

All organisms share a common goal in translocation of a given tRNA to the mitochondrial matrix for translation, yet much about the nature of the import machinery still remains to be fully understood. Although the requirement of external ATP has been shown in all systems studied to date, its precise role at the outer membrane is still unknown, except in plants where ATP is required for the tRNA binding step at the surface of mitochondria. Across the eukaryotes, a fundamental observation is that mitochondrial genomes bear a higher burden of deleterious mutations than that of nuclear genomes[131]. Although evolutionary forces have driven the reduction of organellar genomes, the cell also developed means of recruiting cytosolic factors encoded in the nuclear genome to maintain organellar function. Alluding to the growing prospect that tRNA import may also occur in other organelles, the variations on the theme seen among nucleus and mitochondria tRNA import systems may shed some light on how the cell meticulously coordinates the travel itinerary of tRNAs within its confines.

Acknowledgments

We thank Drs. A. Fassati and T. Yoshihisa for communication of unpublished results and Dr. J. Alfonzo for comments on the manuscript. The work from the authors' laboratories was supported by NIH grants GM27930 (to AKH) and GM084065 to J. Alfonzo.

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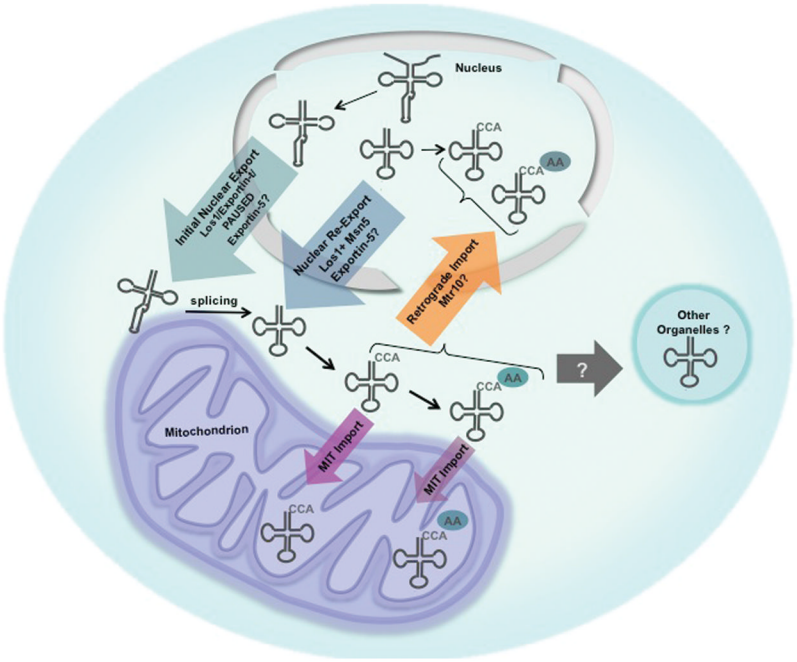

Figure 1.

Movement of tRNAs in eukaryotic cells. Nuclear encoded tRNAs are transcribed and largely processed in the nucleus. In yeast, but not in vertebrate cells, tRNAs encoded by intron-containing genes, are exported (initial tRNA export; teal arrow) to the cytoplasm prior to being spliced. Splicing occurs on the outer surface of mitochondria. tRNAs in the cytoplasm are able to return to the nucleus via retrograde import (orange arrow) and, under nutrient replete conditions, the tRNAs can be re-exported to the cytoplasm (blue arrow). A subset of tRNAs can be imported into the mitochondrial matrix, past the outer and inner mitochondrial membranes (purple arrows). It is unknown whether cytoplasmic tRNAs are imported into other organelles such as chloroplasts (grey arrow).

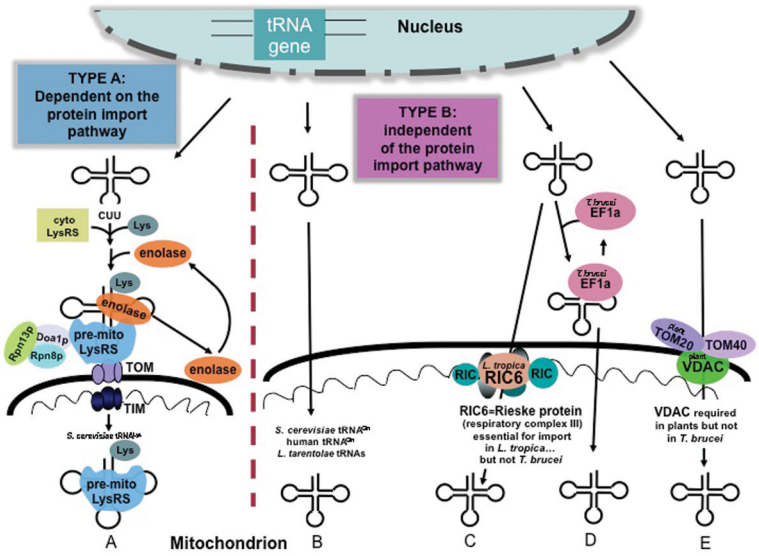

Figure 2.

Two types of tRNA import into mitochondria. Type A: import is strictly dependent on the protein import pathway A) *S. cerevisiae* $tRNA^{Lys}$(CUU) (tRK1) is aminoacylated then recognized by enolase the precursor form of the mitochondrial lysyl tRNA synthetase followed by delivery to the mitochondrial surface Type B: tRNA import occurs independently from the protein import pathway. B) *S. cerevisiae* $tRNA^{Gln}$, human $tRNA^{Gln}$, & *L. tarentolae* tRNAs, D) *L. tropica* $tRNA^{Tyr}$, *T. brucei* tRNA, E) *plant* $tRNA^{Gln}$.

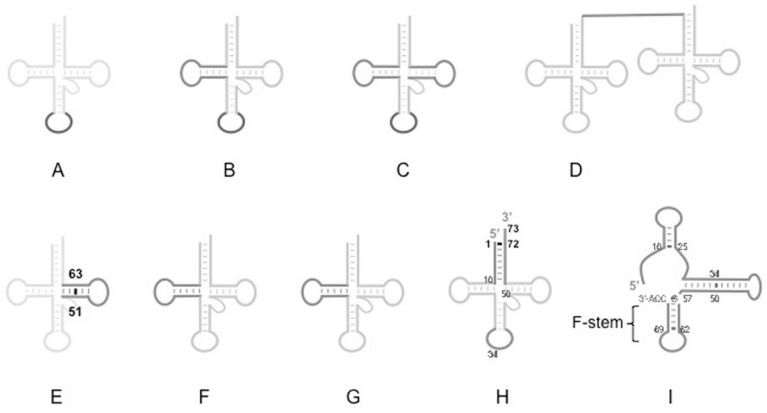

Figure 3.

Sequence determinants for tRNA import into mitochondria: A) anticodon nucleotide of *T. pyriformis* $tRNA^{Gln}$, B) anticodon and D-arm of *N. tabaccum* $tRNA^{Gly}$ C) anticodon, D-arm and TΨC-arm of *N. tabaccum* $tRNA^{Val}$, D) intergenic sequence between the precursor form of the dicistronic $tRNA^{Ser}$ and $tRNA^{Leu}$ of *T. brucei*, E) T-stem base pair 51:63 of *T. brucei* tRNA, F) D-arm of *L. tarentolae* $tRNA^{Ile}$ and $tRNA^{Gln}$, G) D-arm of *L. tropica* $tRNA^{Tyr}$, H) anticodon, acceptor stem base pair 1:72, acceptor nucleotide, 73 of *S. cerevisiae* $tRNA^{Lys}$(CUU), and I) proposed conformational rearrangement of *S. cerevisiae* $tRNA^{Lys}$(CUU) into the F-structure. Dark lines highlight the sequence determinant(s).