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Review

Import of Nuclear DNA-Encoded RNAs into Mitochondria and Mitochondrial Translation

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ABSTRACT

Targeting nuclear DNA-encoded tRNA is a quasi-ubiquitous process, found in a variety of species, although the mechanisms of this pathway seem to differ from one system to another. In all cases reported, this import concerns small non-coding RNAs and the vast majority of imported RNAs are transfer RNAs. If was commonly assumed that the main criterion to presume a tRNA to be imported is the absence of the corresponding gene in mitochondrial genome, in some cases the imported species seemed redundant in the organelle. By studying one of such "abnormal" situation in yeast *S. cerevisiae*, we discovered an original mechanism of conditional regulation of mitochondrial translation exploiting the RNA import pathway. Here, we provide an outline of the current state of RNA import in yeast and discuss the possible impact of the newly described mechanism of translational adaptation

INTRODUCTION

Mitochondria are intracellular organelles found in all eukaryotic cells responsible for a large number of essential and non-essential processes, i.e., respiration, ATP-generation, synthesis of amino acids, oxidation of fatty acids, regulation of reactive oxygen species, apoptosis, etc.[1,2] Their particularity is the presence of a mitochondrial genome, mtDNA, which, although different in organization and size among species, has the property to code for only a minor part of macromolecules present in the organelle. The vast majority of mitochondrial proteins are nuclear DNA encoded and imported into mitochondria by mechanisms studied in details.[3-5] Less is known about RNA import, although this pathway may now be considered as quasi-universal, imported RNAs being found in mitochondria of protozoans, plants, fungi and animals.[6-9] In contrast with protein mitochondrial import, RNA import mechanisms as well the nature of imported RNAs appear to differ form one system to another, which suggests that this pathway may have appeared independently several times during evolution. Furthermore, for a number of reported cases, the function of the imported RNA species is far to be evident. This report aims to compare such “non-evident” cases and to show, on the example of yeast *S. cerevisiae*, as comprehension of the import mechanism may lead to a discovery of an unexpected function.

EVIDENT AND NON-EVIDENT FUNCTIONS

The main mitochondrially imported RNA species are transfer RNAs (tRNAs), but several other small non-coding RNAs are supposed to be imported in mammalian mitochondria (RNase P and MRP RNA components and ribosomal RNA 5S).[7] It was commonly agreed that the main criterion that permit to affirm that a given RNA is imported is the absence of the corresponding gene in mitochondrial genome (while present in other organisms). So far, there exist several examples where the presence of the imported RNA in mitochondria seems redundant. Such a situation may be found in mammals. Indeed, if massive amounts of 5S rRNA are mitochondrially imported[10,11] (amounts that, in theory, might fit all mito-ribosomes[12]), no 5S rRNA was detected in ribosomes isolated from mitochondria.[13,14] This may be explained either by the loss of the RNA during the isolation procedure or by an alternative function of the 5S rRNA in mitochondria. RNase P, enzyme responsible for tRNA processing, is an ribonucleoprotein (RNP), in which the RNA component is essential for enzymatic activity in bacteria and

important for assembly in eukaryotes.[15-19] In mammalian mitochondria, RNase P activity was both associated either with the presence of the RNA (imported) and the protein,[20,21] or with the protein alone,[22-24] so the question about the need of the RNA remains the matter of discussion, especially since the number of imported RNA molecules is extremely low.[21] A similar confused situation is with the RNA component of the nuclease MRP, which participates in processing of the RNA primer during mtDNA replication.[25] Indeed, if low amounts of this nuclear DNA encoded RNA were found in association with highly purified mitochondria and RNA processing function suggested,[26] other reports point at its purely nuclear function.[20] DNA primase activity involved in mtDNA replication was primarily associated with the presence of nuclear DNA-encoded 5.8 S rRNA in mitochondria.[27-29] If this report was never confirmed thereafter in a direct way, more recently large amounts of this nuclear-encoded RNA was indeed found in association with mammalian mitochondria.[30]

Some cases of tRNA import also merit reflection. The coexistence of both the imported $tRNA^{Val}$ (AAC) and the mtDNA-encoded $tRNA^{Val}$(UAC) rises the question about the decoding overlap within *Marchantia polymorpha*,[31] since, theoretically, $tRNA^{Val}$(UAC) might be able to decode all four valine codons by the two out of three rule.[32] Similarly, import of two $tRNA^{Gln}$ isoacceptors in *Saccharomyces cerevisiae* mitochondria (anticodons UUG and CUG) was recently described.[33] If the role of the CUG- one may be explained by the capacity of the mitochondrially encoded $tRNA^{Gln}$(UUG) to read only CAG codons, the role of the imported $tRNA^{Gln}$(UUG) is not clear, since it seems to be redundant with the mitochondrial one. Another striking case of unclear function concerned the other yeast tRNA, nuclear encoded $tRNA^{Lys}$(CUU) (further, tRK1), which was found in association with the mitochondria almost 30 years ago.[34,35] Since the mtDNA- encoded $tRNA^{Lys}$(UUU) (further, tRK3) was always supposed to have a capacity of reading both AAA and AAG codons,[35-37] the role of the imported tRNA in the organelle was unclear. Formerly, its role in other processes than translation, like splicing or replication priming were proposed,[38,39] but no experimental evidence for that existed. More recently, we expressed mutant forms of the second cytosolic $tRNA^{Lys}$ (tRK2) that were able to be mitochondrially imported and to decode the *amber*-stop codon (UAG) and found that the imported tRNA corrected mitochondrial translation of a mutant mitochondrial *COX2* gene where such a stop codon was introduced.[40] This result suggested that the imported cytosolic-type tRNA participated in mitochondrial translation, but did not give any direct proof that the naturally imported tRK1 has a translational activity. Only recent understanding of the details of tRK1 import mechanism permitted to approach the problem in a direct way.[41]

DIFFERENT MECHANISMS OF IMPORT?

The pathway of RNA import into mitochondria has a unique particularity to be at the same time quasi-ubiquitous and to use very different mechanisms in different species. The only common feature of all described RNA import events was the necessity of energy (ATP hydrolysis and, eventually, the intermembrane electrochemical potential).[6,7,9] Beside this fact, the other properties of RNA import mechanisms reveal incredible disparities.

In trypanosomes importing the totality of their mitochondrial tRNAs from the cytoplasm, a multi-protein bi-part complex associated with both outer and inner mitochondrial membranes was reported to specifically discriminate various tRNA species.[42-45] This complex is partially constituted from known subunits of the respiratory chain and unknown ones.[45-48] No specific targeting soluble factors were found. In plants, importing a subset of mitochondrial tRNAs (from few to a dozen), the porine protein (Voltage-Dependent Anion Channel, VDAC) was implicated in the translatcation, but also Tom proteins constituting the GIP (General Insertion Pore).[49,50] Additionally, the existence of non-essential targeting cytosolic factors was suggested (aminoacyl-tRNA synthetases or other, nonidentified proteins).[51-53] In yeast $tRNAs^{Gln}$ and $tRNA^{Lys}$ (tRK1) seem not to follow the same way to the organelle, since the first was reported to be imported without soluble proteins in vitro,[33] while the second - only in the presence of at least two essential proteins, the cytosolic precursor of mitochondrial lysyl-tRNA synthetase and, more unexpectedly, glycolytic enzyme enolase-2.[54-56] In human cells, import of 5S rRNA seems to have similar requirements that tRK1 import in yeast, so far the soluble factors needed are clearly different.[12] On the other hand, if 5.8 rRNA is to be considered as another candidate as imported species,[29] the mechanism must be different, since in the in vitro conditions permitting specific 5S rRNA import, 5.8S rRNA is not imported.[12]

At first glance, it may seem that the field is yet at its expansion stage and that in the future similarities between the different systems may emerge. On the other hand, it is attractively to suggest that mitochondria are, in general able to import negatively charged nucleic acids and such import may be directed by a variety of molecules, which were adapted for this purpose during evolution and for each particular case in an independent way. This consideration drive us back to the importance to identify the functions of imported RNA species.

FROM THE MECHANISM TOWARDS THE FUNCTION

Import of tRK1 into yeast *S. cerevisiae* mitochondria may be arbitrary divided in two distinct processes - recognition by cytosolic factors that target it towards the mitochondria and translocation of the RNA across the mitochondrial double membrane. tRK1, once aminoacylated by the cognate cytoplasmic aminoacyl-tRNA synthetase (Krs1p), becomes the target of a competition between the cytoplasmic machinery of translation and the first import factor, which was identified as enolase-2.[54] This glycolytic enzyme serves as a chaperone to address the RNA towards the outer mitochondrial membrane and to facilitate its binding to the second import factor, the cytosolic precursor of mitochondrial lysyl-tRNA synthetase (preMsk1p).[55] The exact mechanism of the further translocation is unclear, but the intactness and functionality of the pre-protein import apparatus Tim/Tom is required to achieve the RNA uptake.[56] In this context the central role of the tRK1 import belongs to preMsk1p. This is a precursor of an aminoacyl-tRNA synthetase (aaRS) potentially constituted, as the other aaRS of the IIb class,[57] by two structural domains: the C-terminal one, including the enzymatic active center, and the N-terminal one, normally responsible for the primary binding of the tRNA and then for interaction with the anticodon region of the tRNA. These two domains are linked by a flexible "hinge" region permitting conformational changes of the protein

upon interaction with the substrate.[58] tRK1 import is strictly dependent on interaction with preMsk1p and the capacity of the protein to be imported (the presence of the N-terminal MTS-sequence). On the other hand, it was clear that the manner of this RNA-protein interaction might be different from that expected from a cognate tRNA-aaRS one, since the tRNA is imported already in its aminoacylated form. We therefore analyzed several truncated recombinant versions of preMsk1p and found that the N-terminal domain of preMsk1p with the adjacent hinge-sequence (N-preMsk1p) were sufficient to deliver tRK1 into isolated yeast mitochondria.[41] This result was then confirmed in vivo, since replacement of the native *MSK1* gene by its truncated version resulted in a strain where tRK1 was still imported. This strain, however, was not suitable for functional analysis, since due to the absence of tRK3 - aminoacylation acitivity of N-preMsk1p, it became rapidly rho° (mtDNA loss). To build an exploitable genetic assay, we exploited available sequences of other fungal genomes, and found that one of the filamentous fungi, *Ashbya gossypii*, possessed an ortholog of preMsk1p (AshRS), which the N-terminal domain was significantly shorter than that of the yeast enzyme. Since the sequence of the mtDNA-encoded *A. gossypii* $tRNA^{Lys}$ was relatively close to that of tRK1, we thought that AshRS would be able to aminoacylate tRK3 in yeast mitochondria but would fail to direct cytoplasmic tRK1 into the organelle. Indeed, the strain expressing AshRS instead of preMsk1p contained aminoacylated tRK3 but no tRK1 in the mitochondria, while additional expressing N-preMsk1p restored tRK1 import. This system was exploited to test mitochondrial functions of imported tRK1.[41]

We found that abolishing of tRK1 import has no detectable mitochondrial effect in normal conditions of cultivation, but at elevated temperature (37°C), it leads to perturbation of mitochondrial translation. Besides the expected general weakness of mitochondrial translation in recombinant strains (AshRS is able to aminoacylate tRK3, but certainly less efficiently as Msk1p), a specific decrease of translation of two mitochondrial proteins, Var1p and Cox2p was observed. Analysis of codons frequency in yeast mtDNA revealed that the most of lysine-coding codons are AAA, while AAG is found in less than 6% of cases, in 39 positions. Furthermore, from these 39 AAG codons, 36 were in under-expressed or putative ORFs localised in the introns, and only 3 - in highly expressed genes, namely in *VAR1* (two codons) and *COX2* (one). Since tRK1 has CUU as anticodon, it may decode only AAG codons. Therefore, we might assume that in temperature stress conditions decoding of AAG codons in mitochondria exploit this imported tRNA, and not tRK3. Such need might be explained by the non-functionality of tRK3 in AAG-decoding at 37°C (Fig. 1). One obvious defect that may perturb in a specific way the decoding properties of a tRNA - the base modification of the anticodon bases. Indeed, decoding defects of undermodified tRNA were previously found in human mitochondrial tRNAs as a result of point mutations.[59,60] We therefore checked the state of the anticodon of tRK3 in stress conditions.

tRK3 wobble position is occupied by a 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$).[37] This modified uridine must permit correct decoding of both AAA and AAG codons. To test the state of wobble base modification, we first used the primer extension arrest method, which consists in elongation of a primer hybridized with the 3'-part of the tRNA by reverse transcriptase.[61] The modified bases were shown to cause an arrest of elongation in the vicinity of the modification group (before of after the modified nucleotide,

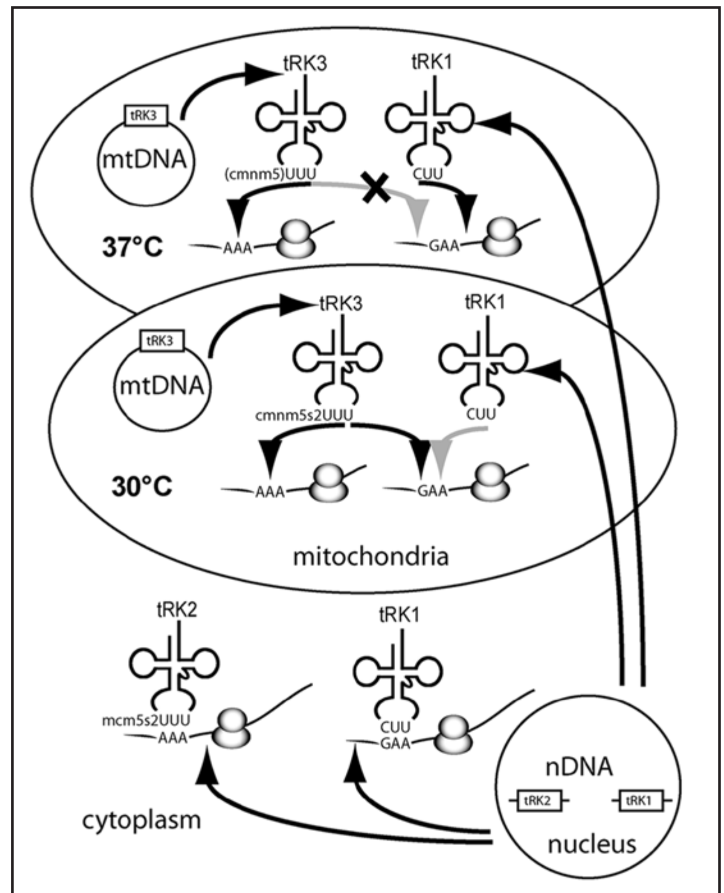

Figure 1. Hypothesis explaining the function of imported tRK1 in yeast mitochondria. mtDNA, mitochondrial DNA; nDNA, nuclear DNA. Solid arrows indicate either the RNA targeting direction, or the effective use of the given tRNA in translation. Gray arrows indicate optional or inhibited functions of tRNAs. The cmnm5-modification in tRK3 at 37°C is drawn in parentheses, since no information concerning this modification state in temperature stress conditions exists.

depending on the case). We indeed observed that at 37°C tRK3 is partially undermodified, since the elongation through the wobble position was more efficient than in tRK3 extracted from the cells grown at 30°C.[41] However, this approach does not give information if the 5-carboxymethylaminomethyl- or 2-thio- group was affected. For that purpose, we used (N-Acroylamino)phenyl-mercuric chloride (APM) containing polyacrylamide gels to separate tRNAs with following Norhtern-hybridization. The presence of APM, able to covalently (but reversibly) link the thio-groups, in the gel leads to a lower mobility of the modified tRNAs with respect to non-modified ones.[62] We observed that in cells cultured at 37°C, a half of tRK3 molecules was non-thiolated, while at 30°C, modification was present in virtually all the tRK3 pool. Therefore, one can suggest that this under-thiolation is the cause of the AAG-decoding defect and that in stress conditions, tRK1 import from the cytoplasm is used to correct this deficiency (Fig. 1).

Another interesting question emerging from this finding is which are the reasons of the under-modification of a given mitochondrial tRNA at 37°C? It cannot be the problem of the enzyme responsible for the 2-thiogroup synthesis (Mtu1p[37,63]), since APM-gel analysis performed with two other mitochondrial tRNAs possessing the same wobble-base modification, $tRNA^{Glu}$ and $tRNA^{Gln}$, did not reveal

any defect at 37°C.[41] Therefore, the main problem might come from the tRNA itself. When we compare the cloverleaf structures of these three tRNA, one obvious particularity of tRK1 is the presence of a "bulged" U base in the TΨC-arm (Fig. 2). Furthermore, taking into account the properties of AshRS (see above), we can assume that in *A. gossypii* no tRNA^{Lys} import occur. This suggests that no tRNA(Lys) import is needed as well, since mitochondrial translation is not affected by temperature stress. Indeed, we also did not observe any bulge elements in the TΨC-arm of *A. gossypii* mitochondrial tRNA^{Lys} (Fig. 2). We can hypothesize therefore that this element might locally destabilize tRK3 at elevated temperatures, thus preventing its correct interaction with the modification enzyme, but not affecting aminoacylation. Additional physico-chemical tests must be performed to verify this hypothesis.

MAY IT BE EXPLOITED?

The mechanism we described herein completes the panoply of functions of mitochondrially imported RNAs. One can see that, besides the evident situation when the mitochondrial genome lacks non-coding RNA genes essential for any mitochondrial function and this lack is complemented by importing the needed molecule from the cytoplasm, another possibility would be that RNA import complement a conditional defect of mtDNA-coded molecules. In all the cases cytosolic tRNAs that are, in a number of features, very different from mtDNA-coded ones,[64,65] seem to perfectly fit mitochondrial translation machinery. This evidence, together with accumulating data suggesting a high degree of flexibility of the RNA import, make this pathway very attractive as a tool for correcting pathological mtDNA mutations.

Mutations in human mtDNA are an important cause of human muscular and neurodegenerative diseases.[66,67] More than 300 different point mutations and rearrangements localized in tRNA, rRNA and protein-coding genes were already characterized. The most of these pathologies with complex clinical manifestations are incurable by classical medicamentous approaches. RNA import pathway seems to procure an interesting tool to develop a new gene therapy approach.[68] At this moment two successful attempts were reported. By expressing mutant importable yeast tRNAs^{Lys} in human cultured cells, we partially cured mitochondrial deficiency caused by a mutation in human mitochondrial tRNAs^{Lys}, associated with the syndrome MERRF (Myoclonic Epilepsy and Red Ragged Fibres).[69] More recently, it was reported that caveolin-dependent uptake of a multiprotein complex RIC responsible for tRNA mitochondrial import in *Leishmania*, by human cultured cells induced tRNA import of cytoplasmic human tRNAs, which cured mitochondrial deficiencies caused by two tRNA^{Lys} mutations (MERRF and another one, associated with the Kearns Sayre Syndrome; KSS).[70] It seems remarkable that similar positive effects were achieved by two alternative approaches: in the first case a cryptic tRNA import mechanism present in human cells as exploited to target in the organelle recombinant heterologous tRNAs, while in the second one - a heterologous tRNA import apparatus was introduced in the human cell to target in the organelle host cytoplasmic tRNAs. Other similar approaches may be developed, like import of suppressor tRNAs correcting non-sense or missense mutations in protein-coding genes, import of tRNAs with altered aminoacylation identities and the use of imported RNAs (tRNAs or 5S rRNA) as vectors to deliver in the mitochondria

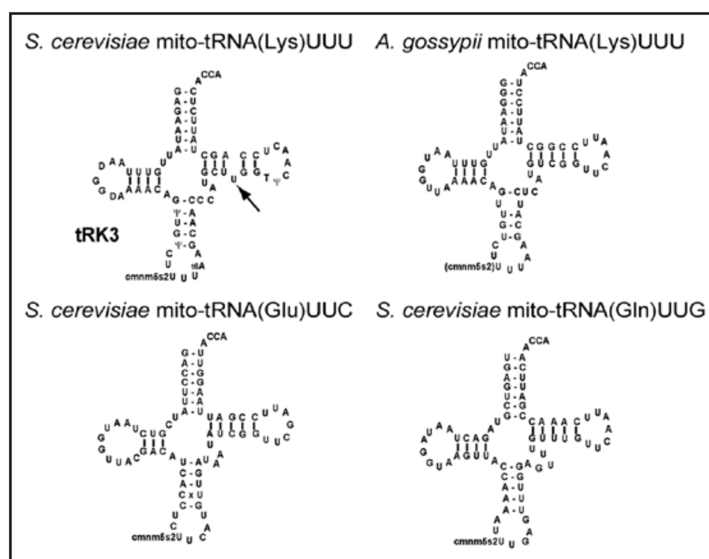

Figure 2. Comparison of secondary structures of *S. cerevisiae* and *A. gossypii* mitochondrial tRNAs bearing the cmnm5s2-modification. Cloverleaf structures were generated by the tRNAscan SE software available at http://rna.wustl.edu/tRNAscan-SE/. The cmnm5s2-modification in *A. gossypii* mitochondrial tRNA^{Lys} is drawn in parentheses, since no RNA sequencing was performed and the presence of this modification is only presumed (by analogy with other fungal mitochondrial tRNA^{Lys}). The arrow indicates the position of a bulged U in the TΨC-arm of tRK3.

oligoribonucleotides with therapeutic activities. Continuing to study the mechanisms of RNA import into mitochondria and assignment of functions to the imported RNA species may therefore become an appreciable help for biomedicine.

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