Chapter 4
Mitochondrial Targeting of RNA and Mitochondrial Translation

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Abstract Mitochondrial translation depends on the macromolecular components imported from the cytosol, which include translation factors, ribosomal proteins, aminoacyl-tRNA synthetases, and a variable number of small noncoding RNAs. The lasts are essentially tRNAs, but other small RNAs, like mammalian 5S rRNA, are also concerned by the RNA mitochondrial targeting pathway. If their importance in mitochondrial translation was demonstrated in each case where it was addressed, the precise function of these molecules differs from one system to another: in many cases they complement lacking mtDNA encoded counterparts, in others can fulfill conditional functions, finally they can complement the lack of needed mitochondrial enzymatic activities. In any case, it appears that the innated capacity of mitochondria to import small RNA molecules is supplied by specific

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additional protein, often performing their "second job" to deliver the needed RNA in the organelle. This mechanism, still not understood in details, remains the unique natural pathway of nucleic acids delivery in mitochondria, and is therefore of a significant interest as a tool permitting to target this organelle with potentially therapeutic molecules and thus addressing a very important bulk of human pathologies linked with dysfunctions of mitochondrial translation machinery.

4.1 Introduction

This chapter focuses the role of mitochondrially targeted cytosolic RNA molecules in the organellar translation. Mitochondria import the majority of macromolecules from the cytosol, including almost a thousand of proteins and a various number of small noncoding RNAs. Mitochondrial targeting of RNAs is nowadays to be considered as a quasi-ubiquitous process, found in almost all studied biological systems (for review, see Entelis et al. 2001c; Rubio and Hopper 2011; Salinas et al. 2008). In all studied cases, naturally imported RNAs are small and noncoding: they are essentially tRNAs, but larger RNAs (like 5S rRNA, MRP, or RNase P RNA components) or smaller ones (like microRNAs) were described as mitochondrially targeted. A major part of these molecules clearly refer to protein synthesis machinery (tRNAs, 5S rRNA, RNase P). So far, different cases of RNA import of even similar types of molecules may have different functional implications—sometimes they are clearly essential, sometimes only conditionally important, in some cases their implication in mitochondrial translation is still not evident. The first part of the chapter tries to review these different cases with the emphasis on still unclear functional issues or contradictory data.

Mitochondrial DNA mutations are the recognized source of neuromuscular pathologies (Ruiz-Pesini et al. 2007). This large group of "mitochondrial diseases" includes mutations in virtually all mtDNA encoded genes and often have a strong impact on mitochondrial translation (Smits et al. 2010). One of their main features is the phenomenon of heteroplasmy, meaning the simultaneous presence of mutant and wild-type genomes in the same cell, the level of heteroplasmy determining the pathogenic effect of the mutation. All these pathologies have no efficient classical therapy and many attempts are currently done to develop alternative gene therapy strategies. Two main approaches were proposed—the "allotopic" one was to express in the nucleus the lacking or therapeutically active molecule and to address into mitochondria; and the "antigenomic" one, which aims to decrease the heteroplasmy below the threshold pathogenic level by addressing into the organelle specific recombinant molecules (Smith et al. 2004). If both strategies may use imported protein, nucleic acids delivery into mitochondria appears as more attractive. Therefore, in the second part of the chapter, we shall focus here RNA targeting into mitochondria, since many recent studies were aimed to exploit this pathway as a tool to correct dysfunctions of the mitochondrial translation machinery.

4.2 Imported tRNAs and Mitochondrial Translation

4.2.1 Plants

The situation in higher plants appears as the most evident—up to now, in all cases mitochondrially imported RNA species were limited to tRNAs and, also in all cases, imported tRNA species strictly corresponded to those whose genes were absent in mtDNA (Dietrich et al. 1992; Kumar et al. 1996; Salinas et al. 2008; Schneider and Marechal-Drouard 2000). The mechanism of this pathway is actively studied. Aminoacyl-tRNA synthetases, another component of the translation machinery, were formerly implicated in tRNA mitochondrial targeting (Dietrich et al. 1996; Small et al. 1992), while at the mitochondria level, Voltage Dependent Anion Channel (VDAC) and/or TOM complex were involved in tRNA translocation across mitochondrial membranes (Salinas et al. 2006, 2008).

There still is no evident evolutionary explanation why a given tRNA gene was conserved in mtDNA and the other—not, as well why there is a so significant variation in the imported tRNAs among different plant species, ranging from few to more than a half of the mitochondrial set. One could assume therefore that the emergency of tRNA import was polyphyletic and occurred many times during evolution, maybe in a sporadic way (Kumar et al. 1996). The similarity of mitochondrial and nuclear genetic code in plants may, at some extent, explain this situation: indeed nuclear-encoded and mitochondrially addressed tRNAs not only can function both in cytosolic and organellar translation, but also cannot become potentially toxic nonsense/missense suppressor tRNAs in the cytosol.

The question arises if there exist any regulatory mechanism which would permit to optimize mitochondrial translation by differential tRNA import efficiency. A priori there is no limitation for that, taking into account that mitochondrial subset of tRNA is a minor one comparing to the cytosolic pool. Indeed, in the case of a lower plant cells, in *C. reinhardtii,* mitochondrial abundance of nuclear DNA encoded tRNA was found to be in correlation with mtDNA genes codon usage (Vinogradova et al. 2009), which may reflect the existence of a kind of retrograde control of RNA import or, alternatively, a controlled system of RNA degradation in the organelle.

4.2.2 Trypanosomes

The group of *Trypanosomatidae* represents an extreme case, where all or almost all tRNAs are nuclearly encoded (Hauser and Schneider 1995; Rubio et al. 2000; Schneider 1994; Schneider and Marechal-Drouard 2000; Simpson et al. 1989). The need of these molecules for mitochondrial translation appears as evident, since no mtDNA-coded tRNA genes were ever identified while mitochondrial translation of the kinetoplast DNA (trypanosomal equivalent of mtDNA) is rather

standard. The situation on these protozoans was recently exhaustively described in (Schneider 2011), therefore, we focus here only few translation-related issues.

In such trypanosomatids as *Trypanosoma brucei* or *Leishmania tarentolae*, cytosolic and mitochondrial pools of tRNAs are not identical, the exception concerning the initiator $tRNA^{Met}$ and the $tRNA^{Sec}$, both found only in the cytosolic compartment of the cell (Geslain et al. 2006; Tan et al. 2002b). This fact refers directly to the absence of functional need of these tRNA species in the mitochondrial compartment. Mitochondrial initiation of translation does not require special eukaryotic initiator tRNA since a portion of mitochondrially imported cytosolic elongator $tRNA^{Met}$ is formylated by specific $tRNA^{Met}$-formyltransferase (Charriere et al. 2005; Tan et al. 2002a). This formylated elongator tRNA is then recognized by the mitochondrial bacterial type initiation factor. This is just an example of the higher extent of mitochondrial translation flexibility, which is observed also in other species, as described below. Also, as in many other cases, it is difficult to explain how such mechanism was retained in evolution. It clearly demonstrates that many features of mitochondrial translation may have sporadic origins, and therefore being species/genera-specific. On the other hand, it also illustrate strictly classical functional explanation of RNA import in terms of translation processes, without involving "alternative functions" of imported RNA molecules, as for $tRNA^{Sec}$, whose presence in trypanosomatid mitochondria would be obsolete, in the absence of selenoproteins.

4.2.3 Yeast

In ascomycetes yeast *Saccharomyces cerevisiae* the situation with imported tRNAs is all but simple, taking into account that mtDNA appeared to encode all the set of tRNAs, in theory able to fulfill translational activity (Foury et al. 1998). So far two cases of tRNA import were independently described. The first concerns one out of two cytosolic $tRNA^{Lys}$, $tRNA^{Lys}{}_1$ $\left(tRNA^{Lys}{}_{CUU}, \text{ or } tRK1\right)$. This cytosolic tRNA was partially found in the mitochondrial compartment of the cell in 1979 (Martin et al. 1979), while the second $tRNA^{Lys}$ $\left(RNA^{Lys}{}_{UUU}, tRK2\right)$ resided only in the cytosol. The mechanism of tRK1 delivery into yeast mitochondria was studied in details and involved several targeting factors (Enolase-2 as an RNA chaperone and the cytosolic precursor of mitochondrial lysyl-tRNA synthetase pre-Msk1p) and the TOM/TIM translocators (Entelis et al. 2006; Kamenski et al. 2007b; Kolesnikova et al. 2010; Tarassov et al. 1995), issues recently reviewed elsewhere (Rubio and Hopper 2011; Salinas et al. 2008; Schneider 2011). Up to more recent studies its function in the mitochondria was not clear. Indeed, mtDNA codes for another $tRNA^{Lys}$ (tRK3), which, due to its modification of the wobble position ($cmnm^5S^2$ U) was predicted to decode both AAA and AAG lysine codons in the organelle. Several alternative functions (like participating in splicing or mtDNA replication priming) were proposed for mitochondrial tRK1 (reviewed in Entelis et al. 2001c), so far these hypotheses never found experimental evidence. Finally, a peculiar mechanism was uncovered. It was shown that mitochondrially encoded tRK3 anticodon wobble base was undermodified (underthiolated) at

higher temperatures, and, as a consequence loses the ability to efficiently decode the very rare mitochondrial AAG codons, while the imported CUU anticodon bearing tRK1 complements this deficiency (Kamenski et al. 2007a; Tarassov et al. 2007) (Fig. 4.1). This mechanism was the first case of mitochondrial translational

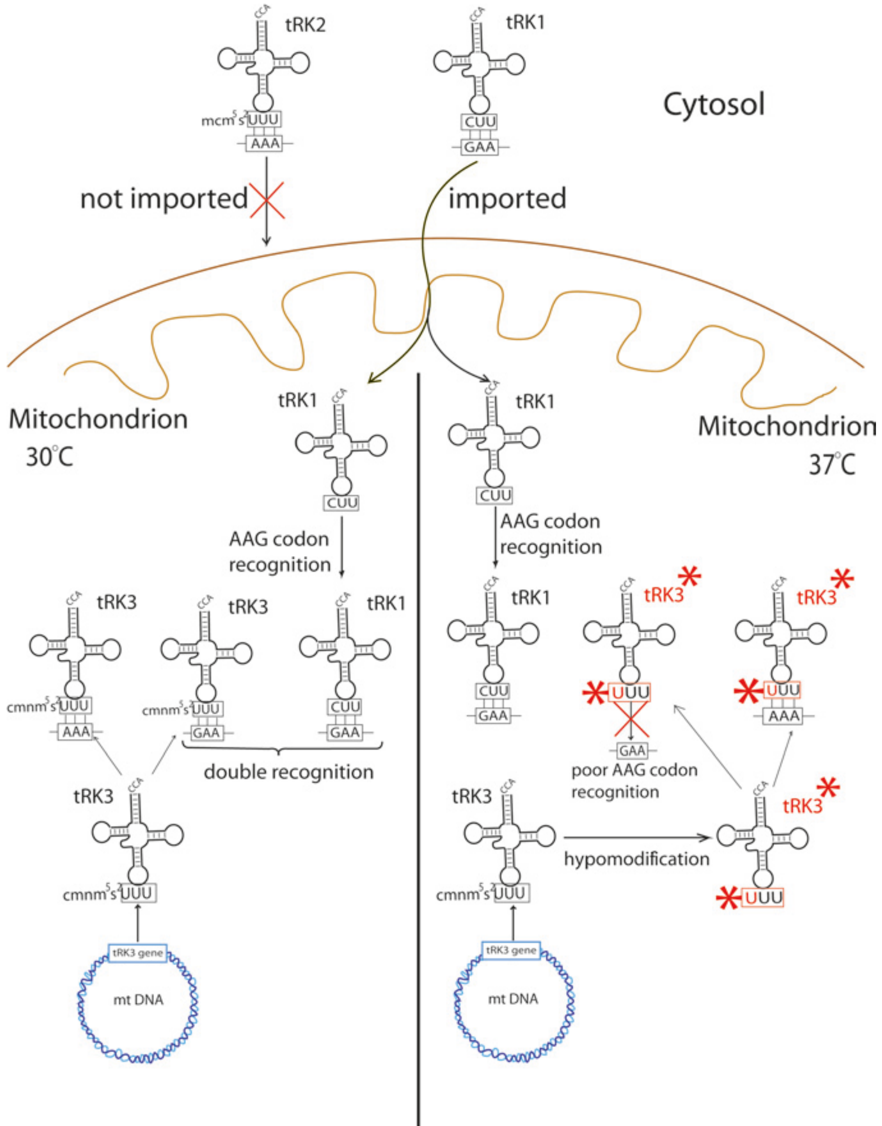

Fig. 4.1 tRNA import into yeast mitochondria is a mechanism of translational adaptation to stress conditions. At the *left* side of the scheme, normal conditions of growth (30 °C), when both lysine codons can be read by mtDNA encoded tRK3. At the *right* side—at higher temperature tRK3 wobble position of the anticodon in undermodified (underthiolated) which prevents efficient decoding of the AAG lysine codon, while imported tRK1 becomes essential for mitochondrial translation

adaptation to stress conditions. So far many questions remains to be solved. tRK1 is imported in mitochondria in a constitutional manner, and not only at higher temperature, while its function in mito-translation becomes detectable only at elevated one. So it is not clear if it participates in translation in a permanent way and if it is more or equally efficient to decode AAG codons in normal conditions. In this context it is worth to stress that tRK1 is aminoacylated by the cytosolic lysyl-tRNA synthetase (KARS), imported into mitochondria in aminoacylated form and cannot then be reacylated in the organelle by the mature mitochondrial synthetase (MSK1), the last one able to charge only tRK3 (Tarassov et al. 1995). This obviously limits the efficiency of tRK1 use in mitochondrial translation. It is still not evident if there exist any regulatory mechanisms governing this targeting. Indeed, it was demonstrated that differential deregulation of the proteasomal degradation of proteins (ubiquitin/26S proteasome system, UPS) may have various effects on tRK1 import (either increasing of decreasing it) (Brandina et al. 2007), which may reflect existence of such a regulation. So, once more, the function of the imported tRNA species was strictly classical while no evidence of any other “alternative” function was ever found.

The second described case of tRNA import in yeasts concerns tRNA^{Gln} species. Formation of Glutaminyl-tRNAs can, in principle, proceed either by a specific glutaminyl-tRNA synthetase (QRS), as in eukaryotic cells and many bacteria, or by misaminoacylation of the corresponding tRNA^{Gln} by the nondiscriminating glutamyl-tRNA synthetase (ERS) with subsequent transamidation of the aminoacyl residue in Glu-tRNA^{Gln} to generate Gln-tRNA^{Gln}, as it is found in the majority of bacteria and all the archea (Feng et al. 2005; Ibba and Soll 2004). The last pathway is also used in mitochondria, so far in yeast amidotransferase activity has not been characterized for long time and, to explain how the glutamine decoding system could function, a new mechanism involving tRNA import was proposed (Rinehart et al. 2005) (Fig. 4.2a). Following this scheme, mtDNA encoded tRNA$^{Gln}{}_{UUG}$, due to a specific wobble base modification (mcm^{5}S^{2}U or cmnm^{5}S^{2}U, cited differently in different reports), can decode only CAA glutamine codons (hypothesis based on earlier studies, reviewed in Yokoyama and Nishimura 1995). The two isoacceptor tRNAs^{Gln} (anticodons CUG and UUG) would be imported from the cytosol, along with the cytosolic QRS, which proceeds the aminoacylation in the “eukaryotic” fashion permitting decoding of the other two glutamine codons (CAG and CAA). This hypothesis responds to the question how glutamination may occur when no amidotransferase is present. It is also in agreement with the idea that mitochondrial enzyme, mERS, cannot generate Glu-tRNA^{Gln}, the required intermediate for the transamidation pathway (Rinehart et al. 2005). However, more recently, a new heterotrimeric complex with amidotransferase activity (GatA/GatF/GatB) was characterized, which, once assembled in mitochondria, can modify the mtDNA encoded tRNA^{Glu} misacylated by nondiscriminating cytosolyc ERS, partially imported into the organelle (Fig. 4.2b) (Frechin et al. 2009). Such a mechanism eliminates the functional need of tRNA^{Gln} import, and indeed, the latter study failed to detect any considerable amount of cytosolic tRNAs^{Gln} in yeast mitochondria. Furthermore, the incapacity of the modified U in the wobble position of the mitochondrial

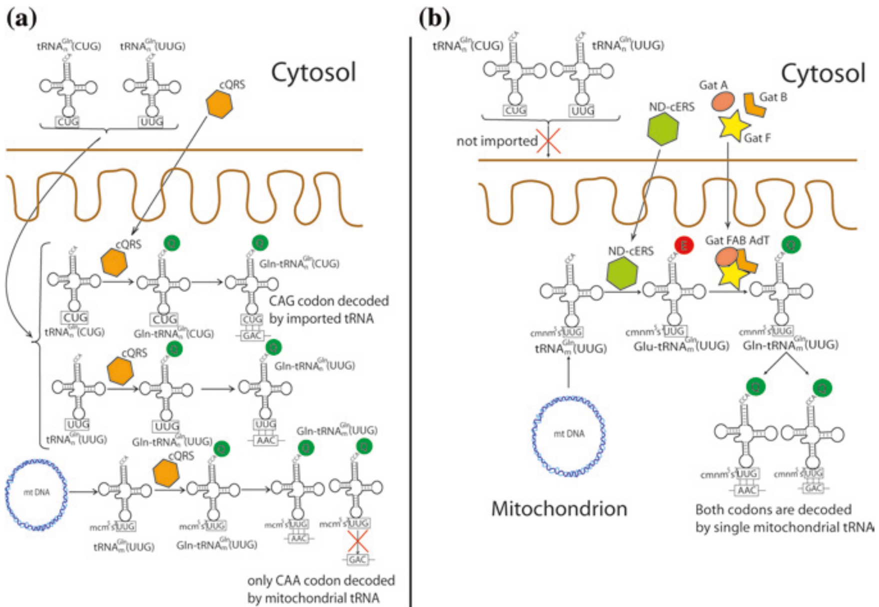

Fig. 4.2 tRNA^{Gln} in yeast mitochondria. **a** The model proposing simultaneous import of both two cytosolic tRNA^{Gln} and the cytosolic QRS. **b** The model proposing the generation of mitochondrial tRNA^{Gln} by the nondiscriminating cytosolic ERS and tripartite amidotransferase (Gat FAB AdT), both imported into mitochondria

tRNA^{Gln}$_{UUG}$, to pair with both A and G in the codon's first position is not supported by many reports (for example, Kurata et al. 2008; Umeda et al. 2005). Still remains the possibility that both mechanisms may coexist, but the former one being minor in terms of efficiency.

4.2.4 Mammalians

For a number of years it was assumed that mammalian mitochondria do not import any tRNA (Enriquez and Attardi 1996a, b; Enriquez et al. 1996), since no significant amounts of cytosolic tRNAs were detected in these organelles by classical hybridization methods. So far, a more recent study suggested that such pathway may, however, exist. It was proposed that a system which resembles to that proposed for yeast (see above) to import tRNAs^{Gln} into mitochondria would also exist in human cells (Rubio and Hopper 2011; Rubio et al. 2008) basing on the same reasoning as for the yeast system, the authors suggested that the modified wobble U34 of the mtDNA encoded tRNAs^{Gln} would affect decoding CAG codon, according to commonly agreed codon-anticodon recognition rules (Agris et al. 2007; Sprinzl and Vassilenko 2005). So, to fulfil complete set of decoding,

mitochondria must import corresponding nuclear-encoded tRNA^{Gln} isoacceptors (CUG and UUG anticodons) from the cytosol (see the scheme corresponding to the yeast system, Fig. 4.2a). Indeed, these two cytosolic tRNAs were detected in purified rat and human mitochondria by a more sensitive RT-PCR approach (Rubio et al. 2008). These data are in agreement with the fact that mammalian mitochondria were already described as able to import tRNAs in artificial way (Entelis et al. 2001b; Kolesnikova et al. 2002), and also import other RNA molecules in vivo (Magalhaes et al. 1998; Smirnov et al. 2008a; Yoshionari et al. 1994), so their innate capacity to internalize RNA is out of doubt. So far, the fact that these tRNAs are detectable only by RT-PCR indicates on tiny amounts of them in mitochondria. Furthermore, the transamidation pathway, allowing to complement the absence of mitochondrial QRS, was described (Nagao et al. 2009). This last study permitted to demonstrate that human mitochondrial ERS can misaminoacylate the mtDNA encoded tRNA^{Gln} by a glutamate, which is modified by a heterotrimeric transamidase hGatCAB imported into mitochondria. This mechanism makes the functional issue of the cytosolic tRNAs^{Gln} import not evident. The possibility remains open that both pathways are coexisting but the tRNA-based having a minor functional input.

In the context of this chapter, one can also cite the results of a systematic analysis of human mitochondrial transcriptome, where not only cytosolic tRNA^{Gln} was also detected, but also tRNA^{Lys} and tRNA^{Leu} ones (Mercer et al. 2011). However, there is no hint for the moment if this was due to contamination problems or corresponds to the in vivo situation, since no plausible function for cytosolic tRNA^{Lys} and tRNA^{Leu} in human mitochondrial compartment was ever assigned.

4.3 Import of 5S rRNA in Mammalians and Mitochondrial Translation

Found for the first time in beef liver mitochondria, nuclear DNA encoded 5S rRNA appeared to be nearly the most abundant ribonucleic acid species inside the organelles (Yoshionari et al. 1994; Magalhaes et al. 1998; Entelis et al. 2001a). Its presence in mitochondrial matrix was somewhat unexpected, since 5S rRNA genes have disappeared from mitochondrial genomes of animal cells, and this component was believed to be replaced by new proteins, so typical for mitochondrial ribosomes (Smits et al. 2007). Furthermore, no 5S rRNA was detected in large-scale purified mammalian mitoribosomes (Sharma et al. 2003). On the other hand, a recent series of works, focused on function of this small but important molecule, showed clearly that 5S rRNA, being an extremely conservative ribosomal component, administers the clockwork-like mechanism of all main functional sites of ribosomal machine (Smith et al. 2001; Kiparisov et al. 2005; Kouvela et al. 2007). This fact suggests that the total loss of 5S rRNA cannot possibly occur, if only the mitoribosome has not suffered some substantial structural reorganization of its

protein component before (what is probable only in very dynamic systems, exemplified by some protists). How did eukaryotic cells solve the problem of 5S rRNA loss from mitochondrial genomes? One of possible ways, as mentioned above, consists in direct substitution of this RNA with new ribosomal proteins forming the central protuberance (yeast, trypanosomatids) (Sharma et al. 2009; Smirnov et al. 2008a). Still, the obvious existence of cytosolic 5S rRNA import pathway in mammalian cells, as well as recent data discussed below, suggest that in the latter case, the role of the lost component may be performed by its cytosolic ortholog.

5S rRNA targeting into mammalian mitochondria mechanisms were recently described. This pathway includes RNA protein interaction involving at least two cytosolic precursor of mitochondrial proteins, preMRP-L18, and rhodanese and involves, as in the case of yeast tRNA mitochondrial import, structural rearrangements of the imported RNA molecule (Smirnov et al. 2008b, 2010, 2011) (Fig. 4.3). In the context of this chapter, it is worth to note that preMRP-L18 protein belongs to the L5-family of proteins which are direct interactants with 5S rRNAs in all known ribosomes. As a matter of fact, no MRP-L18 homologue was ever found in yeast mitochondria where no 5S rRNA is imported. This fact is to be considered as an indirect evidence on the involvement of the imported

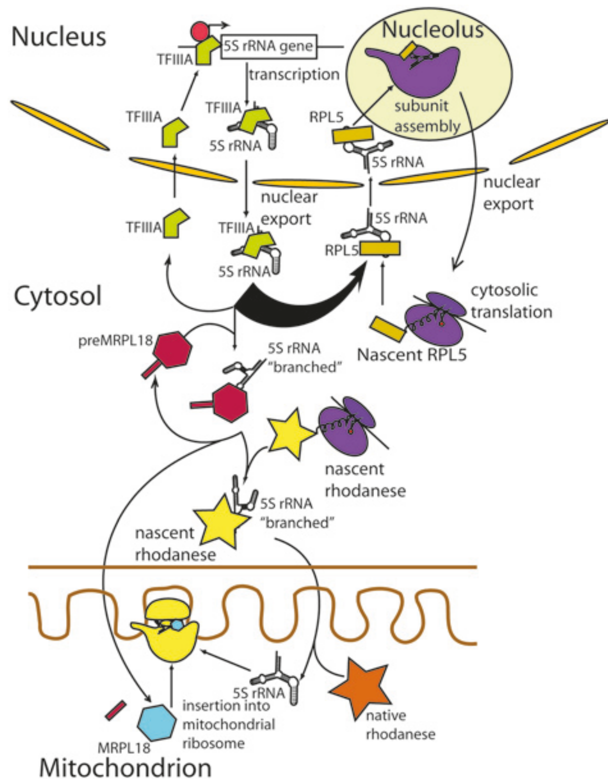

Fig. 4.3 5S rRNA import and cellular translation in mammalians. The intracellular distribution of 5S is ruled by a succession of RNA–protein interaction and chaperone events, which withdraw a minor portion of the cytosolic pool of the RNA from the RPL5 dependent nuclear re-export and further cytosolic translation to address it toward mitochondrial matrix where it is hypothesized to become a part of mitochondrial translation machinery

RNA molecule in mitochondrial ribosome, and is not in direct contradiction with the previously described “5S rRNA-less mitoribosome” lacking also many ribosomal proteins described thereafter (Dennerlein et al. 2010; Kolanczyk et al. 2010; Richter et al. 2010; Rorbach et al. 2008; Soleimanpour-Lichaei et al. 2007; Wanschers et al. 2012). Further analysis revealed that, indeed, a sub-population of mitochondrially localized 5S rRNA co-purified with mitochondrial ribosomes, being essentially associated with monosomal fraction, but not with the fraction of the large ribosomal subunit. This result may mean that the association of 5S rRNA with the mitochondrial ribosomes is leaky and the RNA molecule is easily lost upon subunits dissociation, which would explain why it was not previously detected. This idea was also in a good agreement with the fact that inhibition of 5S rRNA import by siRNA-downregulation of targeting factors (either rhodanese or MRP-L18) strongly affected mitochondrial translation (Smirnov et al. 2011). It is clear that additional functional and structural studies are needed to validate the “ribosomal” function of the imported rRNA, but all currently available data are in favor of it. Once more, as in the case of tRNA import in yeast mitochondria, the primary function of the imported RNA seems to be exploited in the organelle, while no evidence for any other alternative function was found.

4.4 Import of RNase P RNA in Mammalians and Mitochondrial Translation

Another case of RNA import that might be indirectly related to mitochondrial translation concerns the RNA component of 5′-end tRNA processing enzyme, RNase P in mammalians. RNases P were long time considered as obligatory containing a ribozyme (Joyce 1989). Since many species (but not all) do not possess corresponding genes in their mitochondrial genomes, it was suggested that they import this 200–300 bases long RNA from the cytosol. In the case of mammalian cells, the first experimental evidence of the presence of RNase P RNA in the mitochondria was reported more than 20 years ago (Doersen et al. 1985), which corroborated with a further study (Puranam and Attardi 2001). So far, the amount of this RNA associated with mitochondria was extremely low, which raised a debate about the existence of the RNase P RNA import pathway, along with that of MRP RNA, a related RNA species [mitochondrial RNA processing (MRP) enzyme RNP complex, proposed to participate in the initiation of mtDNA replication] (Chang and Clayton 1989; Topper et al. 1992). Furthermore, more recently RNA-free (only proteinaceous) mitochondrial RNase P was characterized in several species, including mammalians (Pavlova et al. 2012; Gutmann et al. 2012; Gobert et al. 2010; Holzmann et al. 2008; Rossmanith and Potuschak 2001). This discovery made unclear the role of the tiny amounts of RNase P RNA (H1 RNA) in mammalian mitochondria. So far, another study suggested a new possible function of this RNA. Indeed, RNA import into mammalian mitochondria,

including three RNAs hypothesized to be targeted into the organelle, namely 5S rRNA, MRP RNA, and RNase P (RNA H1), was reported to depend on a protein associated with the inner mitochondrial membrane or intermembrane space, PNPT1 (PNPase, polynucleotide phosphorylase). Inhibition of PNPT1 resulted in a decrease of RNA import. tRNA processing was also analyzed and it was demonstrated that in conditions of PNPT1 downregulation single tRNA genes produced correctly maturated tRNA, while processing of tandem ones was affected. It was then proposed that RNA-containing RNase P may be required only for maturation of the laters (Wang et al. 2010). This defect is expected to affect not only the very tRNA maturation (and therefore its functionality in translation), but also mRNA one, since tRNA secondary structures are thought to serve as signals of primary multicistronic transcript cleavage (Ojala et al. 1981). More recently, analysis of a case of pathological mutations in PNPT1 gene (causing a respiratory chain deficiency), demonstrated that when PNPase was reduced, translation in mitochondria was also affected, while overexpression of a wild-type gene fully restored the deficiency (Vedrenne et al. 2012). In the context of this chapter, it is noteworthy to mention that in PNPT1 mutants not only RNA H1 import appears as affected (von Ameln et al. 2012), but also 5S rRNA one (Vedrenne et al. 2012), which may be the main reason for translational deficiency. At this moment, the exact meaning of the H1 RNA presence in mammalian mitochondria is still subject to debate, although coexistence of both RNA-containing and RNA-free RNases P remains a plausible possibility.

4.5 Import of Micro RNAs and Mitochondrial Translation

Several recent studies, essentially systematic ones, have found microRNAs (miRNAs) in mitochondria of liver cells, myoblasts, HeLa cells etc. (Bandiera et al. 2011; Barrey et al. 2011; Bian et al. 2010; Kren et al. 2009). If the question of their origin is still open, at least for some of them nuclear coding and mitochondrial import was hypothesized. The mechanisms of this targeting were still not studied. This type of small noncoding RNA molecules participate usually in regulation of mRNA translation and stability (Fabian et al. 2010). There is still very little information on possible involvement of imported miRNAs in mitochondrial translational regulation. So far, it was already reported that miR-181c miRNA species would be nuclear-encoded, mitochondrially targeted, and regulating mitochondrial cytochrome oxidase (COX) subunit 1 mRNA translation (Das et al. 2012). One can expect that further examples of such regulation may appear in the future. In this context it is also crucial to determine with precision the real origin of these RNAs, since the mitochondrial targeting for molecules having homology with mtDNA is sometimes thorough to prove and requires specific model systems of in vitro and in vivo import, as it was done for tRNAs or 5S rRNAs for yeast, plant, or mammalian systems. Besides the mechanistic aspect of potential microRNA import into mitochondria, it will also be challenging to identify the exact functions of a given

imported microRNA in mitochondrial translation. Indeed, since in other biological systems they were essentially implicated in cap-dependent translation regulation (Fabian et al. 2010), which is not existing in mitochondria, one can reasonably expect the existence of other, still not discovered mechanisms.

4.6 Import of RNA as a Tool to Correct Dysfunctions in Mitochondrial Translation

Mutations in the mtDNA are an important cause of human diseases, which affect essentially (but not exclusively) the nervous system and the muscles. Diseases associated with deleterious mutations of the mtDNA are often severe neuromuscular disorders, such as for example the syndromes MERRF, MELAS, CPEO, KSS, or LHON. Each of these diseases is to be considered as "rare" (<1:5,000–10,000), but their global socio-medical input is very important and often underestimated. Among the over 350 mtDNA pathogenic mutations characterized to-date in the relatively small human mitochondrial genome (16,569 base pairs), more than 140 are located in tRNA genes, over 70 in protein-coding genes, and a few in the rRNA genes, the rest 120 being deletions of variable size (Ruiz-Pesini et al. 2007). Most of these mutations affect mitochondrial translation, either in specific (one mRNA) or nonspecific (nonfunctional tRNAs or rRNAs) manner. To-date, these diseases do not have efficient curative therapies. The gene therapy approach therefore represents an open possibility, which may be followed in two directions. The first one, commonly referred to as "allotopic expression" (Smith et al. 2004), is to express the gene of interest in the nucleus and to target its product into mitochondria where it would replace its mutant counterpart. The second direction, referred to as "anti- genomic" (Taylor et al. 2001), is to inhibit the replication of mutant mtDNA molecules, thus favoring the replication of wild-type genomes. This approach is based on the heteroplasmy of most deleterious mtDNA mutations, i.e., within each cell mutated mtDNA molecules coexist with wild-type ones. As clinical symptoms are detected only above a relatively high proportion of mutant mtDNA, even partial inhibition of the propagation of mutant versions of mtDNA is expected to permit a significant rescue of mitochondrial functions. Here below we describe how both strategies were experimentally tested by exploiting the RNA mitochondrial pathway, in each case observing improvement of mitochondrial translation affected by mtDNA mutations.

4.6.1 Allotopic Strategy

The first description of successful allotopic strategy using RNA import refers to the yeast model, where a nonsense mutation in mtDNA encoded COX2 subunit was functionally complemented by an engineered mitochondrially targeted

cytosolic tRNA (Kolesnikova et al. 2000). This study, validating the idea of exploiting the RNA import for correction of mtDNA mutations effects, demonstrated that eukaryotic type tRNAs can be functional in mitochondrial translation. A similar approach was further applied to two different pathogenic mutations in human mitochondrial tRNAs genes. The first one concerned a point mutation A8344 > G localized in the $tRNA^{Lys}$ gene, often associated with the MERRF syndrome (Myoclonic Epilepsy with Red Ragged Fibers). In this study (Kolesnikova et al. 2004), we used the fact that human mitochondria are able to import the yeast $tRNAs^{Lys}$ (tRK1, see above) or their mutant derivatives (Entelis et al. 2002, 2001b; Kolesnikova et al. 2002). Several versions of specifically designed importable tRNA versions were indeed shown to partially correct all mitochondrial functions affected by the MERRF mutation. In the context of this chapter, it is noteworthy that this mutation in the $tRNA^{Lys}$ affects modification of the anticodon, which leads to a recognition of AAA or AAG lysine codon with different efficiencies (the last one is less efficiently read) and consecutive abortive or inefficient translation (Kirino et al. 2004; Yasukawa et al. 2001). Mitochondrially targeted cytosolic $tRNA^{Lys}$ was shown to reverse this defect of translation and, as the functional consequence, to improve other mitochondrial functions, both in model immortalized cybrid cells or in patient derived primary fibroblasts (Kolesnikova et al. 2004).

The second, more recent successful attempt to apply allotopic strategy using RNA import concerns another point mutation in the mitochondrial $tRNA^{Leu}$ gene, A3243 > G, causing the MELAS disease (Mitochondrial Encephalopathy, Lactic Acidosis, Stroke-like episodes). In this case no naturally imported $tRNA^{Leu}$ was available neither in human nor in yeast cells, so a set of chimeric tRNAs with switched aminoacylation identity was created based on tRK importable versions. It was found that introduction of leucinylation identity elements [essentially localized in the anticodon (Giegé et al. 1998)] in the context of yeast cytosolic $tRNAs^{Lys}$ did not affect their capacity to be mitochondrially targeted into yeast, but more important, also into human mitochondria. It was then demonstrated that the imported recombinant tRNA was able to be correctly aminoacylated by the Leucine (either in the cytosol or in the organelle) and to participate in mitochondrial translation. Transient or stable expression of the importable tRNA in model cybrid cells bearing the MELAS mutation lead to a significant rescue of mitochondrial translation, and consequently other mitochondrial functions (respiration, electron transfer complexes activity, etc.) (Karicheva et al. 2011).

Several alternative strategies, but which also can be termed as "allotopic", were developed by another team, studying the RNA mitochondrial import mechanisms in a trypanosomatid *Leishmania tropica*. An important part of the results of this team (Goswami et al. 2006) were subject of controversy which is still not resolved (Schekman 2010), therefore we shall not discuss here the mechanistic aspects of this pathway, but in the context of the current chapter, is would be worthy to mention recent development of this study leading to propose a therapeutic use of RNA import. All these studies exploit the mitochondrial inner membrane anchored RIC (RNA Import Complex) to deliver RNAs with therapeutic capacities into mitochondria (Adhya 2008). If RIC was described as facilitating tRNA delivery

into *Leishmania* mitochondria, it was also proposed that it can be internalized by cultured human cells in a caveolin-1-dependent manner and to be then inserted in human mitochondrial membranes (Mahata et al. 2006). This insertion was described to promote import of cytosolic tRNAs into human mitochondria, which permitted to complement translation defects caused either point mtDNA mutation in either $tRNA^{Lys}$ (MERRF) or 2 kb deletion (KSS, Kearns-Seyre syndrome). A related method was more recently used to address *any* type of mtDNA mutation, comprising single and multiple deletions, or even more generally many types of mitochondrial dysfunction, as altered ROS production. To this end the RIC reconstituted from recombinant proteins was complexed with synthetic transcripts covering significant or all coding portions of mammalian mtDNA, by introducing into these transcripts a previously identified "import signature" (a short hairpin structure present in all imported *Leishmania* tRNAs), and addressing it into affected cells or tissues (Jash and Adhya 2011, 2012; Mahato et al. 2011; Jash et al. 2011).

A recent development of allotopic strategy described exploited the newly identified RNA import mitochondrial factor, PNPT1 (PNPase, polynucleotide phosphorylase). This inner membrane localized protein was formerly shown to be the part of RNA mitochondrial delivery machinery and its specificity relayed to recognition of a conserved hairpin structure in the imported H1 RNA molecule (Wang et al. 2010). This "import signature", referred to as RP sequence, was fused to either $tRNA^{Lys}$ or $tRNA^{Leu}$ expressed in nucleus, which promoted their import into human mitochondria and corrected in a significant manner translation defects due to MERRF or MELAS mutations, correspondingly (Wang et al. 2012) (Fig. 4.4). As a matter of fact, the same RP import signature permitted to address into mitochondria much larger RNA molecules in the PNPase-dependent manner, like COX2 mRNA expressed in the nuclueus (Wang et al. 2012).

Allotopic strategy can also be imagined by using the artificially induced protein-driven RNA import, which is not strictly corresponding to the natural RNA import pathway focused in the chapter, still being worth to be mentioned here. Indeed, covalent (Seibel et al. 1995; Vestweber and Schatz 1989) or noncovalent (Sieber et al. 2011b) complexing of nucleic acids with mitochondrially imported proteins or even peptides can promote their co-internalization into the organelles, and thus can be used for mitochondrial addressing functionally active molecules, as tRNAs, or even mRNAs, into mitochondria bearing pathogenic mutations.

4.6.2 Antigenomic Strategy

Reducing the heteroplasmy level of mtDNA mutations below the pathogenic threshold was attempted by many strategies, including addressing into mitochondria peptide nucleic acids (PNAs), specific restriction enzymes, using mitochondrial substrates and, on the level of the organism, specific diets (reviewed in Smith and Lightowlers 2010; Smith et al. 2004), all of these approaches are valuable but still have many technical limitations. The first validation of RNA import as a tool for

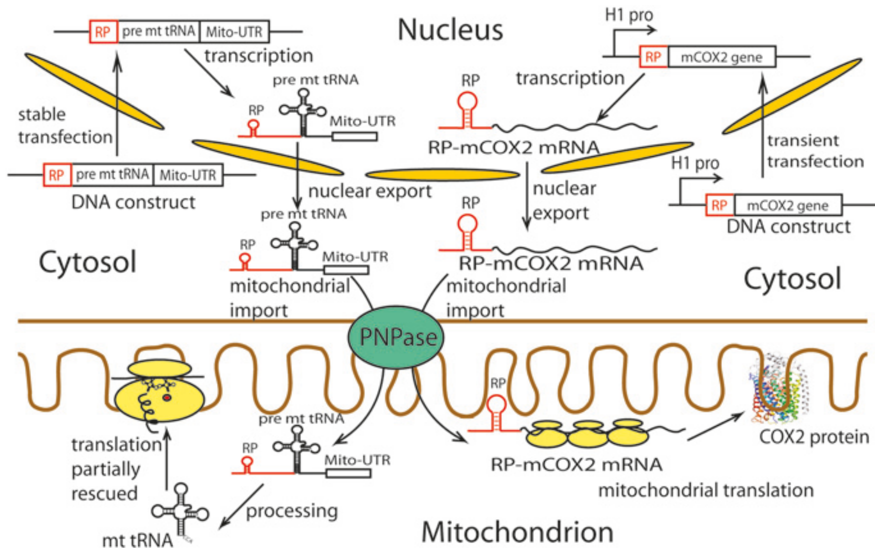

Fig. 4.4 Two allotopic gene therapy models exploiting *PNPase* -promoted RNA import. At the *left* side is schematically presented the tRNA import into human mitochondria, which permits complementation of tRNA mutations in mtDNA (MERRF, MELAS). RP, RNA import signature of RNase P H1 RNA fused to the pre-tRNA, which targets the RNA into mitochondrial. At the *right* side—a similar approach but when RP sequence was fused to an mRNA (COX2 respiratory subunit)

antigenomic strategy was recently described by our team (Comte et al. 2013), the model concerned a large 8 kb long deletion from base 8363 to 15438 and including several structural genes and 6 tRNA ones. This heteroplasmic deletion provokes the KSS disease and is characterized by a significant, though not very dramatic, changes in mitochondrial translation pattern, which may be explained by a 2–3 lower copy number of the genes localized within the deletion. The rationale of the approach was to target mitochondria with short oligoribonucleotides complementary to the new sequence generated by the mutation which could affect replication of only mutant mtDNA molecule. Two types of RNA molecules were used as vectors to address "therapeutic" oligoribonucleotides into mitochondria; the first one was based on 5S rRNA, where the so-called β-domain, dispensable for mitochondrial targeting (Smirnov et al. 2008b), was replaced by the oligonucleotides corresponding to either the H- or L-strands of mtDNA region surrounding the deletion (Fig. 4.5). The second RNA-vector was based on mini-RNAs resulting form SELEX experiments held on importable tRNA sequences, which represent a simple two-hairpin structures where the linker can be replaced by oligonucleotides of interest (Kolesnikova et al. 2010). It was demonstrated that both types of chimeric RNA molecules were addressed into mitochondria of cultured human cells and that their import was accompanied by a reproducible decrease of the ratio between mutant and wild-type mtDNA. This shift of heteroplasmy (transient in the case of transient transfection and stable when

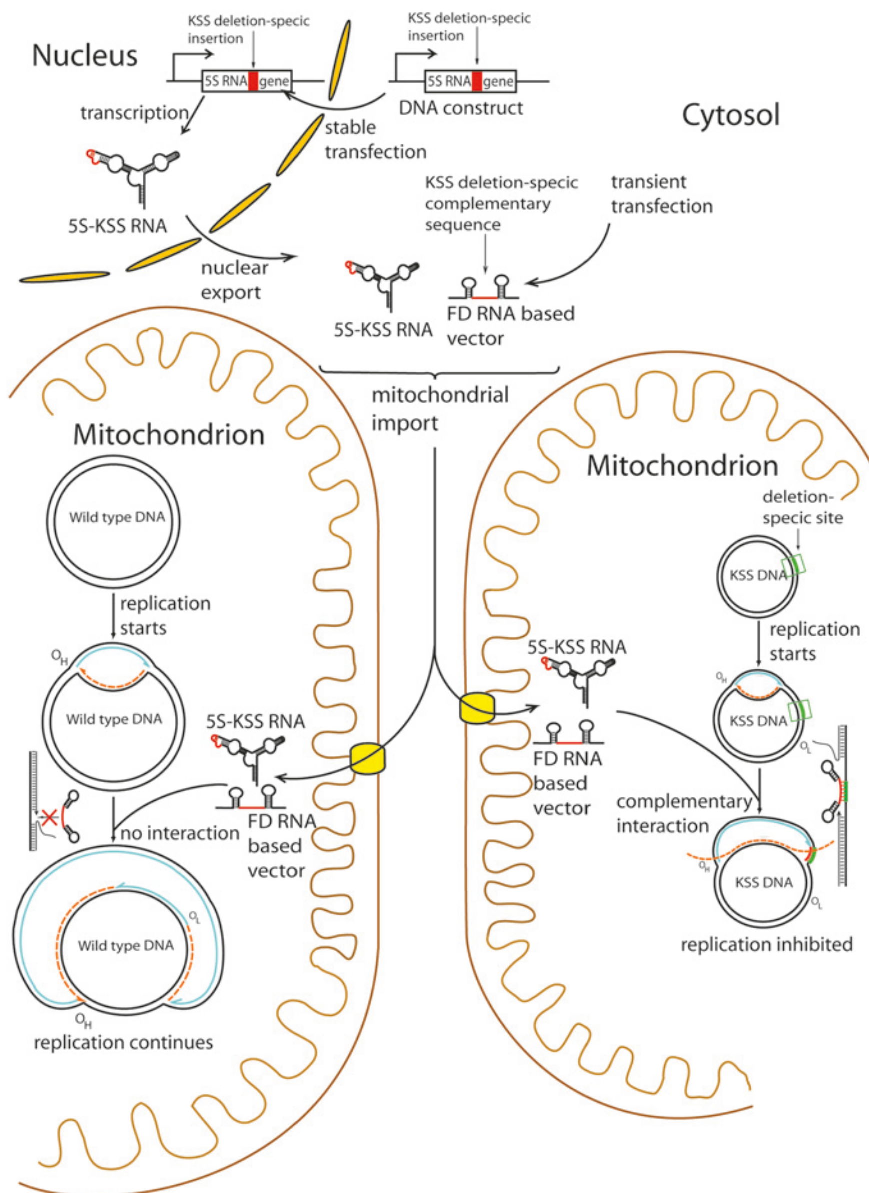

Fig. 4.5 Antigenomic gene therapy models exploiting RNA mitochondrial import. In both cases, a large deletion associated with the KSS disease was addressed. At the *upper left* side—the use of natural import of 5S rRNA, whose recombinant versions were stably expressed in cybrid cells. The antigenomic oligonucleotide replacing the 5S rRNA β-domain is in *red*. At the *upper right* side—FD short tRNA derivatives were used for transient transfection of cybrid cells. At the *bottom*, the schema illustrates the specificity of anti-replication effect of the recombinant mitochondrially targeted RNAs: no effect on wild-type mtDNA replication (*left*) and inhibition of KSS mutant mtDNA (*right*)

recombinant RNAs were stably expressed) resulted in a restoration of a normal mitochondrial translation patters, thus producing a curative effect (Comte et al. 2013).

4.7 Concluding Remarks

To summarize the main issues of this chapter, one can remark that RNA targeting into mitochondria is, in the majority of studied cases, intimately linked to mitochondrial translation, either in direct (tRNAs, rRNA) or nondirect (regulation of translation by miRNA) manner. As it was stressed many times (Sieber et al. 2011a; Schneider 2011; Rubio and Hopper 2011; Salinas et al. 2008), RNA import, even sharing a number of common features among species, still is considered as emerged many times upon evolution. One can suggest that innate capacity of mitochondria to import short RNAs was used to either complement or to optimize the organellar biosynthetic apparatus. It is interesting to note that if the proteins participating in this pathway vary enormously among species and often are "moonlighting" (performing their secondary jobs, i.e., enolase, aminoacyl-tRNA synthetases, rhodanese, ribosomal protein MRP-L18, PNPase, etc.), each time when the imported RNA function inside the organelle was investigated in details, it was rather a classical one in translation (tRNA, 5S rRNA) and not alternative. The functionality of these imported molecules and the flexibility of the overall process make the RNA import pathway an extremely promising tool for the future gene therapy of mitochondrial pathologies, and the first model systems described here above are well illustrating this idea. Indeed, RNA mitochondrial import might be altered in order to either send in the organelles fully functional translation-related molecules to replace the mutant ones or to promote a reduction of mutant mtDNA to correct translation defects.

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