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par

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Modélisation de la thérapie génique du syndrome mitochondrial MELAS exploitant la voie d'adressage des ARNt dans les mitochondries

Soutenue le 26 octobre 2010 devant la commission d'examen :

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« Génétique Moléculaire, Génomique, Microbiologie »

Моему отцу, Зие Рамизовичу Каричеву

Oh God! How long is art,
Our life how short! With earnest zeal
Still as I ply the critic's task, I feel
A strange oppression both of head and heart.
The very means how hardly are they won,
By which we to the fountains rise!

Faust, Johann Wolfgang von Goethe.*

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* Ах, боже мой, наука так пространна,
А наша жизнь так коротка !
Мое стремленье к знанью неустанно,
И все-таки порой грызет меня тоска.
Как много надо сил душевных, чтоб добраться
До средств лишь, чтоб одни источники найти…

Фауст, Иоганн Вольфганг Гёте

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ABBREVIATIONS

A	adenine
aaRS	aminoacyl-tRNA synthetase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BB	breakage buffer
BET	ethidium bromide
bp	base pair
BSA	bovine serum albumin
C	cytosine
Ci	Curie
COX	cytochrome *c* oxidase
cpm	counts per minute
CS	citrate synthase
Da	Dalton
DAB	diaminobenzidine
DAPI	4', 6 – diamidine-2'-phenylindole dihydrochloride
DIFP	diisopropyl phosphorofluoridate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EB	elution buffer
EDTA	ethylenediaminetetraacetic acid
EF-G2	elongation factor G2
EF-Tu	elongation factor Tu
F14	Ham's F-14 medium
FACS	fluorescence activated cell sorting
$FADH_2$	flavin adenine dinucleotide hydroquinone form
FCCP	p-trifluoromethoxycarbonylcyanidephenylhydrazone
FCS	fetal calf serum
G	guanine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hmtLeuRS	human mitochondrial leucyl-tRNA synthetase
IDPs	import directing proteins
IPTG	isopropyl β-D-1-thiogalactopyranoside
kbp	kilo base pair
K_d	dissociation constant
K_M	Michaelis constant
KRS	yeast cytoplasmic lysyl-tRNA synthetase
LB	lysogeny broth, Luria-Bertrani
LeuRS	leucyl-tRNA synthetase
LysRS	lisyl-tRNA synthetase
MELAS	Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like episodes
MERRF	Myoclonic Epilepsy and Ragged Red Muscle Fibers
MLS	mitochondrial leading sequence
MSK	yeast mitochondrial lysyl-tRNA synthetase
mt	mitochondrial

NaAc	Na acetate
NADH	nicotinamide adenine dinucleotide reduced form
NaP	Na-phosphate buffer
NBT	nitroblue tetrazolium
OXPHOS	oxidative phosphorylation
PAAG	polyacrylomide gel
PAGE	polyacrylomide gel electrophoresis
PCR	polymerase chain reaction
PFA	paraformaldehyde
PMSF	phenylmethanesulfonyl fluoride
POLR	RNA polymerase
Q_0	quinone
redox	reduction oxidation
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
rxn	reaction
SDH	succinate-ubiquinone oxidoreductase
SDS	sodium dodecyl sulfate
SOC	super-optimal broth with catabolite repression
T	thymine
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TBS	Tris-Buffered Saline
TCA	trichloroacetic acid
TE	Tris-EDTA buffer
TMPD	NNN'N'-tetramethyl-p-phenylenediamine
Tris	tris (hydroxymethyl) aminomethane
tRK1	yeast cytoplasmic $tRNA^{Lys(CUU)}$
tRK2	yeast cytoplasmic $tRNA^{Lys(UUU)}$
tRK3	yeast mitochondrial $tRNA^{Lys(UUU)}$
tRNA	transfer RNA
u	enzyme unit
U	uridine
UV	ultra-violet
V_{max}	maximal rate of reaction
X-Gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside
$\Delta\Psi$	mitochondrial *trans*membrane potential

THE THESIS ORGANISATION

The first chapter (**INTRODUCTION**) describes mitochondrial organization and function, and is followed by an overview of mitochondrial diseases and developed gene therapy models for their treatment.

Results of the thesis work are provided and discussed in the second chapter of this manuscript (**RESULTS AND DISSCUSION**). This chapter includes three publications of the author. **Publication 1** describes the main results of the thesis and is dedicated to the development of gene therapy model for MELAS disease. **Publication 2** describes the results of the collaborative research, in which the author took part, and is dedicated to study of mitochondrial disorders associated with the defect in post-transcriptional modification of mitochondrial tRNAs. **Publication 3** describes the current state of knowledge on tRNA import mechanism and function in yeast and its possible application to gene therapy of human mitochondrial diseases.

The third chapter (**MATERIALS AND METHODS**) contains detailed description of all experimental procedures. The section **REFERENCES** comprises all sources cited in this thesis manuscript. Finally, the section **APPENDIX** contains the list of pathogenic mutations reported in mitochondrial DNA and the cloverleaf structures of mitochondrial tRNAs.

I INTRODUCTION

I.1 Overview of mitochondrial functions

Mitochondria are intracellular organelles present in all eukaryotic organisms. They fulfil many functions vital for cellular homeostasis, from important metabolic functions, ATP production, iron metabolism, and fatty acid oxidation, to participation in cell signalling events *via* apoptosis and sensitive modulation of mitochondrial metabolism to respond to cellular bioenergetics demand.

Nowadays, mitochondria are considered as a dynamic, adaptable organellar network, which is genetically regulated in its morphological and spatial organization by fusion and fission events. Mitochondria have two membranes that separate four distinct compartments: outer membrane, intermembrane space, inner membrane and matrix. The inner membrane is highly folded into cristae, which house the system of oxidative phosphorylation (OXPHOS), producing key energy source of the cell – ATP (Fig. 1.1). This system consists of five megadalton protein complexes: four redox complexes, including NADH : coenzyme Q oxidoreductase *(*Complex I), succinate dehydrogenase (Complex II), ubiquinol: cytochrome *c* oxidoreductase (Complex III) and cytochrome *c* oxidase (Complex IV); and ATP synthase (Complex V). The respiratory chain transfers electrons from NADH and $FADH_2$, which are generated through the glycolysis and/or oxidation of fatty acids and subsequent oxidation in the citric acid cycle, to molecular oxygen, the terminal electron acceptor. The energy released during this process is used to generate proton gradient by pumping protons from the matrix to the intermembrane space. This gradient is used by ATP-synthase to produce ATP from ADP and inorganic phosphate. This process was intensively investigated over the past 50 years and for a long time mitochondria were considered in the first place as “energy powerhouse of the cell”.

Nevertheless, mitochondria are also implicated in a number of other essential functions. These organelles represent a source of iron-sulphur clusters (ISCs) and heme - prosthetic groups, that are utilized by proteins throughout the cell in various crucial processes, reviewed in (Lill et al., 2006). Several steps of steroid synthesis also occur in mitochondria (Payne and Hales, 2004). Together with endoplasmic reticulum, mitochondria store calcium (Ca^{2+}) and participate in intracellular calcium homeostasis and signalling, particularly in those for programmed cell death (apoptosis).

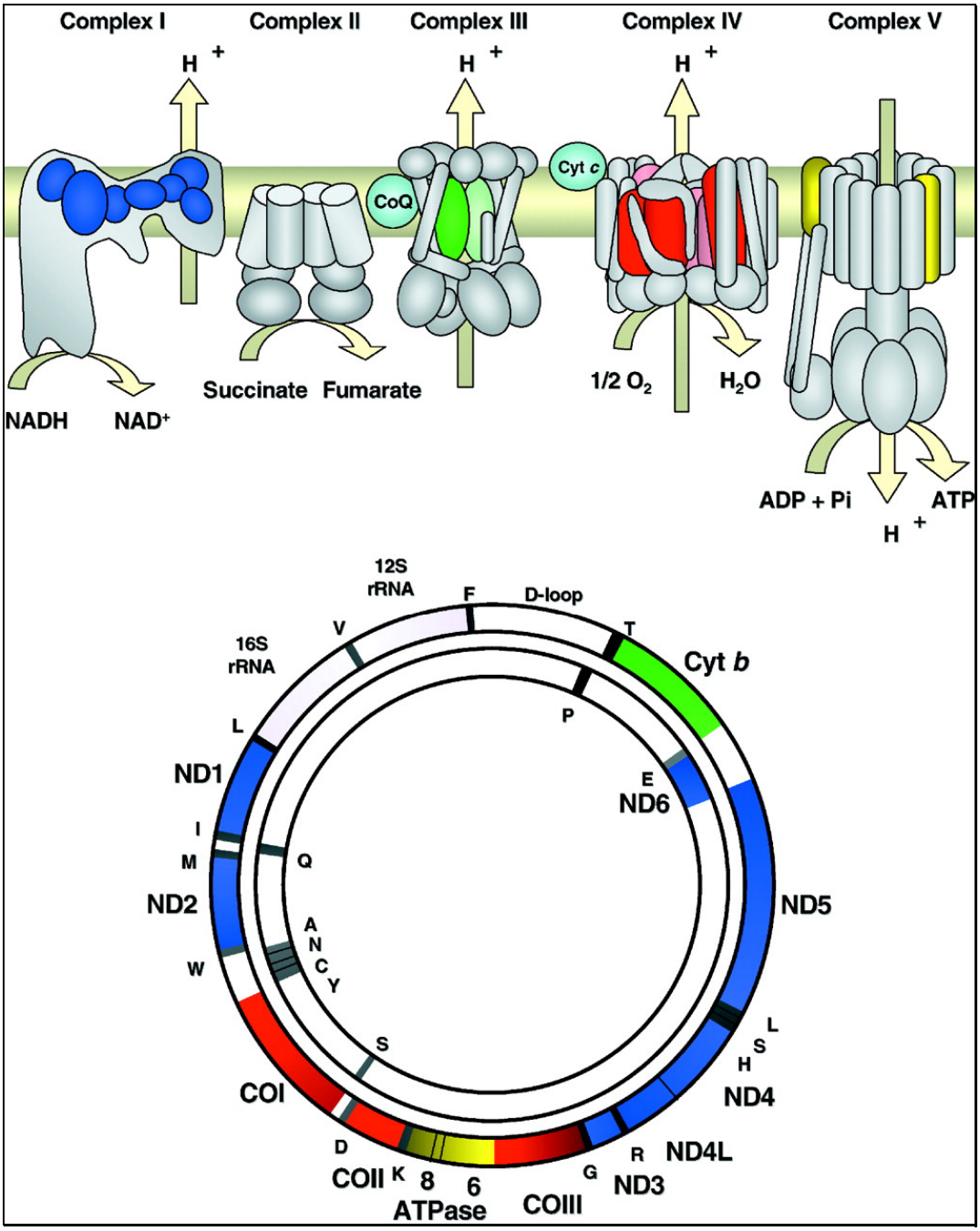

Fig. 1.1. Mitochondrial respiratory chain and human mtDNA (Zeviani and Di Donato, 2004)

mtDNA-encoded protein subunits of respiratory chain complexes and their corresponding genes in mtDNA are shown in colour. Single letters on mtDNA genome map represent mt-tRNA genes according to standard aminoacid code.

Apoptosis is the mechanism used to eliminate unwanted cells under three major circumstances: (i) development and homeostasis, (ii) as defence mechanism against genetically damaged and hence potentially tumorigenic cells, and (iii) natural senescence and aging. This phenomenon was identified in most multicellular eukaryotic organisms. Mitochondria play a key role in triggering or amplifying apoptotic events (Desagher and

Martinou, 2000). Mitochondrial factors that can trigger apoptosis are Ca^{2+} overload, increased exposure to reactive oxygen species (ROS) or severe decline in energetic capacity. Several pro-apoptotic proteins are located in the mitochondrial intermembrane space. Among them, the cytosolic release of cytochrome *c* leads to cascade activation of caspases, bringing within several hours to cell shrinkage, chromatin degradation, nuclear fragmentation, loss of plasma membrane integrity and finally cell death. Cytochrome *c* release is believed to occur through the opening of mitochondrial permeability transition (PT) pore, which is accompanied by the arrest of oxidative phosphorylation and collapse of mitochondrial *trans*membrane potential ($\Delta\Psi$).

Thereby, given its fundamental role in the cell processes, defects in mitochondrial function can have disastrous consequences. The potential as well as the complexity of mitochondrial dysfunction is increased by the existence of the mitochondrial genome. Its organization, reproduction and function will be discussed in details in the next chapter.

I.2 Mitochondrial DNA, its organization, function and propagation

Mitochondria contain their own DNA, mitochondrial DNA (mtDNA), which is believed to be a remnant of once free-living α-proteobacterium. Though all the mitochondrial genomes examined so far contain vastly fewer genes (by 1-3 orders of magnitude) than their hypothetical ancestor, their size and content are highly variable among different eukaryotic species (reviewed in (Adams and Palmer, 2003)). Over the course of mitochondrial evolution, large portion of genes could have been eliminated because of no need in symbiotic life, many others have been functionally transferred to the nucleus (often protein-coding genes); others have been replaced by pre-existing nuclear genes of similar function (often tRNA-coding genes). Plants have hundreds or even thousands kilobase-pair long mtDNA, while human mtDNA consists of 16,6 kbp. The number of mitochondrial protein genes varies from 3 to 67 in different species, while tRNA gene content varies from 0 to 27. Despite of numerous variations, several genes, those coding for 12S rRNA, 16S rRNA, cytochrome *b* and cytochrome-*c* oxidase subunit I (COXI) are "universally" present in all mitochondrial genomes. Three other genes, *MT-ND1*, *MT-ND4* and *MT-ND5* are so-called "*de facto* universal" mitochondrial genes, since the only two lineages lacking them (yeast and protists apicomplexans) have completely lost the function of the entire complex I. There are several

hypotheses explaining why do mitochondria retain separate genetic system, whose maintenance and expression require numerous proteins involved in DNA replication, repair, recombination, transcription, RNA processing, translation, and gene regulation to be addressed into mitochondria. A widely discussed hypothesis explaining why all genes have not been transferred to the nucleus is that some highly hydrophobic proteins are difficult to be imported across the mitochondrial membranes and sorted to correct intramitochondrial location. Indeed, COXI and cytochrome *b* are the two most hydrophobic proteins present in mitochondria. A second hypothesis for retention of several genes in the mitochondrion is that their products are toxic if present in cytosol. A third - consists in a need of quick, direct and energy state coupled regulation of genes coding for key proteins of electron transport chain. Finally, another hypothesis states that it is non-standard genetic code which prevents further gene transfer in the nucleus. These hypotheses with the references to supporting experimental data are presented in more details in the review by (Adams and Palmer, 2003).

Human mtDNA is a compact double stranded DNA molecule of 16,569 base pairs (Fig. 1.1). Up to 1000 copies of mtDNA can be presented in a cell depending on tissue. These multiple mtDNA molecules are organized within mitochondrial matrix as DNA–protein assemblies called nucleoids, which are thought to be composed of 2–8 copies of mtDNA and associated proteins (Legros et al., 2004). These organizing units of mtDNA are apparent as foci located at discrete spatial intervals within the mitochondrial network (Garrido et al., 2003). Human mitochondrial genes are located on both strands of mtDNA molecule, the heavy (H) and light (L) strand, and do not contain intronic regions. Human mtDNA codes for 13 proteins essential for assembly of respiratory chain complexes, 12S rRNA, 16S rRNA and 22 tRNAs required for mitochondrial translation. The other respiratory chain proteins, as well as the majority of factors needed to maintain, replicate and express mitochondrial DNA are expressed from nuclear DNA and are further imported into mitochondria from cytoplasm, keeping the organelle entirely dependent upon the nucleus. MtDNA is continuously replicating, its replication seems to proceed independently of that of the nucleus, and occurs in dividing as well as non-dividing cells (Schapira, 2006). MtDNA polymerase γ (POLG) is a single DNA polymerase present in mammalian mitochondria. With a help of Twinkle DNA helicase, it is responsible for replication of mtDNA. MtDNA is transcribed polycistronically from two H strand promoters (HSP1 and HSP 2) and one L strand promoter (LSP).). MtDNA transcription is required for the initiation of replication. The basic human mitochondrial transcription machinery consists of mtRNA polymerase (POLRMT), mitochondrial transcription factors. Mitochondrial translation machinery comprises mtDNA-encoded rRNAs

and tRNAs as well as many proteins coded for by the nuclear genome, including translation factors, mitochondrial ribosomal proteins (MRPs) and mitochondrial aminoacyl-tRNA synthetases (mt-aaRS).

Mitochondria use a genetic code that has several distinct differences from the universal code (Fig. 1.2A). Mitochondria of all vertebrates use universal arginine codons AGG and AGA, in addition to UAA and UAG, for termination. On the other hand universal UGA stop codon serves as a codon for tryptophan. Additionally, AUA codon has been reassigned to methionine from isoleucine (Barrell et al., 1980). Furthermore, mitochondria use a simplified decoding mechanism that allows translation of all codons with only 22 tRNAs instead of 31 predicted by Crick's wobble hypothesis. The expansion of the wobble rule is achieved due to post-transcriptional modifications of the 1st nucleotide of tRNA anticodon (wobble base). Thus, the codon-anticodon pairing relationships in animal mitochondrial systems can be classified into three categories (Fig. 1.2B) (Watanabe, 2010). In the first category, a single species of tRNA can read all four codons (four-way wobble). It is well known that tRNAs possessing an unmodified U at the wobble position can read all four codons in a codon box and the same holds true for tRNAs with an unmodified A of tRNA^{Arg} or m^{7}G at the wobble position of tRNA$^{Ser(GCU)}$. The second category of pairing relationships is one in which the codon box is divided 3:1. Within a box, a codon ending with G is read by a tRNA possessing an unmodified C at the wobble position, whereas the remaining three codons ending with U, C and A are read by a tRNA possessing an unmodified G at the wobble position (three-way wobble). In the third category, the codon box is divided 2:2. In this case, two codons ending with U and C are read by a tRNA possessing an unmodified G or queuosine (Q) at the wobble position, whereas the other two codons ending with A and G are read by a tRNA possessing either a modified uridine (5-carboxymethylaminomethyl-2-thiouridine (cmnm^{5}s^{2}U), 5-methylaminomethyluridine (mnm^{5}U), 5-taurinomethyluridine (τm^{5}U) and 5-taurinomethyl-2-thiouridine (τm^{5}s^{2}U)) or 5-formylcytidine (f^{5}C) at the wobble position (two-way wobble).

Mitochondrial mRNAs contain almost no 5' untranslated regions (5'-UTR), they contain neither the cap structure as eukaryotic cytoplasmic mRNAs, nor the Shine-Dalgarno sequence observed in prokaryotes. Moreover, mechanism of mitochondrial translation initiation is not fully understood. Finally, mammalian mitochondria use a single tRNA^{Met} for both elongation and initiation phases, whereas in the mitochondria of most lower eukaryotes as well as in prokaryotic and eukaryotic cytoplasmic translation systems there are two specialized tRNA^{Met} species (Mikelsaar, 1983).

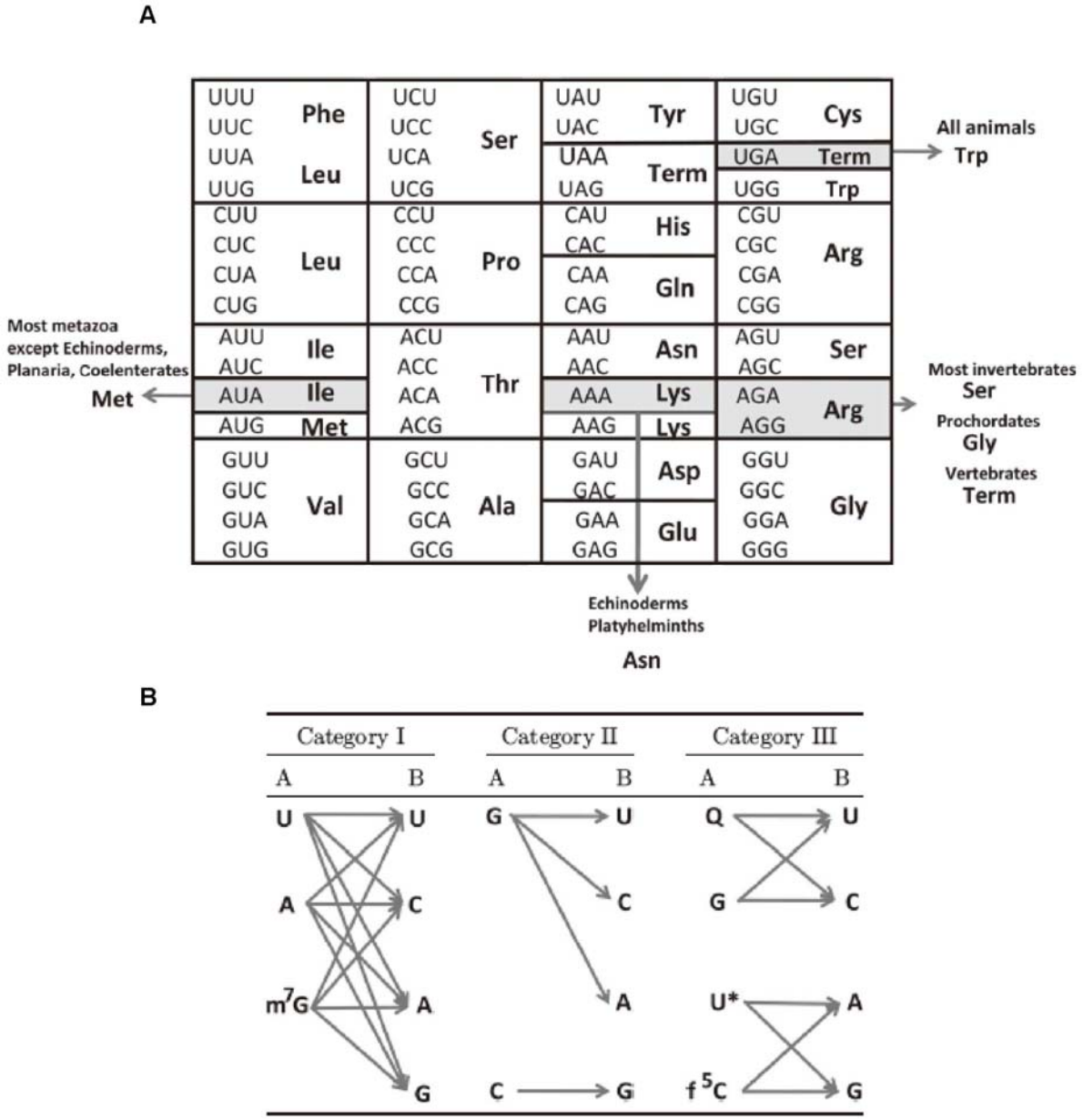

Fig. 1.2. Genetic code and an expanded mitochondrial wobble rule (Watanabe, 2010)

(A) Standard genetic code is shown inside the box, variations in animal mitochondrial genetic code – outside the box. (B) Possible pairings between the wobble nucleotide of tRNA [A] and the codon third nucleotide of mRNA [B] found in animal mitochondria. m^7G stands for 7-methylguanine, f^5C – for 5-formylcytidine, and U* - for 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$), 5-methylaminomethyluridine (mnm^5U), 5-taurinomethyluridine (τm^5U) or 5-taurinomethyl-2-thiouridine (τm^5s^2U).

Mitochondria possess different types of DNA repair activity similar to nuclear DNA repair systems (for review and further references see (Larsen et al., 2005)). Base excision repair (BER) is relatively well described in mammalian mitochondria and seems to play an important role in protecting the mitochondrial genome from oxidative stress and mutation. On the contrary, mitochondria of mammalian cells seem to lack nucleotide excision repair (NER) mechanisms. An evidence of direct reversal (DR) repair, mismatch repair (MMR) and recombinational repair (RER) by homologous recombination or non-homologous end joining (NHEJ) was reported. Thus, mitochondrial extracts from human cells were shown to catalyze homologous recombination between closed plasmid substrates (Thyagarajan et al., 1996), as well as rejoining both cohesive and blunt-ended linearized plasmid DNA (Lakshmipathy and Campbell, 1999). Recombination intermediates have been identified in patients with deletions in mtDNA (Poulton et al., 1993) and in human heart muscle (Kajander et al., 2001). An intramolecular and intermolecular recombination was shown *in vivo* on mice (Bacman et al., 2009) and on human cells (D'Aurelio et al., 2004).

It is agreed that mtDNA has a mutation rate 10-20 times higher than that of nuclear DNA (Lynch et al., 2006), which is thought to be mainly caused by the lack of protective histones and the proximity to damaging reactive oxygen species (ROS) generated by respiratory chain. Mutations in mitochondrial genes in one or more of mtDNA copies lead to co-existence within one cell of mutated and wild-type copies of mitochondrial genome, a situation called heteroplasmy. The cell can usually tolerate quite high levels of mutant mtDNA, as the wild-type mtDNA copies can produce sufficient numbers of gene products necessary for effective mitochondrial function, meaning no disease phenotype is observed. However, once a certain threshold level of mutant mtDNA is passed, a loss of mitochondrial function becomes phenotypically evident, leading to disease. This threshold varies depending on particular mutation and tissue type, but is generally about 70-90% of mutant genomes. Nevertheless, evidence of respiratory chain dysfunction at very low levels of heteroplasmy (<10% of m.3243A>G MELAS mutation) has also been reported in such highly energy-dependent tissues as skeletal muscle (Chinnery et al., 2000b) or brain (Dubeau et al., 2000).

MtDNA is maternally inherited, it is transmitted through the egg's cytoplasm, whereas sperm mitochondria constitute a minor fraction of the zygote's cohort and are rapidly eliminated after fertilization (Sutovsky et al., 1999). Therefore, mutations segregate along maternal lineages without any benefit of homologous recombination with mtDNA of paternal origin. Distribution of mtDNA sequence variants is largely a stochastic process. Analysis of the distribution of pathogenic mtDNA mutations in the offspring of carrier mothers shows

that the risk of inheriting a pathogenic mutation increases with the proportion in the mother, but there is no bias toward transmitting more or less of the mutant mtDNAs (reviewed in (Shoubridge and Wai, 2007). This implies that there is no strong selection against oocytes carrying pathogenic mutations and filter for oocyte quality based on oxidative phosphorylation capacity.

I.3 Mitochondrial diseases

Mitochondrial diseases are a clinically heterogeneous group of disorders that arise as a result of dysfunction of mitochondrial respiratory chain. Common clinical features include ptosis, external ophthalmoplegia, proximal myopathy and exercise intolerance, cardiomyopathy, sensorineural deafness, optic atrophy, pigmentary retinopathy, and diabetes mellitus. The central nervous system findings are often fluctuating encephalopathy, seizures, dementia, migraine, stroke-like episodes, ataxia, and spasticity. Estimated prevalence of all mitochondrial diseases is ~11.5/100000 (Chinnery, 2000).

Mitochondrial myopathies were firstly described in the early 1960s, when systematic ultra structural and histochemical studies revealed excessive proliferation of mitochondria in muscle of patients with weakness or exercise intolerance (Shy and Gonatas, 1964). The first biochemical evidence of a mitochondrial dysfunction – loss of coupling of oxidation and phosphorylation – was reported in case of non-thyroidal hypermetabolism (Luft syndrome) (Luft et al., 1962). The association of mitochondrial diseases with particular mutations came in 1988. A mitochondrial missense mutation was found to be the reason of maternally transmitted Leber's hereditary optic neuropathy (Wallace et al., 1988). The presence of mtDNA deletions was shown in patients with spontaneous mitochondrial encephalomyopathies (Holt et al., 1988). Since then hundreds of mtDNA mutations associated with numerous mitochondrial diseases have been described.

As previously discussed, mitochondrial function is under the dual control of the mitochondrial genome and of the nuclear genome. Therefore, genetic classification of mitochondrial diseases distinguishes disorders due to mutations in mtDNA and disorders due to mutations in nuclear DNA (Table 1.2.). MtDNA mutations can be subdivided on large-scale rearrangements (deletions or duplications) and point mutations both in non-coding RNA and protein coding genes.

Table 1.2. Genetic classification of human mitochondrial disorders (Chinnery, 2000)

Primary Mitochondrial DNA Disorders [1]		**Inheritance Pattern** [2]
Rearrangements (large-scale partial deletions and duplications)	Chronic Progressive External Ophthalmoplegia (CPEO)	S or M
	Kearns-Sayre Syndrome (KSS)	S or M
	Diabetes Mellitus and DeaFness (DMDF)	S
	Pearson marrow-pancreas syndrome	S or M
	Sporadic tubulopathy	S
Point mutations	Protein-encoding genes:	
	Leber Hereditary Optic Neuropathy (LHON) (G11778A, T14484C, G3460A)	M
	Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa (NARP)/Leigh syndrome (T8993G/C)	M
	Transfer RNA genes:	
	Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS) (A3243G, G3244A, A3251G, A3252G, C3256T, T3271C, T3291C)	M
	Myoclonic Epilepsy and Ragged Red Muscle Fibres (MERRF) (A8344G, T8356C)	M
	CPEO (A3243G, T4274C)	M
	Myopathy (T14709C, A12320G)	M
	Cardiomyopathy (A3243AG, A4269G, A4300G)	M
	DMDF (A3243G, C12258A)	M
	Encephalomyopathy (G1606A, T10010C)	M
	Ribosomal RNA genes:	
	Nonsyndromic sensorineural deafness (A7445G)	M
	Aminoglycoside induced nonsyndromic deafness (A1555G)	M
Nuclear Genetic Disorders		**Inheritance Pattern**
Disorders of mtDNA maintenance	Autosomal dominant CPEO (with 2° multiple mtDNA deletions):	
	Mutations in adenine nucleotide translocator *(ANT1*)	AD
	Mutations in DNA polymerase γ *(POLG1*)	AD or AR
	Mutations in Twinkle helicase *(C10ORF2*)	AD
	Mitochondrial neurogastrointestinal encephalomyopathy (with 2° multiple mtDNA deletions): Mutations in thymidine phosphorylase *(TP*)	AR

	Myopathy with mtDNA depletion: Mutations in thymadine kinase *(TK2*)	AR
	Encephalopathy with liver failure: Mutations in deoxyguanosine kinase *(DGUOK*)	AR
Primary disorders of the respiratory chain	Leigh syndrome:	
	Complex I deficiency - mutations in complex I subunits *(NDUFS2,4,7,8* and *NDUFV1*)	AR
	Complex II deficiency - mutations in complex II flavoprotein subunit *(SDHA*)	AR
	Leukodystrophy and myoclonic epilepsy: Complex I deficiency - mutations in complex I subunit *(NDUFV1*)	AR
	Cardioencephalomyopathy: Complex I deficiency - mutations in complex I subunit *(NDUFS2*)	AR
	Optic atrophy and ataxia: Complex II deficiency - mutations in complex II flavoprotein subunit *(SDHA*)	AD
Disorders of mitochondrial protein import	Dystonia-deafness: Mutations in deafness-dystonia protein DDP1 *(TIMM8*)	XLR
Disorders of assembly of the respiratory chain	Leigh syndrome:	
	Complex IV deficiency - mutations in COX assembly protein *(SURFI*)	AR
	Complex IV deficiency - mutations in COX assembly protein *(COX10*)	AR
	Cardioencephalomyopathy: Complex IV deficiency - mutations in COX assembly protein *(SCO2*)	AR
	Hepatic failure and encephalopathy:	
	Complex IV deficiency - mutations in COX assembly protein *(SCO1*)	AR
	Complex IV deficiency - mutations in protein affecting COX mRNA stability *(LRPPRC*)	AR
	Tubulopathy, encephalopathy, and liver failure: Complex III deficiency - mutations in complex III assembly *(BSC1L*)	AR
	Encephalopathy: Complex I deficiency - mutations in the complex I assembly protein *(B17.2L*)	AR
Disorders of RNA metabolism	Leigh syndrome:	
	Complex IV deficiency *(LRPPRC*)	AR
	Multiple complex defects *(EFG1*)	AR
Disorders of the lipid membrane	Ataxia, seizures, or myopathy: Coenzyme Q10 deficiency *(COQ2*)	AR
	Barth syndrome (Taffazzin)	XLR

1. Mitochondrial nucleotide positions refer to the L-chain and are taken from the Cambridge reference sequence.
2. M = maternal
S = sporadic
AD = autosomal dominant
AR = autosomal recessive
XLR = X linked recessive

I.3.1 Nuclear genes mutations

Although 72 of 85 subunits of OXPHOS system are encoded by nuclear DNA, mutations of the corresponding genes have only rarely been described. Those that have been described generally manifested in the neonatal period of early infancy. Mutations in nuclear coded complex I subunits were associated with Leigh syndrome and leukodystrophy, and those of complex II subunits with Leigh syndrome, ataxia, paraganglioma, and phaeochromocytoma. Several diseases have been shown to be due to mutations in genes coding for proteins involved in Krebs citric acid cycle, β – oxidation, and urea cycle.

On the contrary, numerous mutations in nuclear genes which products are involved in the maintenance and replication of mtDNA, in the biosynthesis and assembly of mitochondrially encoded proteins of respiratory chain, as well as in the import of its nuclear encoded subunits can result in phenotypes similar to those associated with primary mtDNA mutations (reviewed in (Smits et al., 2010)). A number of mitochondrial disorders are caused by defects in mitochondrial dNTP metabolism due to mutations in the nuclear genes. Among them mutations of adenine nucleotide translocator-1 (ANT-1), regulating the adenine nucleotide pool within mitochondria, cause autosomal dominant CPEO with ragged red fibres and multiple mtDNA deletions in skeletal muscle (Kaukonen et al., 2000). Mutations in deoxyguanosine kinase (DGUOK) and thymidine kinase (TK2), catalyzing the first steps of the salvage pathways of pyrimidine and purine deoxynucleosides, respectively, result in mtDNA depletion syndrome, including severe infantile form (Mandel et al., 2001; Saada et al., 2001). Several mutations causing autosomal dominant CPEO are described in the gene of Twinkle DNA helicase protein responsible for unwinding the mtDNA replication fork, and thought together with mitochondrial transcription factor A (TFAM) and mitochondrial single-stranded DNA-binding proteins (mtSSB) to stabilise mtDNA (Spelbrink et al., 2001). Inhibition of Twinkle results in mtDNA depletion (Tyynismaa et al., 2004). Mutations of

mtDNA polymerase γ (POLG) essential for mtDNA replication have been associated with a range of clinical phenotypes including PEO.

Until now, no mutations leading to mitochondrial disease have been identified in genes coding for the protein components of mitochondrial transcription. On the other hand, mutations in tRNA modifying enzymes have been recently associated with mitochondrial disease. In general, mt-tRNAs are shorter than bacterial or eukaryotic cytoplasmic tRNAs, they have larger variation in the size of the D- and T-loops, and lack multiple conserved nucleotides involved in classical tertiary interactions creating the L-shape. Post-transcriptional modifications seem to play more important role in the proper tertiary structure formation and functioning of mt-tRNAs compared to cytosolic tRNAs. Certain mt-tRNAs have shown aberrant structures and decrease of function when lacking post-transcriptional modifications (Helm, 2006). Pseudouridilation is the mostly frequently found modification in tRNAs, however its exact function is not clear. The missense mutation in pseudouridylate synthase gene (*PUS1*) has been detected in patients with mitochondrial myopathy and sideroblastic anaemia (MLASA) (Bykhovskaya et al., 2004). A defect in PUS1 impairs both cytosolic and mitochondrial translation, resulting in clinical phenotypes of variable severity presumably depending on different compensation mechanism in both cell compartments. Recently, 9 different mutations in *TRMU* gene coding for mitochondria-specific tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase were detected in patients with acute liver failure in infancy accompanied with lactic acidemia (Zeharia et al., 2009). TRMU is responsible for 2-thiolation of the wobble base in mt-tRNA^{Lys}, mt-tRNA^{Gln}, mt-tRNA^{Glu}. This modification is essential for codon-anticodon interaction and confers the tRNA an efficient ribosome binding. The amount of the thio-modified mt-tRNAs was found to be severely reduced in patient fibroblasts, which was accompanied by two-times reduction of total mitochondrial translation level (Zeharia et al., 2009).

Mitochondrial translation factors mtEFG1, mtEFTu and mtEFTs have been also associated with mitochondrial disease, leading to severe hepato(encephalo)pathy, rapidly progressive encephalopathy, and encephalomyopathy or hypertrophic cardiomyopathy, respectively (reviewed in (Smits et al., 2010)). Often mutations of corresponding genes result in marked global translation defect.

In total, 19 mitochondrial aminoacyl-tRNA synthetases have been identified, two of them, mtLysRS and mtGlyRS, are encoded by the same genes as cytosolic enzymes (Bonnefond et al., 2005). Two mitochondrial aminoacyl-tRNA synthetases, mtArgRS and mtAspRS, were shown to be implicated in mitochondrial disorders. Mutations in intronic

regions of respective *RARS2* and *DARS2* genes were found to be associated with severe forms of encephalopathy (Edvardson et al., 2007; Isohanni et al., 2010; Scheper et al., 2007). Both mutations were shown to affect aminoacylation properties of enzymes and levels of respective tRNAs. Impairment of mitochondrial protein synthesis was confirmed on yeast model of *RARS2* mutation (Tzagoloff and Shtanko, 1995). In contrast, *DARS2* mutations for instance are not likely to affect the OXPHOS system (Scheper et al., 2007).

Two syndromes have been related to defects of mitochondrial protein import. Mutation in the gene of translocase subunit TIMM8A have been reported in patients with Deafness Dystonia syndrome (Roesch et al., 2002) and Mohr-Tranebjaerg syndrome (MTS) (Blesa et al., 2007). Dilated cardiomyopathy with ataxia (DCMA) has been associated with the mutation in one of the components of TIM23 complex (Davey et al., 2006).

Several mutations have been identified in nuclear genes for mitochondrial proteins involved in the assembly and maintenance of Complex III (de Lonlay et al., 2001), Comlpex IV (Pecina et al., 2004) and Complex V (De Meirleir et al., 2004), associated with different clinical phenotypes, including Leigh syndrome, myopathy, encephalopathy, lactic acidosis, progressive course with early deaf, iron overload and etc. Defects in fusion proteins MFN 2 (mitofusin 2) and OPA1 (Optic Atrophy 1) cause a reduction in mitochondrial membrane potential and activity of OXPHOS complexes and are associated with Charcot-Marie-Tooth 2A and dominant optic atrophy, respectively (reviewed in (Liesa et al., 2009)). DNML1 (dynamin 1-like) protein involved in fission of mitochondrial membranes and Tafazzin protein required for the metabolism of the inner membrane phospholipid cardiolipin have been also associated with mitochondrial disease (McKenzie et al., 2006; Waterham et al., 2007).

Abnormalities of mtDNA or OXPHOS activity have been identified in several different neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease and some other pathologies (reviewed in (Schapira, 2006).

I.3.2 MtDNA deletions and duplications

The size of the deletion can vary from a single base to several kilobases and be located on any part of the mtDNA molecule (reviewed in (DiMauro, 2004). The most common deletion is 5 kb long and spans the region between the genes for cytochrome *b* and COXII,

thus encompassing several tRNA and protein genes. Large-scale deletions of this type have been typically associated with three disease phenotypes, including chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre syndrome, and Pearson's marrow-pancreas syndrome. However, the pathological expression of deletions is not restricted to these phenotypes. The prevalence of single deletion disorders is estimated at 1-2 per 100 000. Deletions exist in heteroplasmic form, the proportion of deleted molecules varies between tissues, and the degree of heteroplasmy can shift over time. Small deletions associated with a variety of clinical presentations have been described in genes coding for subunits of cytochrome-*c* oxidase, cytochrome *b* and complex I.

Some patients have duplications of mtDNA, which although might not be pathogenic themselves, could be an intermediate step in the generation of deletions (Bouzidi et al., 1998; Manfredi et al., 1995; Poulton et al., 1995).

I.3.3 MtDNA point mutations

Point mutations associated with human diseases have been described in protein coding genes, tRNA and rRNA genes. Their clinical expression is wide and includes phenotypes such as MELAS (myopathy encephalopathy lactic acidosis and stroke-like episodes), MERRF (myoclonic epilepsy and ragged red fibres), NARP (neuropathy ataxia and retinitis pigmentosa), MILS (maternally inherited Leigh syndrome), and LHON (Leber hereditary optic neuropathy). In addition, oligosymptomatic syndromes arising from mtDNA point mutations can include diabetes mellitus, cardiomyopathy, sensorineural deafness and myoglobinuria. The correlation between genotype and phenotype in mitochondrial diseases has always been recognized as complex. For instance, many patients with LHON have homoplasmic mutations, and unaffected family members can have the same homoplasmic mtDNA mutation. Another tRNA pathogenic mutation causing an encephalomyopathic phenotype and present in heteroplasmic state in affected members of the family, was found in homoplasmic state in asymptomatic family member (McFarland et al., 2004). The same mutation can result in multiple phenotypes, and the same phenotype can be caused by several different mutations. Multiple independent factors can influence the clinical expression of mutation, including tissue distribution, levels of heteroplasmy, nuclear background, and varying dependence of organs on the energy produced by OXPHOS system. Thus a lot remains to be discovered on the molecular consequences of these mutations.

Among different mitochondrial point mutations more than 170 have been identified in mtDNA-encoded tRNA genes, with $tRNA^{Lys}$ and $tRNA^{Leu}$ genes being hot-spots (see APPENDIX, Table A.1 and Fig. A.1). The carrier frequencies of m. 3844A>G mutation in $tRNA^{Lys}$ gene associated with MERRF syndrome and m.3243A>G mutation in $tRNA^{Leu}$ gene associated with MELAS are about 10/100 000 (Chinnery et al., 2000a; Majamaa et al., 1998; Uusimaa et al., 2007). Up to now, no efficient treatment for these disorders is known. However, important progress has lastly been made in developing various gene therapy models. Our laboratory was the first who proposed and validated the use of tRNA "allotopic" expression approach to complement this kind of mtDNA mutations on the model of MERRF m. 3844A>G mutation (Kolesnikova et al., 2004). To enlarge this therapeutic approach from mutations in $tRNA^{Lys}$ gene to mutations in other tRNA genes, we have chosen the most frequent one, the MELAS m.3243A>G mutation in $tRNA^{Leu}$ gene. To develop the therapeutic approach it is important to understand how m.3243A>G mutation leads to disease phenotype, which will be discussed in details in the next chapter.

I.3.3.1 MELAS syndrome

This disease was firstly described by Pavlakis and colleagues in 1984 (Pavlakis et al., 1984) as "mitochondrial myopathy, encephalopathy, lactic acidosis, and recurrent cerebral insults that resemble strokes (MELAS)" with "the following features: ragged red fibers evident on muscle biopsy, normal early development, short stature, seizures, and hemiparesis, hemianopia, or cortical blindness". The substitution of conservative adenine to guanine in position 3243 of $tRNA^{Leu(UUR)}$ gene was the first mutation associated with MELAS (Goto et al., 1990). Several other mutations in mitochondrial RNA genes can be also associated with MELAS syndrome, but more rarely. From the other side, the m.3243A>G mutation itself often results in additional to MELAS clinical phenotypes including CPEO (Chronic Progressive External Ophthalmoplegia), DMDF (Diabetes Mellitus and DeaFness) and others (MITOMAP, 2010) (Fig. 1.3).

In all cases described m.3243A>G mutation is present in heteroplasmic state, but the relative proportion of mtDNA carrying mutation (heteroplasmy level) and its pathogenic expression threshold are often individual and vary strongly in different tissues (Shanske et al., 2004). Clinical studies on MELAS patients, performed mostly on skin fibroblast cultures

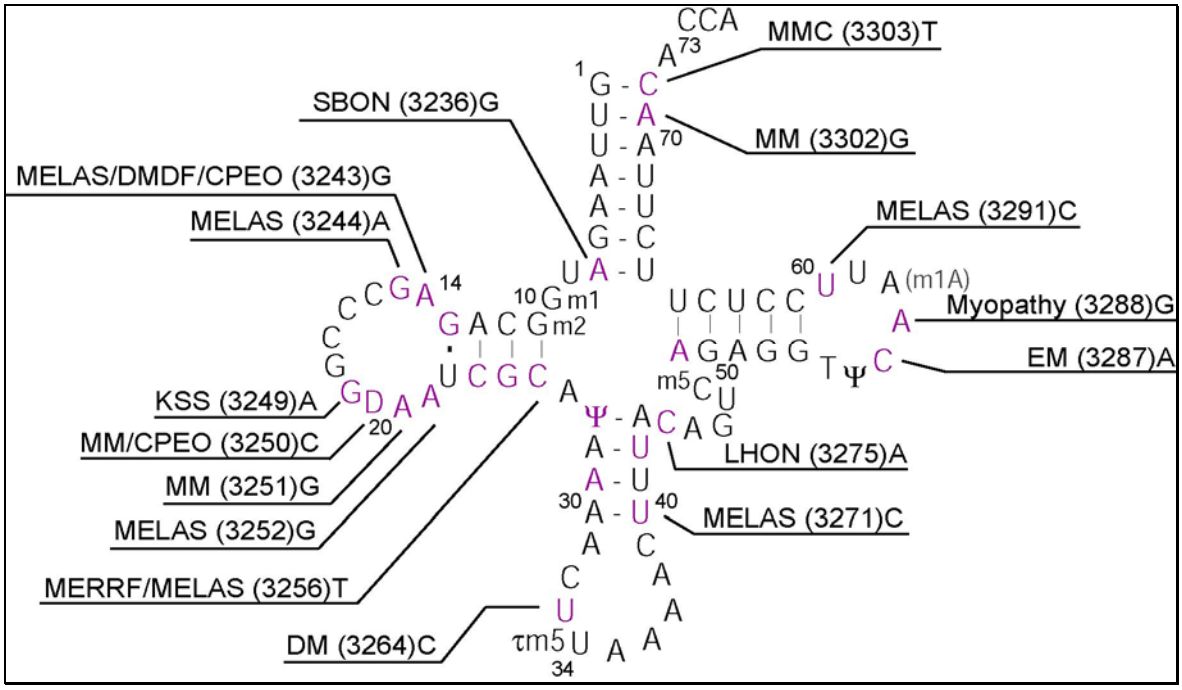

Fig. 1.3. Pathogenic mutations in human mt-tRNA$^{Leu(UUR)}$ gene (*MT-TL1*)

In violet are shown nucleotides of mt-tRNA$^{Leu(UUR)}$ for which pathogenic mutations in corresponding positions of mtDNA (shown in parentheses) were reported. Diseases associated with certain mutations are outlined. SBOP, Sporadic Bilateral Optic Neuropathy; MM, Mitochondrial Myopathy; DM, Diabetes Mellitus; EM, EncephaloMyopathy; MMC, Maternal Myopathy and Cardiomyopathy; for other disease abbreviations see Table 1.2. Positions of tRNA are numbered according to standard nomenclature. Post-transcriptional modifications: 5-taurinomethyluridine (τm^5U), 5-methylcarboxymethyl-2-thiouridine (mcm^5s^2U), 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$), 1-methylguanosine (m^1G), 2-methylguanosine (m^2G), 7-methylguanosine (m^7G), 1-methyladenosine (m^1A), 5-methylcytidine (m^5C), pseudouridine (Ψ), dihydrouridine (D), 5-methyluridine (T) and N6-threonylcarbamoyladenosine (t^6A).

or skeletal muscle biopsies, revealed severe deficiency of respiratory chain with complex I affected at the first place (Koga et al., 2000; Mariotti et al., 1995). Recent post-mortal analysis of OXPHOS protein complexes levels in different tissues of MELAS patient (Fornuskova et al., 2008) have shown that steady-state level of complex I in skeletal muscle and heart was decreased to 30% and 20% of control, respectively; complex IV level was decreased to 60% of control in muscle, but not in heart specimen; while relative levels of other complexes in these tissues were normal. The most pronounced reduction of OXPHOS protein complexes levels was observed in frontal cortex sample, where complex V was undetectable, complexes I and IV levels were reduced to 10% and to 20% of the control value, respectively.

The majority of studies on the pathogenic mechanism of m.3243A>G mutation were carried out on well-established model of *trans*mitochondrial cybrid cell lines (King and Attardi, 1989). Such cell lines are generated by the fusion of mtDNA depleted immortalized cells (usually osteosarcoma 143B or HeLa cells) with cytoplasts (enucleated cells bearing

mitochondria with mutant mtDNA) from patients. Different research groups have shown that M.3243A>G mutation leads to numerous structural and functional defects of mt-tRNA$^{Leu(UUR)}$, which affect its processing, post-transcriptional modification, folding, stability or aminoacylation level, but the precise pathogenic mechanism remains controversial, maybe because much of the data has been generated from cybrid cells, all of which are mostly anaeuploid and genetically unstable.

Besides the localization within the tRNA^{Leu} gene, m.3243A>G MELAS mutation overlaps with the binding site for mitochondrial transcription termination factor, mTERF (Kruse et al., 1989). mTERF is believed to regulate transcription termination from mtDNA heavy-strand in order to generate a short transcript, including two tRNAs and both 12S and 16S rRNAs. *In vitro* experiments have shown that binding affinity of partially purified mTERF for the MELAS template is indeed strongly reduced (Chomyn et al., 1992) and cause severe impairment of 16S rRNA transcription termination (Hess et al., 1991). However, an analysis of mtDNA-depleted human cells repopulated with mitochondria from MELAS patients (Chomyn et al., 1992; King et al., 1992) did not reveal any significant difference from the controls in the relative steady-state levels of the two rRNA species, encoded upstream of the termination site, and of the mRNAs encoded downstream. Later, *in vivo* footprinting analysis of the mtDNA segment within the tRNA$^{Leu(UUR)}$ gene that binds mTERF failed to reveal any difference in occupancy of sites or qualitative interaction with the protein between mutant and wild-type mtDNAs (Chomyn et al., 2000).

The m.3243A>G mutation occurs at position 14 in the consensus structure of tRNA (Fig.1.3). The base at this position is invariant in all bacterial and cytosolic eukaryotic tRNAs and is typically involved in tertiary folding of classical tRNAs (Goddard, 1977). However, it was suggested that in human mt-tRNA$^{Leu(UUR)}$ the residue A14 plays not only a structural role, but also interacts directly with human mtLeuRS in the process of recognition (Sohm et al., 2003). Finally, tRNA$^{Leu(UUR)}$ affected by MELAS mutation lacks several post-transcriptional modifications. Methylation of the G at position 10 in the consensus tRNA structure is reduced by 50% in mt-tRNA$^{Leu(UUR)}$ from MELAS cybrid cells (Helm et al., 1999). Taurine modification of the U (5-taurinomethyluridine) at the wobble position of anticodon of mt-tRNA$^{Leu(UUR)}$ was found to be reduced in cybrid cells as well as in patients carrying m.3243A>G MELAS mutation (Helm et al., 1999; Kirino et al., 2004; Yasukawa et al., 2005; Yasukawa et al., 2000). Modification of wobble uridines in tRNAs is responsible for specific and efficient codon recognition. Indeed, it was shown that an artificial mt-tRNA$^{Leu(UUR)}$ of the wild-type sequence, including all modified bases except for 5-taurinomethyluridine at the

wobble position, was severely defective in decoding UUG but not UUA codons (Kirino et al., 2004). In addition, heteroplasmic mutation, m.12300G>A, identified in the gene coding for mt-tRNA$^{Leu(CUN)}$ was shown to suppress m.3243A>G respiratory defect in MELAS cybrid cells (Kirino et al., 2006). This mutation converts the anticodon sequence of mt-tRNA$^{Leu(CUN)}$ from UAG to UAA identical to that of mt-tRNA$^{Leu(UUR)}$. Mass spectrometric analysis of the suppressor mt-tRNA$^{Leu(CUN)}$ from the phenotypically revertant cells carrying the m.12300G>A mutation revealed that the wobble uridine acquires *de novo* taurine modification. Taken together, these results strongly indicate that UUG codon-specific translational defect caused by the wobble modification deficiency in mt-tRNA$^{Leu(UUR)}$ plays an important role in the molecular pathogenesis of MELAS.

Decreased aminoacylation level of mt-tRNA$^{Leu(UUR)}$ was shown independently by several research groups. Mutant mt-tRNA$^{Leu(UUR)}$ was found almost exclusively in deacylated form in lung carcinoma cybrids bearing about 99% of M.3243A>G mutation (El Meziane et al., 1998). Osteosarcoma cybrid cell lines nearly homoplasmic for the M.3243A>G mutation as well exhibited a strong (70-75%) reduction in the level of aminoacylated tRNA(Leu(UUR)) and a decrease in mitochondrial protein synthesis rate (Borner et al., 2000; Chomyn et al., 2000; El Meziane et al., 1998; King et al., 1992; Park et al., 2003). Native m.3243A>G mutant tRNA$^{Leu(UUR)}$ was 25-fold less efficiently aminoacylated *in vitro*, compared to native wild-type tRNA$^{Leu(UUR)}$ (Park et al., 2003). A quite different picture has emerged from a study in HeLa cell based cybrids, and was then supported by findings from patient-derived lymphoblastoid cell lines and some other osteosarcoma cybrid studies. In HeLa background, mt-tRNA$^{Leu(UUR)}$ aminoacylation seemed to be only slightly affected (Yasukawa et al., 2000), even though there was a clear drop in the steady-state level of the mutant tRNA compared to wild-type. In patient-derived lymphoblastoid cells bearing 70% of mutation the abundance of mt-tRNA$^{Leu(UUR)}$ was unaffected, as was the overall amount of mitochondrial protein synthesis, yet the relative amount of incorporation of leucine into some mitochondrial translation products was differentially decreased (Flierl et al., 1997). Analysis of muscle biopsies from MELAS patients revealed a 4-times decrease of mt-tRNA$^{Leu(UUR)}$ aminoacylation level, accompanied by a general decrease of its amount (Borner et al., 2000). However, not all biopsy samples demonstrated the same phenomenon. In some of them, the mutant tRNA appeared to be expressed as efficiently as the wild type one, yet showing evidence of a specific defect in aminoacylation. In others, the mutant tRNA was under-expressed but aminoacylated efficiently. Moreover, in one sample no abnormality at all was detected (Borner et al., 2000).

The observed extent of mitochondrial translational defect in cells bearing MELAS m.3243A>G mutation was variable. 143B cells carrying high percentage of mutation (>90%) have shown strongly decreased synthesis of mtDNA-encoded polypeptides (Chomyn et al., 1992; King et al., 1992). Large polypeptides ND2, COXI, II and III were particularly affected, while ND4L+ATPase8 and ND6 were either unchanged or even increased. The qualitative patterns of mitochondrial translation products in these two studies were identical to those of wild-type cells. In the contrast, Dunbar and colleagues have shown the specific decrease of ND6 polypeptide, accompanied by the appearance of abnormally migrating protein band in 143B cells harbouring from 60% to 95% of m.3243A>G mutation (Dunbar et al., 1996). Jassen and colleagues reported that the overall rate of mitochondrial protein synthesis in 143B based cybrid cells with m.3243A>G mutation which was comparable with those of wild-type cybrids (Janssen et al., 1999). No correlation with the number of UUR codons was found, mutant cells displayed four additional but weak protein bands and synthesis of ATPase8 was significantly increased. [^{3}H]-Leucine incorporation was slightly lower (~15%) in mutant cells *versus* wild-type cells, indicating on eventual misincorporation of amino acids at UUR codons. Analysis of B-lymphoblastoid cell lines originated from blood lymphocytes of MELAS patients harbouring up to 70% of M.3243A>G mutation did not reveal any significant decrease of mitochondrial protein synthesis (Flierl et al., 1997). On the other hand, the specific decrease of leucine incorporation was observed in distinct polypeptides, ND3 and ND6, and fingerprint analysis revealed different cleavage patterns for ND5, COXI and ND4 subunits, showing an alteration of their primary amino acid sequence. In the other study (Sasarman et al., 2008), mitochondrial protein synthesis in myoblasts isolated from MELAS patient was 70% of control, however translation of ND5, and especially ND6 (rich in UUG codons), was disproportionately decreased and additional anomalously migrating translation products were detected. Endoproteinase fingerprint analysis showed clear evidence of amino acid misincorporation in three polypeptides: COXIII, COXII and ATP6. Almost complete lack of assembly of complexes I, IV and V, and a slight decrease of assembled complex III were observed. A similar defect of respiratory chain assembly was reported in the frontal cortex of a m.3243A>G MELAS patient (Fornuskova et al., 2008) and was mentioned in the beginning of this chapter.

Microarray analysis of gene expression in skeletal muscle from MELAS patients comparing to controls revealed significantly different expression level for 128 transcripts from 7084 analysed (van Eijsden et al., 2008). This analysis indicated on stimulation of the complement system, stimulation of protein synthesis and degradation, both stimulation and

inhibition of programmed cell death. In addition, the same authors showed higher amount of oxidatively damaged proteins and increased expression of ROS producing enzymes in muscle of m.3243A>G carries comparing to controls. Significant difference in gene expression was observed between the asymptomatic and symptomatic carriers of m.3243A>G. The authors hypothesised that dysfunctional proteins are replaced by new proteins, while stimulation of apoptosis and activation of complement system lead to muscle regeneration in asymptomatic patients, while in symptomatic patients, where protein damage can not be repaired or removed, muscle pathology becomes manifest.

Finally, prominent apoptosis tightly linked to mitochondrial proliferation and high mutation load have previously been reported in individual muscle fibres of MELAS patients (Aure et al., 2006).

I.4 Modelling of gene therapy for mitochondrial diseases

Even if mitochondrial diseases up to date remain incurable, several therapeutic approaches are currently used with a relative success (DiMauro and Mancuso, 2007). Treatment of seizures by anticonvulsant medication and surgical procedures may be efficient and therefore are frequently used. Low carbon diet, administration of ROS scavengers, electron acceptors, metabolites and cofactors (CoQ10, vitamin E, idebenone, dihydrolipoate, l-carnitine, creatine, riboflavin, thiamine, folic acid) are the mainstay of real-life therapy. Removal of noxious metabolites, for example, buffering of neurotoxic lactic acid by bicarbonate or more specifically acting dichloracetate (DCA), is also reasonable.

As alternative, the important progress has been made in developing gene therapy approaches. As it was previously discussed, mitochondrial diseases may be of nuclear DNA or mitochondrial DNA mutation origin. The treatment of mitochondrial dysfunction due to nuclear gene defects is addressed by conventional methods applicable to most nuclear gene defects. In this chapter I will describe the proposed models and challenges of therapy of mitochondrial diseases caused solely by mutations in mtDNA. These approaches can be arbitrary subdivided into several groups: (1) introduction of non-mutated mtDNA gene or entire copy of wild-type mtDNA into mitochondria, (2) expression of non-mutated gene in the nucleus (allotopic expression) and further targeting of its protein or RNA product to mitochondria, and finally (3) alteration of the ratio between mutant and wild-type and mtDNA, favouring the last one.

I.4.1 Strategies based on the delivery of DNA into mitochondria

Gene therapy designed to introduce a wild-type copy of gene into mitochondrial matrix may be termed as "direct" mitochondrial gene therapy. However, for the present the delivery of DNA into mitochondria remains challenged. First, transfection is difficult since three membrane barriers should be bypassed. Second, even assuming that DNA will be introduced, gene expression will not be stable without integration in mtDNA, which could be possible by homologous recombination, but the last one appears to be a rare event in mitochondria (see I.2). Alternatively, any imported DNA must contain the correct *cis*-acting elements to promote its replication and maintenance. But to date these elements are not fully characterized and even the mechanism of mtDNA replication itself remains controversial. Taken into account the small size of mtDNA (16.5 kbp) and its simple organization comparing to nuclear DNA it might be possible to introduce into mitochondria the entire copy of wild-type mtDNA carrying all needed *cis*-acting elements discussed above.

Physical methods of introducing DNA into mitochondria (mitochondrial transfection) have been recently described (D'Souza et al., 2007). Electroporation was employed to introduce DNA up to several kilobase pairs into isolated yeast mitochondria and has been used to transform mammalian mitochondria as well. Biolistic bombardment has achieved success in isolated yeast mitochondria, living yeast cells and most recently in algae. In mammalian systems, physical methods like electroporation and biolistic bombardment are as yet restricted to the use of isolated mitochondria therefore making their application in clinical treatment dependent on some sort of intracellular mitochondrial delivery strategy.

Delivery of short oligonucleotides (up to 322 bp) coupled to mitochondrial leader sequence (MLS) peptide into isolated mitochondria as well as in mitochondria of cultured human cells was demonstrated in several studies (Flierl et al., 2003; Geromel et al., 2001; Seibel et al., 1995; Vestweber and Schatz, 1989). Flierl and colleagues demonstrated that peptide-DNA complexes were imported into the mitochondrial matrix through the outer and inner membrane import channels and localized in mitochondrial matrix of living mammalian cells.

It has been known for several years that isolated plant mitochondria can spontaneously import low level of naked linearized DNA (Koulintchenko et al., 2003). More recently, isolated mammalian mitochondria were also shown to import linear DNA, which was acting as a template for DNA synthesis and RNA transcription (Koulintchenko et al., 2006). Now these findings should be reproduced on living cells.

Developing of nuclear gene therapy was possible due to the availability of a wide variety of gene delivery systems. The most efficient of them are viral systems, which are currently in clinical trials. The viral life cycle was described as closely associated with mitochondrial function and moreover certain viral particles can display a marked mitochondrial localization (Beatch and Hobman, 2000; Valentin et al., 2005). However no much interest could be observed in application of viral systems to mitochondrial gene therapy. In contrast to nuclear therapy, non viral delivery systems were intensively studied in the field.

Several mitochondria-specific non-viral DNA delivery systems were developed. Among them DQasomes, liposome-based carrier MITO-Porter and other types of mitochondriotropic liposomes were proposed (D'Souza et al., 2005; Yasuzaki et al., 2010). DQasomes, vesicles formed of mitochondriotropic quinolinium compounds, could be loaded with DNA, deliver and liberate it in proximity of mitochondria. Further uptake of DNA in mitochondria *via* the mitochondrial import machinery requires the presence of mitochondrial leading sequence (MLS). Thus fluorescent oligonucleotide conjugated to a MLS peptide and loaded in DQasomes had been shown to co-localize with mitochondria of cultured cells (D'Souza et al., 2005). Moreover, DQasomes were efficient in delivery of linear plasmid DNA conjugated to an MLS to mitochondria. But it remains unclear if DNA would be able to cross the inner mitochondrial membrane and reach the matrix.

Another approach to correct mtDNA defects could be repopulation of diseased phenotype cells with healthy mitochondria. An interesting observation has been done that active transfer of functional mitochondria or mtDNA from adult stem or somatic cells can rescue aerobic respiration in mammalian cells with non-functional mitochondria (Spees et al., 2006). Theoretically, a woman carrying mtDNA mutation could have her oocytes “cleansed” *in vitro* of the cytoplasm with mitochondria and, consequently, mutated mtDNA. Such partial replacement of the cytoplasm (5-10%) is actually used to “rejuvenate” aged oocytes and to improve the success of *in vitro* fertilization. Healthy mitochondria can be transferred by direct microinjection or cytoplast fusion, the last one widely used to generate cellular models of mtDNA deficiencies. Studies in mice and primates with mtDNA disease have proved feasibility of the reconstitution of a healthy embryo by pronuclear transfer, when pronuclei carefully removed from fertilized oocyte bearing mtDNA mutation were fused to the cytoplasts from zygote containing only normal copies of mtDNA (Sato et al., 2005; Tachibana et al., 2009). The reconstructed oocytes with the mitochondrial replacement were capable of supporting normal fertilization, embryo development and produced healthy

offspring. More recent experiments show the possibility of such germ line therapy technique in humans (Craven et al., 2010).

I.4.2 Strategies based on the allotopic expression of non-mutated mitochondrial genes in the nucleus

Many efforts to correct mtDNA defects relied on expression of mitochondrial genes in nucleus and further import of their products in mitochondria. Transfer of mitochondrial genes during evolution to nucleus and existence of mitochondrial protein and RNA import pathways provided the solid basis for developing of this "indirect" approach to mitochondrial therapy termed as allotopic expression.

I.4.2.1 Allotopic expression of mitochondrial protein coding genes

The first successful attempt to use protein allotopic expression to rescue respiratory defect was done by Nagley *et al.* on yeast carrying mutation in the mitochondrial *MT-ATP8* gene. Normal copy of *MT-ATP8* gene fused to the MLS of a normally imported protein (*N. crassa* ATPase9) was introduced into nucleus (Nagley et al., 1988). The recombinant ATPase8 was indeed targeted into mitochondria, integrated into the mitochondrial ATP synthase and restoration of oxidative phosphorylation was observed. Manfredi et al. re-coded the wild-type human mitochondrial *MT-ATP6* gene, fused it to the MLS of either COXVIII or ATPase9, and expressed the recombinant protein in human cells bearing a pathogenic mutation in the *MT-ATP6* associated with the NARP syndrome (Manfredi et al., 2002). They found that the protein was mitochondrially imported and was able to restore respiration level of mutant cells. More recent and extensive analysis by Bokori–Brown *et al.* conflicted with these results. Similar allotopic experiments on a cell line carrying the identical mutation in *MTATP6* have shown inability of nuclear-encoded ATPase6 to integrate into mature ATP synthase (Bokori-Brown and Holt, 2006). Several studies have attempted to optimise the allotopic expression and import of highly hydrophobic mitochondrial proteins (Bonnet et al., 2007). For this corresponding mRNA was associated with *cis*-acting elements in the 3'-untranslated regions of transcripts known to localise to mitochondria. The re-coded *MT-ATP6* gene was then associated with the *cis*-acting elements of *SOD2*, while the *MT-ND4* gene was

associated with the *cis*-acting elements of *MT-COX10*. As a result both ATP6 and ND4 proteins were efficiently translocated into the mitochondria and were functional within their respective respiratory chain complexes. This lead to a long-lasting and complete rescue of mitochondrial dysfunction of fibroblasts harbouring the neurogenic muscle weakness, ataxia and retinitis Pigmentosa T8993G ATP6 mutation or the Leber hereditary optic neuropathy G11778A ND4 mutation, respectively. Allotopic expression is a very promising strategy, but it requires re-coding of gene sequence for efficient translation in cytosol, fusion with an appropriate signal peptide providing efficient mitochondrial import and correct integration of the allotopically expressed protein in assembling respiratory complex. Each of these steps should be individually tested for each new protein. Furthermore, this approach is difficult to apply to highly hydrophobic mitochondrial proteins, as cytochrome *b*, since the high hydrophobicity may lead to their sticking to mitochondrial membranes or to inefficient unfolding/folding during translocation (Claros et al., 1995).

A substitution of non-functional (due to mutations) mitochondrial proteins by those from other species is another interesting variety of allotopic approach (also called "xenotopic expression"). Both NADH: ubiquinone oxidoreductase (NADH oxidase, or complex I) and cytochrome *c* oxidase (COX, or complex IV) deficiencies were addressed by this approach.

In humans, NADH oxidase is the most voluminous complex of mitochondrial respiratory chain. It consists of approximately 45 polypeptides, 7 of which are encoded by mtDNA. In contrast, yeast *Saccharomyces cerevisiae* posses a single subunit NADH oxidase (Ndi1). Yeast *NDI1* gene can be stably transfected and functionally expressed in human cells. The resulting protein has been correctly targeted into human mitochondria and restored NADH-dependent respiration and galactose-dependent proliferation of human cells bearing mutation in ND4 gene (Bai et al., 2001). Later, the authors have validated this technique on complex I deficiency in animal model of Parkinson's disease (Marella et al., 2008).

In human mitochondria oxygen is reduced exclusively by cyanide-sensitive cytochrome *c* oxidase, the terminal complex of mitochondrial electron transfer chain. On the other hand, many other species encode cyanide-insensitive alternative oxidases (AOXs). Very recently, it has been shown that expression of a mitochondrially targeted AOX from *Ciona intestinalis* in COX-deficient human cells restored their growth and oxidative phosphorylation (Dassa et al., 2009).

I.4.2.2 Allotopic expression of mitochondrial RNA coding genes

As it was mentioned above, more than 170 mitochondrial mutations have been identified in mtDNA-encoded tRNA genes. Deficiencies of this type could as well be addressed by the approach of allotopic expression. For this non-mutated tRNA genes should be expressed in the nucleus and their products further targeted into mitochondria. The pathway of mitochondrial RNA import, far less understood than the protein one, may now be considered as quasi-universal. Import of nuclear-encoded RNAs into mitochondria has been identified in phylogenetic groups as diverse as protozoans, fungi, animals and plants, however the type and number of imported RNAs vary from species to species (Entelis et al., 2001b; Salinas et al., 2008; Schneider and Marechal-Drouard, 2000). In the majority of cases, transfer RNAs are imported, however import of other small RNAs, for example, 5S rRNA (Entelis et al., 2001a; Magalhaes et al., 1998; Yoshionari et al., 1994), RNase P RNA and RNase MRP RNA (Wang et al., 2010) was also described in mammals. The number of imported tRNAs ranges between a few tRNA species in yeast to all mitochondrial tRNA species in trypanosomatids (Lima and Simpson, 1996; Martin et al., 1979; Rinehart et al., 2005; Schneider et al., 1994).

Over the last 20 years our laboratory was extensively studding mechanism of RNA (tRNA and 5S rRNA) mitochondrial import in yeast and human cells. In yeast *Saccharomyces cerevisiae* one of two lysine isoacceptor tRNAs - tRK1 is naturally imported into mitochondria (Entelis et al., 1998). Functional mitochondrial protein translocation apparatus (Tarassov et al., 1995a), ATP-generation system and several soluble protein factors are required for tRK1 import (Tarassov and Entelis, 1992). Cytosolic tRK1 once aminoacylated by cytoplasmic lysyl-tRNA synthetase (KRS) (Tarassov et al., 1995b) either participates in cytosolic translation, or is recruited by the first import factor enolase (Entelis et al., 2006). This glycolytic enzyme address the tRK1 toward mitochondrial sufrace, where it is bound by the second import factor, peri-mitochondrially synthesized precursor of mitochondrial lysyl-tRNA synthetase (preMsk1p) (Tarassov et al., 1995b) (Fig. 1.4). The exact mechanism of further translocation is unclear, but the intactness and functionality of the pre-protein import apparatus is required to achieve the tRNA uptake (Tarassov et al., 1995a). Import of tRK1 plays a role in the conditional regulation of mitochondrial translation. Cytoplasmic tRK1 with CUU anticodon is used to decode rare lysine AAG codons in the situation when the wobble position of UUU anticodon of mitochondrial $tRNA^{Lys}$ (tRK3) is undermodified at elevated temperature (37°C) (Kamenski et al., 2007). More details on the mechanism of tRK1 import

and its function in mitochondria of *S. cerevisiae* and are provided in Publication 3 (Tarassov et al., 2007).

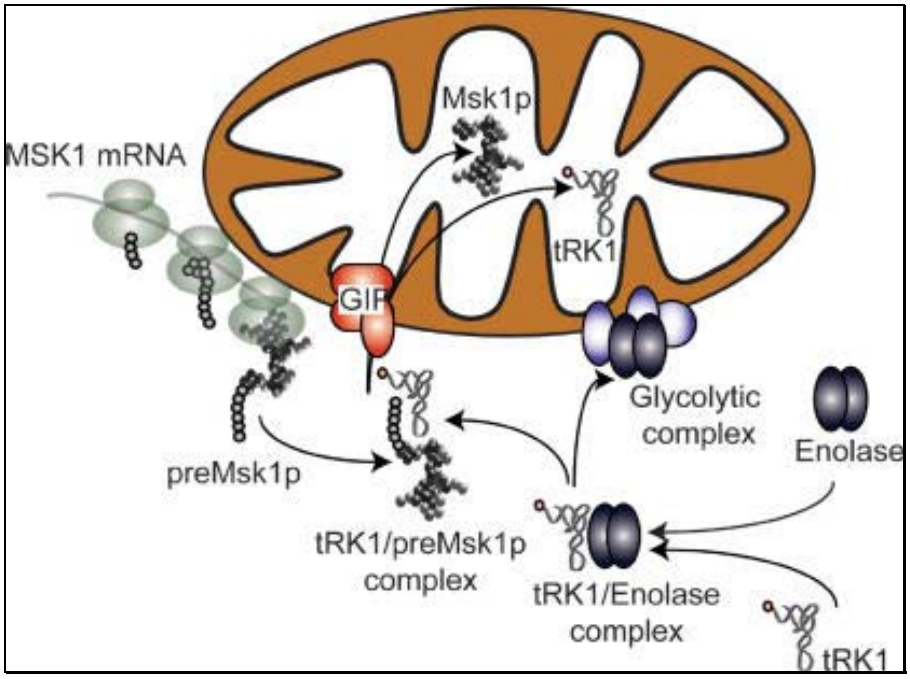

Fig. 1.4. Proposed mechanism of tRK1 import into mitochondria of *S. cerevisiae* (Entelis et al., 2006)

GIP indicates the general insertion pore, the outer membrane pre-protein import complex composed of TOM proteins. Aminoacylated by KRS tRK1 is recognized by enolase (most probably Eno2p) in the cytoplasm and addressed toward the mitochondrial surface, where the tRNA is transferred to the peri-mitochondrially synthesized preMsk1p, while enolase inserts in the hypothetical glycolytic multiprotein complex, whose role may be the channelling of the pyruvate to the organelle. Further targeting of tRK1 to the mitochondrial matrix involves the functional GIP.

Human mitochondria were recently shown to import from the cytoplasm $tRNAs^{Gln}$ (Rubio et al., 2008). Moreover, we have demonstrated that yeast importable $tRNA^{Lys}$ derivatives could be imported into human mitochondria by a similar mechanism to that described in yeast (Entelis et al., 2001a). Their import can be driven by either yeast or human proteins, suggesting the existence of a cryptic tRNA import pathway in human cells. The derivatives of yeast tRNAs were shown to be active in human mitochondrial protein synthesis (Kolesnikova et al., 2000). Finally, it was shown that MERRF (Myoclonic Epilepsy with Ragged-Red Fibres) syndrome caused by m.8344A>G mutation in mitochondrial $tRNA^{Lys}$ gene could be partially rescued by targeting yeast $tRNA^{Lys}$ derivatives into mitochondria (Kolesnikova et al., 2004). Yeast $tRNA^{Lys}$ genes were expressed in cybrid cells and patients' fibroblasts with the m.8344A>G mutation, they were proven to be correctly aminoacylated and imported into mitochondria, where they participated in mitochondrial translation. As a

result, partial rescue of mitochondrial functions such as mitochondrial translation, activity of respiratory complexes, electrochemical potential across the mitochondrial inner membrane and respiration rate was observed (Fig. 1.5.).

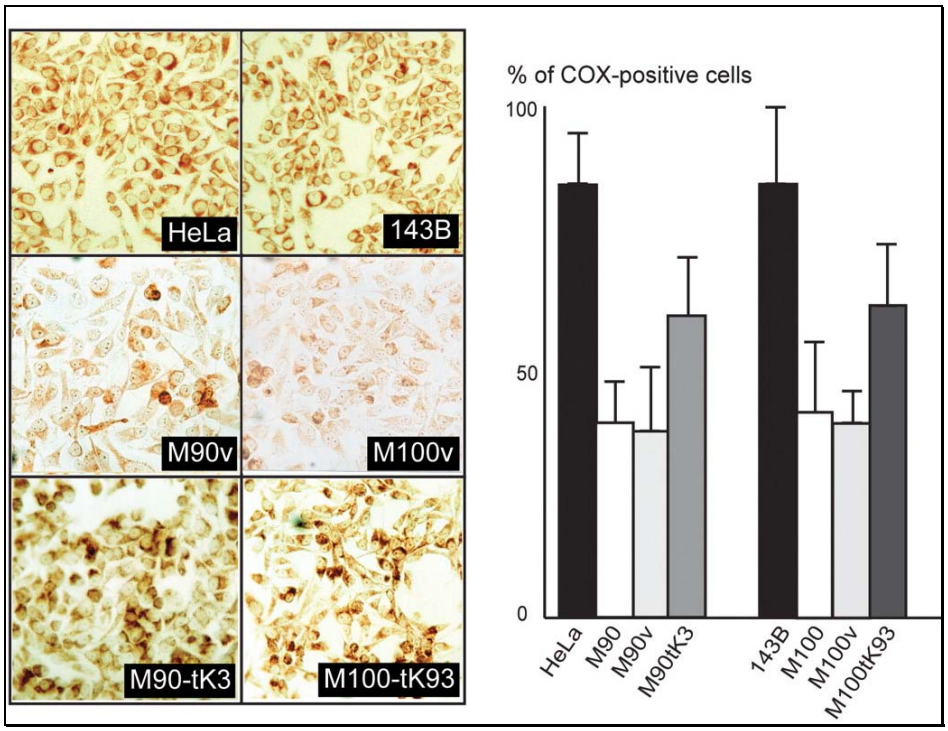

Fig. 1.5. Restoration of COX activity in MERRF cybrid cells (Kolesnikova et al., 2004)

Cytochemical staining for COX activity in MERRF cybrid cells bearing 90% or 100% of m.8344A>G mutation and stably expressing tRK3 (M90-tK3) or recombinant tRK2 (M100-tK93), comparing to MERRF cells transfected with an empty vector (M90v and M100v), 143B and HeLa cells without m.8344A>G mutation.

An alternative way to deliver RNAs in mitochondria of human cells has been proposed by the team of Samit Adhya. They have isolated (Bhattacharyya et al., 2003) and characterized a large (~640 kDa) RNA import protein complex (RIC) from the mitochondrial inner membrane of the parasitic protozoon *Leishmania tropica* (reviewed in (Adhya, 2008)). This complex was shown to be taken up and targeted to mitochondria of cultured human cells through a caveolin-1-dependent endocytotic pathway (similar to that used by viruses) and to induce import of several endogenous cytoplasmic tRNAs, leading to correction of the respiration defect of MERRF cybrid cells (Mahata et al., 2006). Moreover, RIC has been shown to direct in HepG2 mitochondria *in vivo* RNA or RNA/DNA chimeric oligomers (~40 nt) carrying an import signal of *Leishmania* importable $tRNA^{Tyr}$ fused to RNA or DNA antisense sequence complementary to the 5' end of different mitochondrial mRNAs (NDI, COXI, COXII, ATPase6 mRNAs) (Mukherjee et al., 2008). More than 80% reduction of

targeted mRNA level was observed with both types of antisense molecules and was accompanied by the decrease in activity of corresponding respiratory chain complex, general reduction of respiration and lowering of membrane potential in HepG2 cells. The authors clamed the existence in human mitochondria of small RNA-mediated mRNA degradation pathway, which can be employed to treat the heteroplasmic mutations in protein-coding genes.

I.4.3 Indirect complementation of mtDNA mutations

Important progress has recently been made in development of indirect gene therapy models for MELAS disease, caused by m.3243A>G mutation in mt-tRNA$^{Leu(UUR)}$ gene. Partial complementation of m.3243A>G mutation was demonstrated in MELAS cybrid cells over-expressing mitochondrial leucyl-tRNA synthetase (LeuRS) (Li and Guan, 2010; Park et al., 2008). The authors observed increased stability and amount of aminoacylated mt-tRNA$^{Leu(UUR)}$, partial restoration of COXI, COXII and NDI steady-state levels and improvement of respiration of MELAS cybrid cells over-expressing LeuRS. Interestingly, the rate of mitochondrial protein synthesis was almost the same as that in parental mutant cells. Thus authors suggested that mutation suppression occurred *via* a mechanism that increased protein stability rather than translation rate.

In an independent study, it has been demonstrated that over-expression of mitochondrial translation factor EFTu partially rescued the consequences of m.3243A>G mutation in yeast model of MELAS disease (Feuermann et al., 2003). Basing on these early data, Shoubridge and colleagues have recently shown that over-expression of translation factors EFTu and EFG2 partially restored mitochondrial function of myoblasts derived from a MELAS patient, improving their mitochondrial translation, steady-state levels of certain subunits of respiratory chain, assembly and activity of the OXPHOS complexes (Sasarman et al., 2008).

I.4.4 Strategies based on the alteration of the balance between wild-type and mutant mtDNA

The majority of patients that suffer from mtDNA disease carry a mutation in heteroplasmic form, meaning that they harbour mutant and wild-type mitochondrial genomes

at the same time. Thus a very attractive approach to genetic therapy of these disorders would be alteration of the balance between healthy and mutated mtDNA. Moreover one can expect that even a minor shift of heteroplasmy (~ 10%) could be sufficient to reverse pathological phenotype. Several methods are currently being investigated in this field.

Selection of patient cells in the presence of different inhibitors of mitochondrial function or on alternative energy sources can induce changes in heteroplasmy. Thus treatment with the inhibitor of mitochondrial protein synthesis doxycycline, growth on galactose accompanied by treatment with ATP synthase inhibitor oligomycin as well as substitution of glucose by ketone bodies (acetoacetate and *D*-β-hydroxybutyrate) in growth medium resulted in a selection favouring wild-type mtDNA molecules (Manfredi et al., 1999; Santra et al., 2004; Spelbrink et al., 1997). However, it is still difficult to envisage such a treatment applied to patients.

I.4.4.1 Degradation of mutant mtDNA by mitochondria-targeted nucleases

An elegant approach to remove pathogenic mtDNA is by mitochondrial targeting restriction endonucleases able to specifically recognize only mutant mtDNA, thus favouring propagation of solely wild-type copies. This approach was validated on both human and mouse disease models. NARP/MILS mutation T8993G in *ATP6* gene introduced unique *Sma*I restriction site in the mutant mtDNA. Expression of nucleus-encoded *Sma*I endonuclease fused to a MLS peptide and its subsequent targeting into mitochondria of NARP/MILS cybrid cells resulted in the depletion of mutant mtDNA and increase in wild-type mtDNA (Tanaka et al., 2002). A similar method with mitochondrially-targeted *PstI* endonuclease was successfully used on rodent cell lines by the group of Carlos Moraes (Srivastava and Moraes, 2001). More recent study from this group proved the feasibility of this approach on NZB/BALB heteroplasmic mouse model, bearing two populations of mtDNA differed in more than 100 polymorphic sites. Using adenoviral transfection system gene of mitochondrially-targeted *ApaLI* enzyme was delivered in brain and skeletal muscle of mice. Controlled expression of *ApaLI* resulted in a shift towards the NZB mtDNA haplotype, which does not contain *ApaLI* site (Bayona-Bafaluy et al., 2005). However, the use of this method for therapeutic purposes, certainly powerful, is limited by a low frequency of pathogenic mutations able to create specific and unique sites for restriction enzymes. To address this problem “differential multiple cleavage site” model was proposed by Carlos Moraes and

colleagues and tested on NZB/BALB mice with regard to *ScaI* sites; there are five in the NZb compared to three in BALB mtDNA. *ScaI* expression indeed induced a desired shift of heteroplasmy, although high expression of enzyme was accompanied by the negative effect of the mtDNA depletion (Bacman et al., 2007). Another possibility of selective elimination of mtDNA consists in using mitochondrial-targeted zinc finger peptides (ZFPs) engineered specifically to bind mutant mtDNA. Such ZPFs conjugated to a nuclease were shown to selectively degrade mutant mtDNA in cultured cells (Minczuk et al., 2008).

I.4.4.2 Selective inhibition of the replication of mutant mtDNA

Basing on sequence differences between wild-type and mutant mtDNA the replication of the last one could be specifically blocked, thus favouring propagation of a normal copy. This approach termed "antigenomic" requires development of specific mitochondrially-targeted molecules with a higher affinity to mutant mtDNA than to wild-type one. A number of potential antigenomic agents have been investigated.

Peptide nucleic acids (PNA) were one of the first. PNA are synthetic structural homologues of nucleic acids in which the negatively charged phosphate-sugar backbone of the polynucleotide is replaced by an uncharged polyamide backbone consisting of achiral N-(2-aminoethyl) glycine units (Nielsen et al., 1991). The resulting decrease in electrostatic repulsion allows the formation of a PNA–DNA hydrogen- bonded double helix, which is more stable than the one formed by DNA–DNA interaction (Peffer et al., 1993). PNAs are efficiently used for targeting chromosomal genes in a sequence-specific manner in cells both *in vitro* and *in vivo* (Wang & Xu, 2004; Tian et al., 2007; Matis et al., 2009). Because of their artificial backbone, PNAs are resistant to both nucleases and proteases, resulting in a prolonged half-life in cultured cells as well as *in vivo* if injected in mice (Ray & Norden, 2000; Nielsen, 2001; Boffa et al., 2005). It is not surprising that such attractive molecules were also tested in mitochondrial gene therapy. It was demonstrated that complementary PNA oligomers bind to mutant mtDNA and can efficiently inhibit its replication *in vitro* (Taylor et al., 1997). To facilitate their mitochondrial delivery in the living cell PNAs were conjugated either to a mitochondrial-targeting peptide (Chinnery et al., 1999) or to lipophilic cation triphenyl phosphonium (TPP) (Muratovska et al., 2001). Although the targeting of PNA compounds to mitochondria was observed, no modulation of heteroplasmy was detected in MERRF cybrid cells (Muratovska et al., 2001). Detailed investigation have shown that TPP-

PNA molecule was essentially stacked in mitochondrial membranes, therefore was not able to access mtDNA, while only small portion of peptide-PNA was localized in the mitochondrial matrix (Lamla et al., 2010; Ross et al., 2004).

As mentioned above, human mitochondria have innate ability to import 5S rRNA (Entelis et al., 2001a; Magalhaes et al., 1998; Smirnov et al., 2010; Smirnov et al., 2008). Additionally, a number of small artificial RNA molecules can be addressed into mitochondria of human cells (Kolesnikova et al., 2010). These importable RNAs with insertions complementary to the mutant regions of mtDNA can also serve as "anti-replicative" agents. Very recent results obtained in our laboratory confirm the feasibility of this approach (Compte et al., *submitted*).

I.4.4.3 Induction of the mitochondrial fusion

Different mtDNA populations can functionally interact within mitochondrial network of the cell. Generally heterologous mtDNAs are maintained in separate nucleoid populations, but if needed two types of mtDNA can *trans*complement each other to restore wild-type like levels of mitochondrial function and morphology (Gilkerson et al., 2008). To achieve this, different mitochondrial populations can undergo fusion and exchange of organellar contents, even if one or both are respiratory-deficient. Noteworthy, mtDNA molecules themselves do not appear to interact directly, but diffusion of mtDNA-encoded transcripts and/or polypeptides allow for *trans*complementation of heterologous mtDNAs. So, the attractive strategy will be stimulation of mitochondrial fusion in heteroplasmic cells allowing wild-type mtDNA to *trans*complement through mitochondrial network and restore mitochondrial function.

I.4.4.4 Induction of the muscle regeneration

Patients suffering from mitochondrial disorders and bearing high levels of mutant mtDNA in mature muscle, often have very low mutation load in myogenic progenitor satellite cells. So, the idea was to stimulate muscle regeneration by active proliferation of satellite cells and shift the heteroplasmy level. Induction of muscle satellite cells is known to occur as a result of intensive physical training, muscle injury as well as treatment with myotoxic drugs. Regeneration of muscle tissue in patients with heteroplasmic mt-tRNA mutations was indeed

observed as a result of biopsy trauma or treatment with myotoxin and was accompanied in both cases by a decrease of mutant mtDNA and restoration of mitochondrial function (Clark et al., 1997; Shoubridge et al., 1997).

Exercise training is an alternative and *a priori* more natural method to increase muscle regeneration. It is known to promote mitochondrial biogenesis and satellite cell activation. Taivassalo and colleagues carried out a series of experiments on patients with large-scale mtDNA deletion. They observed increased muscle strength and improved muscle oxidative capacity in patients who have undertaken exercise training over 12-14 weeks, but no change or even an increase in proportion of mutant mtDNA was observed (Taivassalo et al., 2001). More recently the same authors have proposed an improvement of this treatment approach, consisting in combination of strength and endurance training. Strength training was aimed to induce satellite cell activation and transfer of wild-type mtDNA to existing muscle, following endurance training should promote mitochondrial biogenesis and though increase the efficiency of expansion of newly incorporated wild-type genomes. Patients with large-scale mtDNA deletion at the end of described exercise training have demonstrated decreased number of COX-deficient fibres and increased level of wild-type mtDNA (Murphy et al., 2008).

OBJECTIVE OF THE THESIS

The main objective of this study was to investigate the possibility to rescue MELAS mutation consequences by allotopic expression of recombinant versions of the yeast importable lysine tRNAs whose aminoacylation identity had been changed from lysine to leucine. To achieve this objective the following goals have been fixed:

1. To construct mitochondrially importable tRNAs with human leucine aminoacylation identity and validate them *in vitro* and *in vivo*;
2. To characterize the model MELAS cybrid cell line and choose the reliable criteria of its mitochondrial function in order to test possible curative effect of the recombinant tRNAs;
3. To study the effect of transient and stable expression of the recombinant tRNA genes in MELAS cybrid cells on mitochondrial function affected by the mutation.

II RESULTS AND DISCUSSION

II.1 Development of gene therapy model for MELAS mutation

II.1.1 Construction and validation of importable tRNAs with leucine aminoacylation identity

To develop anti MELAS therapy, it was needed first to create a set of tRNA molecules able to be aminoacylated with leucine in human cells and imported in their mitochondria in order to substitute for the mt-tRNA$^{Leu(UUR)}$ affected by m.3243A>G mutation and non-functional in mitochondrial translation.

II.1.1.1 *In silico* modelling and construction

Human cells possess two leucyl-tRNA-synthetases, cytoplasmic (hcytLeuRS) and mitochondrial (hmtLeuRS), encoded by two distinct genes, *LARS* and *LARS2*, respectively. They recognize cognate tRNAs by different sets of identity elements (Sohm et al., 2003; Sohm et al., 2004). The only common recognition element for both enzymes, as well as for LeuRS from other organisms is a "discriminator" base A73. The other important identity elements for hcytLeuRS are situated in a long variable arm of cytosolic leucine tRNAs, in their aminoacceptor stem (C3-G70, A4-U69, G5-C68) and D-loop (C20). The major identity elements required for recognition of mitochondrial leucine tRNAs by hmtLeuRS are "discriminator" base A73, A14 base in D-loop (affected by m.3243A>G mutation) and nucleotides of anticodon, while long variable arm is missing in these tRNAs (Fig. 2.1). In our laboratory, three yeast lysine isoacceptor tRNAs (tRKs) able to be imported *in vivo* into mitochondria of human cells have been previously characterized: two cytosolic, tRK1 and recombinant tRK2 (G1-C72; G73; U34), and one mitochondrial, tRK3 (Fig. 2.1) (Kolesnikova et al., 2004). We have compared the sequence of these importable tRNAs with two different sets of human leucine identity elements, and found out that the minimum of mutations was required to introduce in them the recognition elements for hmtLeuRS.

Therefore, we introduced in yeast lysine tRNAs the discriminator base A73 and leucine anticodons, either UAA or CAA, the first one with the expectation that the U in the wobble position would be correctly modified to 5'-taurinomethyluridine (τm^5U), the last one with the purpose to decode UUG codons even if the anticodon will be not modified. As a result, 6 different versions of potentially therapeutic tRNAs (Fig. 2.1), namely tRK1UAA, tRK1CAA, tRK2UAA, tRK2CAA, tRK3UAA and tRK3CAA were constructed and cloned in pUC19 vector as described in Materials and Methods.

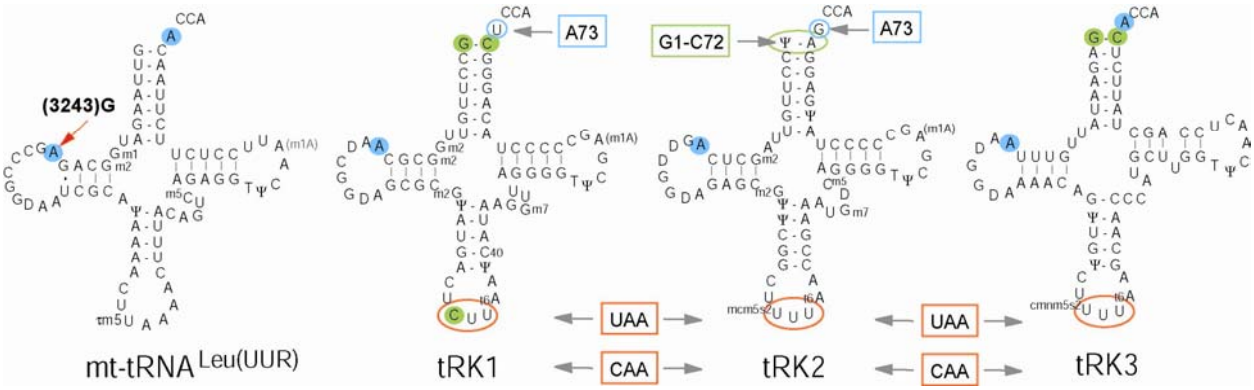

Fig. 2.1. Cloverleaf structures of tRNAs used in this study

From left to right: native human mitochondrial $tRNA^{Leu(UUR)}$, major identity elements of recognition by mtLeuRS are in blue filled circles and MELAS m.3243A>G mutation is indicated by the red arrow; three yeast lysine tRNAs, tRK1, tRK2; tRK3 and their recombinant versions, tRK1UAA/CAA, tRK2UAA/CAA, tRK3UAA/CAA, with determinants of mitochondrial import indicated in green filled circles, identity elements for human mtLeuRS are in blue filled circles, mutations and regions where they were introduced are indicated by arrows and enclosed in blue for leucine aminoacylation identity elements, in green - for import determinants and in red - for leucine anticodons. Post-transcriptional modifications: 5-taurinomethyluridine (τm^5U), 5-methylcarboxymethyl-2-thiouridine (mcm^5s^2U), 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$), 1-methylguanosine (m^1G), 2-methylguanosine (m^2G), 7-methylguanosine (m^7G), 1-methyladenosine (m^1A), 5-methylcytidine (m^5C), pseudouridine (Ψ), dihydrouridine (D), 5-methyluridine (T) and N6-threonylcarbamoyladenosine (t^6A).

Transcripts of these recombinant tRNA genes were produced by *in vitro* T7-transcription from pUC19-tRK plasmids and were further used for *in vitro* and *in vivo* assays. The most of the following *in vitro* experiments were performed with tRK3 versions, tRK3UAA and/or tRK3CAA, as these were the first versions studied in a course of time, while tRK1- and tRK2-based versions were added to the project already on the step of *in vivo* experiments.

II.1.1.2 Analysis of tRNA-binding to the precursor of yeast mitochondrial lysyl-tRNA synthetase

The precursor of yeast mitochondrial lysyl-tRNA synthetase (pre-MSK) is an essential factor of tRK1 import in yeast mitochondria (Tarassov et al., 1995a). It was suggested to function as a carrier of the tRNA towards mitochondria and, maybe, across the mitochondrial membranes *via* the protein import channel. In human cells as well as in the *in vitro* import assay with isolated human mitochondria, pre-MSK could be replaced by its human counterpart, the precursor of human mitochondrial lysyl-tRNA synthetase (Entelis et al., 2001a; Kolesnikova et al., 2002). Furthermore, there is a correlation between capacity of a particular tRNA-version to bind to pre-MSK and efficiency of its import into isolated human mitochondria (Kolesnikova et al., 2002).

Therefore, it was first verified if recombinant tRNAs were able to interact with pre-MSK by gel shift assay. This was essentially performed with tRK3UAA version. For this, tRK3UAA [^{32}P]-labelled-T7-transcipt was incubated with recombinant pre-MSK in increasing concentration (0, 05 - 0, 4 μM), and then formation of the complex was monitored by gel shift assay under native conditions. In case of tRNA-protein complex formation the additional band with mobility lower than that of a free transcript should be observed on an autoradiograph of the gel. These experiments had shown that recombinant tRK3UAA has indeed, the pre-MSK-binding capacity (Fig. 2.2). The dissociation constant (K_d) of complexes tRK3UAA-pre-MSK was 0.27 μM, while K_d of the complex of tRK1-pre-MSK was previously shown to be 0.28 μM. Importantly, the tRK1 transcript in this complex was pre-aminoacylated with lysine by yeast cytoplasmic lysyl-tRNA synthetase (KRS), whereas tRK3UAA was binding to pre-MSK without aminoacylation. This was not so surprising, since pre-MSK is a cytoplasmic precursor of MSK that normally binds to deacylated tRK3 to aminoacylate it. On the other hand, pre-MSK has been shown to bind cytoplasmic tRK1 in a different manner than that of classical aaRS-tRNA binding (S. Dogan, unpublished data). Moreover, several mutant transcripts of tRK1 and tRK2 were previously shown to bind to pre-MSK without aminoacylation (Kolesnikova et al., 2002), suggesting that aminoacylation and/or interaction with KRS is rather needed for inducing correct folding of tRNA and, by this, facilitating its binding to pre-MSK.

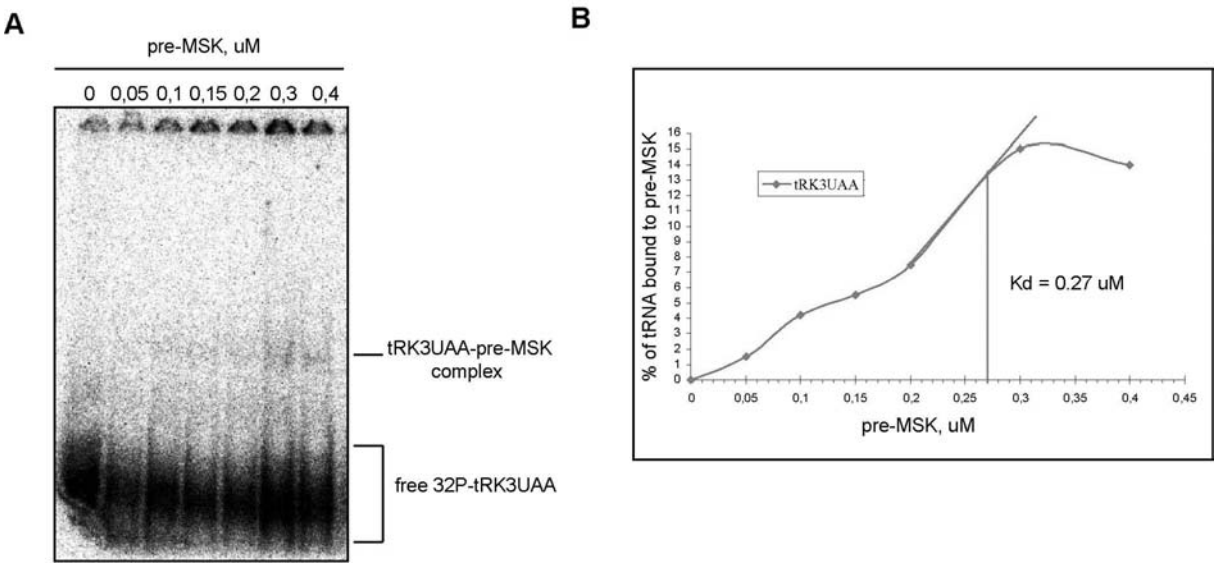

Fig. 2.2. tRK3UAA - pre-MSK complex formation

Autoradiographic detection of the [^{32}P]-labelled-tRK3UAA-pre-MSK complex after native gel electrophoresis (A). Upper retarded band corresponds to the complex; lower band corresponds to the free [^{32}P]-labelled T7-transcript. Dependence of tRK3UAA-pre-MSK complex formation on the pre-MSK concentration (0, 05-0, 4 μM) is shown in panel (B).

II.1.1.3 Analysis of import into isolated human mitochondria

Next step was to verify whether recombinant tRNAs with altered aminoacylation identity elements preserved their ability to be imported into isolated mitochondria of human cells. For this, the tRNA *in vitro* import system developed in our laboratory (Entelis et al., 2001a; Tarassov and Entelis, 1992) was used (Fig. 2.3). It consists in the incubation of radioactively labelled tRNAs or corresponding T7-transcripts with isolated human mitochondria in the presence of human import directing proteins (hmIDPs) and ATP-regeneration system. After 30 min of incubation, the non-imported tRNAs are degraded by RNase treatment, while the imported tRNAs are protected by mitochondrial membranes. A signal corresponding to the full length tRNA on the autoradiograph was considered for quantification of import. Efficiency of import of a particular tRNA (in %) was estimated comparing to its input signal (usually 1% of radiolabelled tRNA added in the reaction). Normally, the import efficiency for tRK1 represents ~1-5% of its initial amount in the import mixture. Import mixture contained human import directing proteins (hmIDPs), but was also supplemented with recombinant yeast pre-MSK to increase tRNA import efficiency.

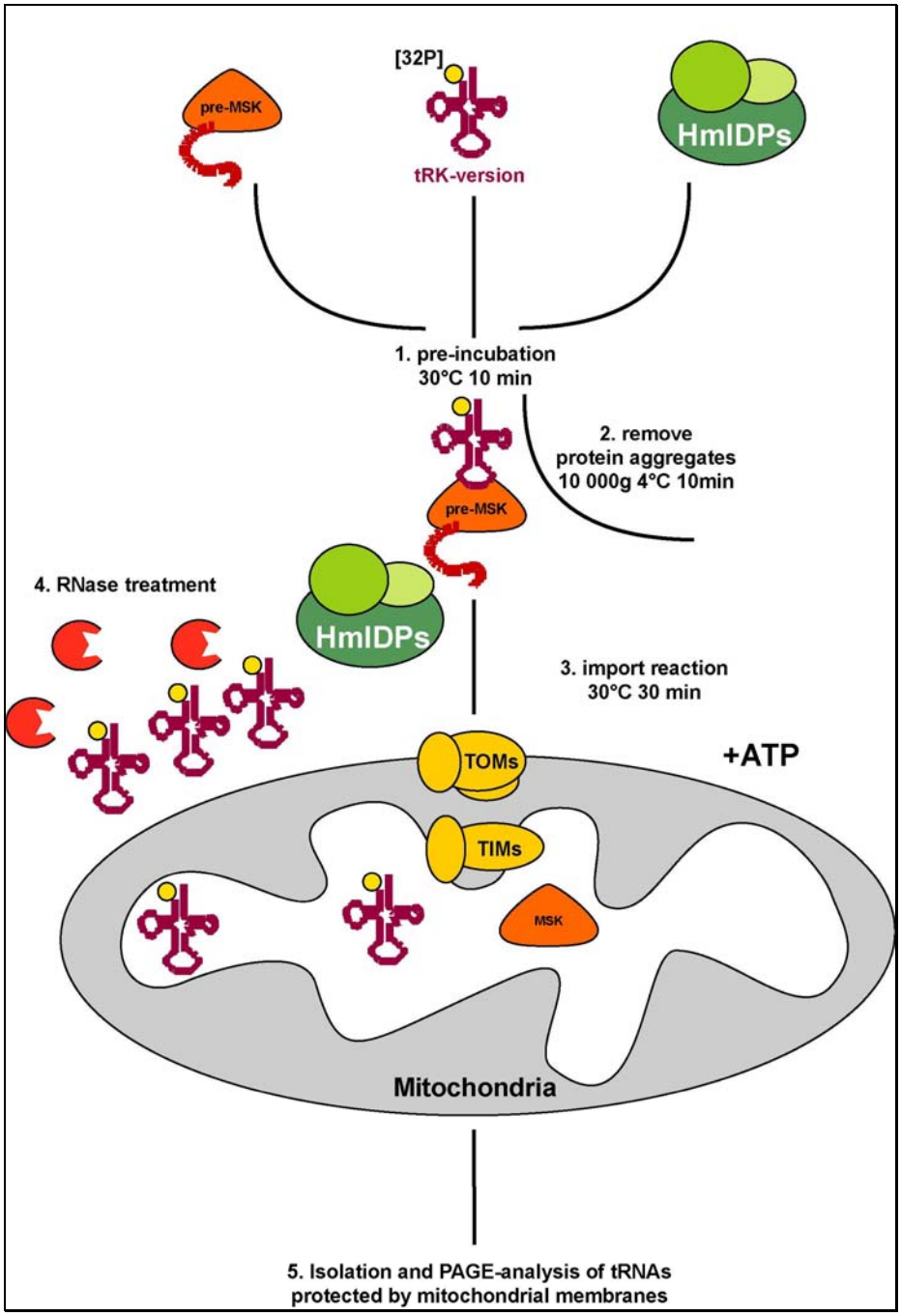

Fig. 2.3. Scheme of *in vitro* import assay (see text for details)

For certain preparations of hmIDPs we have observed partial protein aggregation resulting in non-specific protection of labelled tRNAs and by this, leading to artefactual signals. To avoid this effect, we performed a 10 min pre-incubation of import mixtures with all components except mitochondria, then pelleted unspecific protein aggregates by centrifugation, transferred supernatants into new tubes and initiated import reaction by adding mitochondria. Nevertheless, a control reaction without mitochondria was routinely performed in parallel,

and a value corresponding to the eventual signal without mitochondria was subtracted from values of import signal with mitochondria. tRK1 T7-transcript was used as a positive tRNA import control, and native tRK2 - as a negative non-imported control.

I found out that recombinant tRNAs, tRK3UAA and tRK3CAA, as well as wild type transcript of tRK3 were imported into isolated human mitochondria without any preliminary aminoacylation (Fig. 2.4A). Moreover, the efficiency of their import was comparable to that of tRK1 transcript aminoacylated by KRS. This indicates that, indeed, transcripts of tRK3UAA and tRK3CAA do not require aminoacylation to be imported into mitochondria, probably already being folded in a proper manner to interact with the pre-MSK (as shown in II.1.1.2). Nevertheless, one can not exclude the presence of some aminoacylation activity (lysine or leucine) in the import mixture, coming, for example, from hmIDPs, mitochondria, or even preparation of pre-MSK, which could be contaminated by *E. coli* aaRS. Several approaches have been used to solve this question.

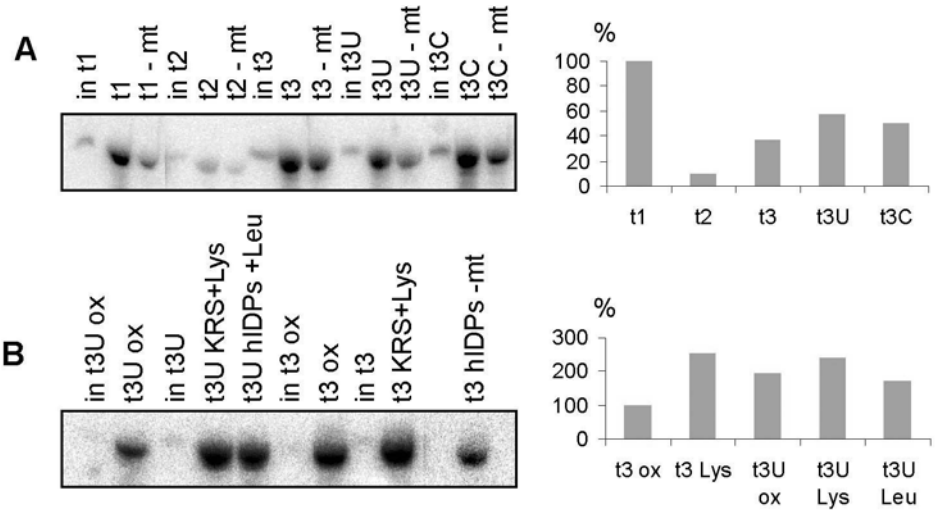

Fig. 2.4. Import of recombinant tRNAs into isolated human mitochondria

Each panel (A, B) consists of autoradiographic detection of [^{32}P]-labelled RNAs after denaturing gel electrophoresis (left) and a histogram showing import efficiencies of individual transcripts (right). Panel (A) illustrates import of T7-transcripts of recombinant tRNAs without pre-aminoacylation: t3 stands for tRK3, t3U - for tRK3UAA, t3C - for tRK3CAA; t1 KRS+Lys corresponds to tRK1 transcript pre-incubated with KRS and lysine (positive import control), t2 - to native tRK2 without pre-aminoacylation (negative import control). Input signal (in) corresponds to 1 % of a transcript used for import reaction, (-mt) stands for a control reaction without mitochondria. The import efficiencies of t2, t3; t3U, t3C are relative to those of t1 KRS+Lys taken as 100%. Panel (B) illustrates import of tRK3 and its recombinant versions in different conditions: (ox) stands for tRNA transcript with oxidized 3'-end, (KRS+Lys) – for pre-incubation with KRS and lysine, (hIDPs+Leu) – for pre-incubation with hIDPs and leucine. The import efficiencies are relative to those of t3 ox taken as 100%.

First, to confirm that import of recombinant tRNAs, indeed, do not require aminoacylation, I performed periodate oxidation of 3'-end of tRNA transcripts, known to prevent any further aminoacylation of tRNA. I compared import of these oxidized transcripts with the import of untreated transcripts upon pre-incubation with KRS and lysine (KRS + Lys), and upon pre-incubation with hmIDPs and leucine (hmIDPs + Leu). Efficient import of oxidized transcripts was observed, which supported the previous result and clearly demonstrated that tRK3 and its mutant versions can be addressed into isolated human mitochondria in deacylated form (Fig 2.4B). The impact of aminoacylating conditions remained less clear. Thus, import of tRK3UAA was not significantly improved by hmIDPs + Leu, or KRS + Lys pre-incubation. No import improvement upon KRS + Lys pre-incubation was expected, since cytoplasmic KRS normally does not aminoacylate mitochondrial tRK3 (S. Dogan, unpublished data). Nevertheless, the import of tRK3 wild-type transcript increased almost three times upon KRS + Lys pre-incubation, comparing to oxidized tRK3. This paradox could be only explained by presence in the import mixture of lysinylation activity from the other source. Therefore, I have verified the hmIDPs fraction used for import experiments for aminoacylation activity *in vitro*, and found out that it could incorporate [^{3}H]-Lys in total beef liver RNA, suggesting the presence of hcytLysRS, or less probably hmtLysRS. However, both human enzymes were previously shown to aminoacylate both cytoplasmic and mitochondrial human $tRNA^{Lys}$ transcripts (Tolkunova et al., 2000). Thus, the observed improvement of tRK3 import efficiency upon presence of lysine does not look absurd. The absence of import increase of tRK3UAA indicates, in this case, onto its inability to be aminoacylated with lysine. On the other hand, no effect of hmIDPs + Leu incubation can be explained by the absence of leucinylation activity in hmIDPs, which has been shown by *in vitro* aminoacylation test. Previously it has been shown that tRK1 must be aminoacylated prior to import into isolated human mitochondria, and aminoacylation can be performed by yeast KRS, as well as by isolated mammalian aminoacyl-tRNA synthetase (aaRS) complex (Entelis et al., 2001a). Nevertheless, the nature of amino acid is not that important (Kolesnikova et al., 2000) and moreover, some mutant transcripts of tRK1 and tRK2, were shown to be efficiently imported into human mitochondria without aminoacylation (Kolesnikova et al., 2002), whereas tRNAs with low or absent pre-MSK binding were never imported. Suggesting that aminoacylation is required to induce conformational change in the tRNA and to facilitate its binding to pre-MSK or its human analogue, which is really essential for import.

Aminoacylation specificity of recombinant tRNAs will be further investigated and discussed in more details.

II.1.1.4 Analysis of aminoacylation by human mitochondrial leucyl-tRNA synthetase

II.1.1.4.1 Purification of the recombinant enzyme

In order to efficiently participate in mitochondrial translation, recombinant tRNAs once imported into mitochondria should be aminoacylated with leucine. In human mitochondria, leucinylation is performed by mitochondrial leucyl-tRNA synthetase (hmtLeuRS). To verify if our recombinant tRNAs were able to be aminoacylate by hmtLeuRS, pET3a-hmtLeuRS expression plasmid was constructed. *E. coli* strain transformed with this plasmid was able to produce recombinant hmtLeuRS without mitochondrial targeting sequence and with six histidines (6-His) tag on its C-terminus. Predicted molecular mass of this recombinant protein was 101 kDa. I have tested several experimental conditions to purify active hmtLeuRs from *E. coli*. The highest production of recombinant protein was observed after inducing its expression at 30°C (Fig. 2.5A), but in this case, most of the protein accumulated in inclusion bodies (Fig. 2.5B) and was inactive in aminoacylation. Thus, I purified hmtLeuRS from *E. coli* native lysate after induction at 10°C. Recombinant protein was eluted from Ni-NTA column in 50 mM and 100 mM imidazole. The most of the recombinant protein was eluted in 50 mM imidazole, but it was contaminated with other proteins (Fig. 2.5C), whereas the minor portion of the protein, eluted in 100 mM imidazole, was more pure (Fig. 2.5D).

The aminoacylation activity of both 50mM and 100 mM elution fractions was then tested by *in vitro* aminoacylation assay. From previously published data, it is known that hmtLeuRS is able to aminoacylate mt-tRNA^{Leu} from a large spectrum of organisms, including yeast and even bacteria (Sohm et al., 2003). Thus, I have first tested the activity of different elution fractions on commercially available *E. coli* total tRNA. Aminoacylation activity was measured in cpm of incorporated [^{3}H]-Leu per μg of hmtLeuRS (cpm/ μg), while amount of hmtLeuRS in aminoacylation reaction was estimated by gel scanning and comparison with known amounts of ladder proteins. Both 50mM and 100 mM elution fractions contained active hmtLeuRS, while leucinylation activity was higher in more pure fractions (Fig. 2.6A) and apparently depended on the lower protein band, therefore being hmtLeuRS.

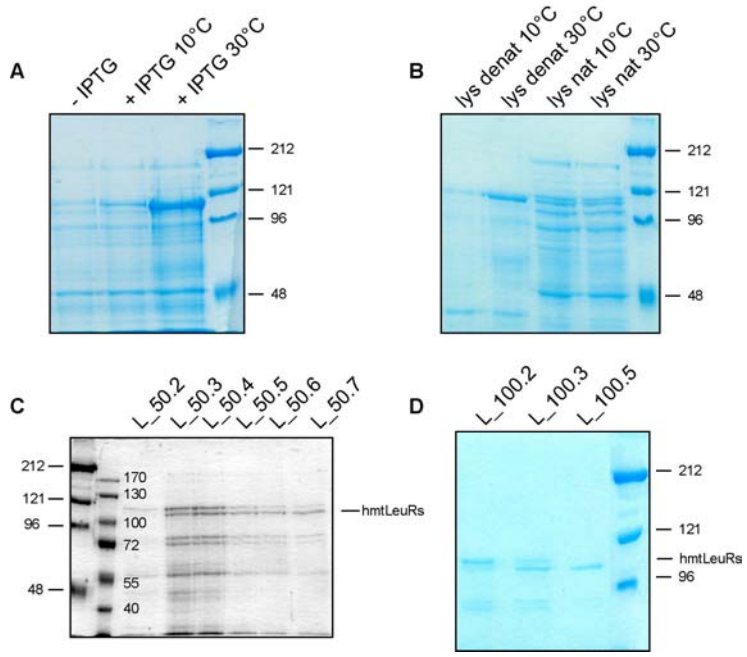

Fig. 2.5. Purification of recombinant hmtLeuRS

SDS-PAGE of (A) *E. coli* protein extract before (-IPTG) and after induction (+IPTG 10°C, +IPTG 30°C) of recombinant hmtLeuRS gene expression; (B) *E. coli* native (nat) and denatured (denat) protein lysates after induction at 10°C or 30°C; (C) fractions of hmtLeuRS eluted from Ni-NTA column in the presence of 50 mM imidazole (L_50.2-7); (D) fractions of hmtLeuRS eluted from Ni-NTA column in the presence of 100 mM imidazole (L_100.2, L_100.3, L_100.5).

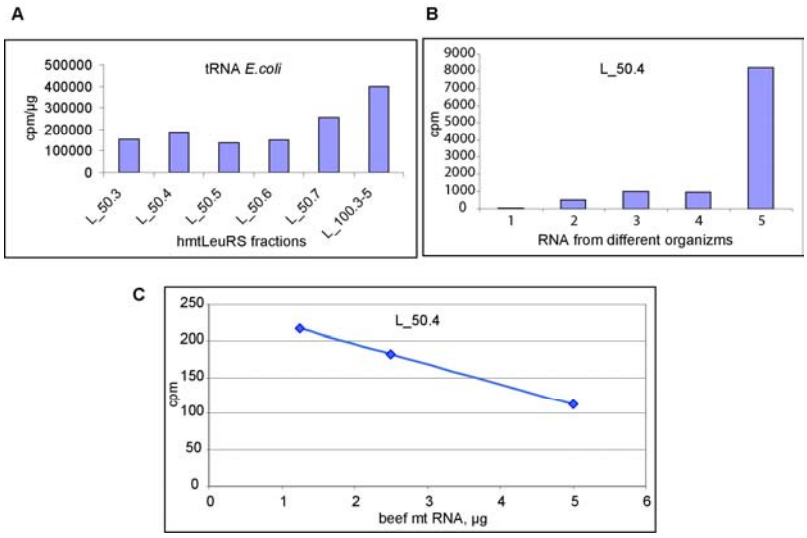

Fig. 2.6. Analysis of aminoacylation activity of recombinant purified hmtLeuRS

Aminoacylation activity of (A) different elution fractions on total *E. coli* tRNA; (B) fraction L_50.4 on RNA from different organisms (1 – high molecular weight fraction mtRNA from beef liver, 2 – low molecular weight fraction mtRNA from beef liver, 3 – total RNA from human cells, 4 – mtRNA from human cells, 5 – total tRNA from *E. coli*). (C) Dependence of aminoacylation efficiency on the amount of beef mtRNA of low molecular weight fraction. Aminoacylation reactions were performed for 15 min in the presence of 0.8 µM [^{3}H]-Leu, 5 µg of RNA preparations, and appropriate amounts of hmtLeuRS fractions.

The absence of leucinylation activity in protein fractions from *E. coli* without pET3a-hmtLeuRS expression plasmid proves that activity of recombinant protein fractions was due to the presence of hmtLeuRS and was not provided by any contaminating proteins of *E. coli.* Besides *E. coli* $tRNA^{Leu}$, recombinant hmtLeuRS was able to aminoacylate $tRNA^{Leu}$ from total or mitochondrial RNA from beef liver and cultured human cells (Fig. 2.6B). A low level of [^{3}H]-Leu incorporation in beef and human RNA maybe explained by their contamination with larger RNAs inhibiting aminoacylation (Fig. 2.6C).

Thereby, we isolated and purified the recombinant protein possessing the expected leucinylation activity of hmtLeuRS. This recombinant protein was further used to analyse aminoacylation of recombinant tRNAs.

II.1.1.4.2 Aminoacylation assays

For aminoacylation of recombinant tRNAs, I used hmtLeuRS from fraction L_100.3-5, as it was the most active and pure one. As a control, the T7-transcript corresponding to wild-type human mt-$tRNA^{Leu(UUR)}$ was used. Often tRNA transcripts missing post-transcriptional modifications are aminoacylated less efficiently than corresponding native tRNAs. For $tRNA^{Leu(UUR)}$ transcript this difference is about 30-45 times, and is explained by failure to fold in a correct tertiary structure (Park et al., 2003). Nevertheless, it was shown that increased Mg^{2+} concentration (from 3 mM to 12 mM) improves aminoacylation of the mt-$tRNA^{Leu(UUR)}$ transcript about 5 times, probably by stabilizing its tertiary structure (Sohm et al., 2003). Therefore, I performed aminoacylation of all transcripts in presence of 12 mM $MgCl_2$. Resulting kinetic parameters of aminoacylation of wild-type mt-$tRNA^{Leu(UUR)}$ and tRK3UAA transcripts by hmtLeuRS are shown in the Table 2.1.

Table 2.1. Kinetic parameters of hmtLeuRS for wild-type mt-$tRNA^{Leu(UUR)}$ and tRK3UAA transcripts

	K_M, μM	V_{max}, pmoles*10^{-3}/min	V_{max}/K_M [a]
Mt-$tRNA^{Leu(UUR)}$	2	13.8	1
tRK3UAA	2	11.8	0.86

a Relative, V_{max}/ K_M of tRNALeu(UUR) is taken as 1.00. Aminoacylation reactions were performed in the presence of 0.8 μM [^{3}H]-Leu, 5 μM of tRNA transcripts, and 100 nM of hmtLeuRS from L_100.3-5 fraction.

The tRK3UAA transcript was aminoacylated with leucine by recombinant hmtLeuRS with the efficiency comparable to that of the wild type human mt-tRNA$^{Leu(UUR)}$ transcript Therefore, one can conclude that aminoacylation specificity of tRK3UAA was successfully changed from yeast mitochondrial lysine to human mitochondrial leucine, at the same time preserving its ability to be imported into isolated human mitochondria (see II.1.1.3).

One should not that in cell, the recombinant tRNAs will be exposed not only to hmtLeuRS, but also to other aaRS, including lysyl-tRNA synthetases, both cytoplasmic and mitochondrial. Taking into account that recombinant tRNAs have yeast tRNA^{Lys} backbone (cytoplasmic for tRK1 and tRK2, and mitochondrial for tRK3), one can reasonably question if in human cells they would not be aminoacylated with lysine, in contrast with our expectation. Cytoplasmic and mitochondrial LysRSs in yeast are encoded by two distinct genes, while in human they are encoded by the same gene and are generated by alternative splicing (Tolkunova et al., 2000). *In vitro* aminoacylation study with tRNA transcripts and recombinant enzymes have shown that yeast cytoplasmic LysRS (KRS) did not aminoacylate mitochondrial lysine tRNA (tRK3). On the contrary, yeast mitochondrial LysRS (MSK) was aminoacylating mitochondrial tRK3 with the same efficiency as cytoplasmic tRK1 (S. Dogan, unpublished data). On the other hand, human cytoplasmic and mitochondrial LysRS were shown to aminoacylate both cytoplasmic and mitochondrial human tRNA^{Lys} transcripts (Tolkunova et al., 2000). Still, the efficiency of aminoacylation of mitochondrial transcript missing post-transcriptional modifications was very poor in both cases. The major identity elements for recognition by LysRS from different organisms, including yeast and human cytosolic and mitochondrial enzymes, are situated in the UUU anticodon (U35, U36) (Francin and Mirande, 2006; Kolesnikova et al., 2002; Sissler et al., 2004). Thus, tRNA transcripts bearing mutations in anticodon region are extremely poor substrates for human and yeast cytosolic LysRS, while a simple introduction of UUU codon in non-cognate tRNAs might bring them the lysine identity. All recombinant tRK1/2/3-based versions have leucine anticodons UAA or CAA, and therefore are very unlikely to be aminoacylated by any LysRS in human cells. Indeed, we have found that tRK3 and tRK3-based versions were not aminoacylated with lysine by KRS.

To resume this section, we have constructed recombinant tRNAs able to be imported into human mitochondria and be aminoacylated with leucine by hmtLeuRS *in vitro*. The next step was to verify if these recombinant tRNAs could be imported and aminoacylated into mitochondria of cultured human cells *in vivo*.

II.1.1.5 Analysis of mitochondrial targeting i*n vivo*

To study the behaviour of *in vitro* validated recombinant tRNAs in cultured human cells, an original approach was developed consisting in cell transfection with fluorescently labelled T7-transcripts (green fluorescence, Alexa Fluor 488) and their subsequent co-localization with the mitochondrial network (red fluorescence, MitoTracker Red) by means of fluorescent confocal microscopy. Subcellular localization of recombinant tRK3UAA was also compared with that of 5S rRNA, naturally imported into human mitochondria. Both transcripts have demonstrated a similar behaviour within the cells. They were already detectable in the cells 3h after transfection as individual green dots, most probably being RNA-Lipofectamine complexes (Fig. 2.7). After 26h, the amount of labelled T7-transcripts in cells significantly increased, however they were still presented in the form of large green dots without any obvious mitochondrial localization. The distribution of the green label drastically changed 76h after transfection; the amount of large green dots was reduced, and T7-transcripts were now mostly dispersed within the cell, displaying clear co-localization with the mitochondrial network. The co-localization coefficients (*c*) for 5S rRNA and tRK3UAA, calculated using data from the scatter diagrams were similar, 12 ± 1.4 % and 13 ± 3%, respectively, indicating on mitochondrial import of tRK3UAA.

The partial nature of co-localization was expected, since all the RNA substrates studied so far, including 5S rRNA, were never imported into mitochondria more than at 10% when compared to the total cellular pool. Therefore, the results obtained by co-localization studies appear to provide correct information about the import and validate the use of the approach. Nevertheless, the mitochondrial localization observed is a proof of RNA targeting toward mitochondria, but not of its translocation across mitochondrial membranes.

II.1.1.6 Analysis of mitochondrial import *in vivo*

It was then tested whether recombinant tRNAs were able to be imported inside mitochondrial matrix in human cells. For this, MELAS cybrid cells were transfected with corresponding T7-transcripts, total and mitochondrial RNA were isolated 24h after transfection and analyzed by Northern-hybridization with [^{32}P]-5'-labelled oligonucleotide probes (Fig. 2.8A).

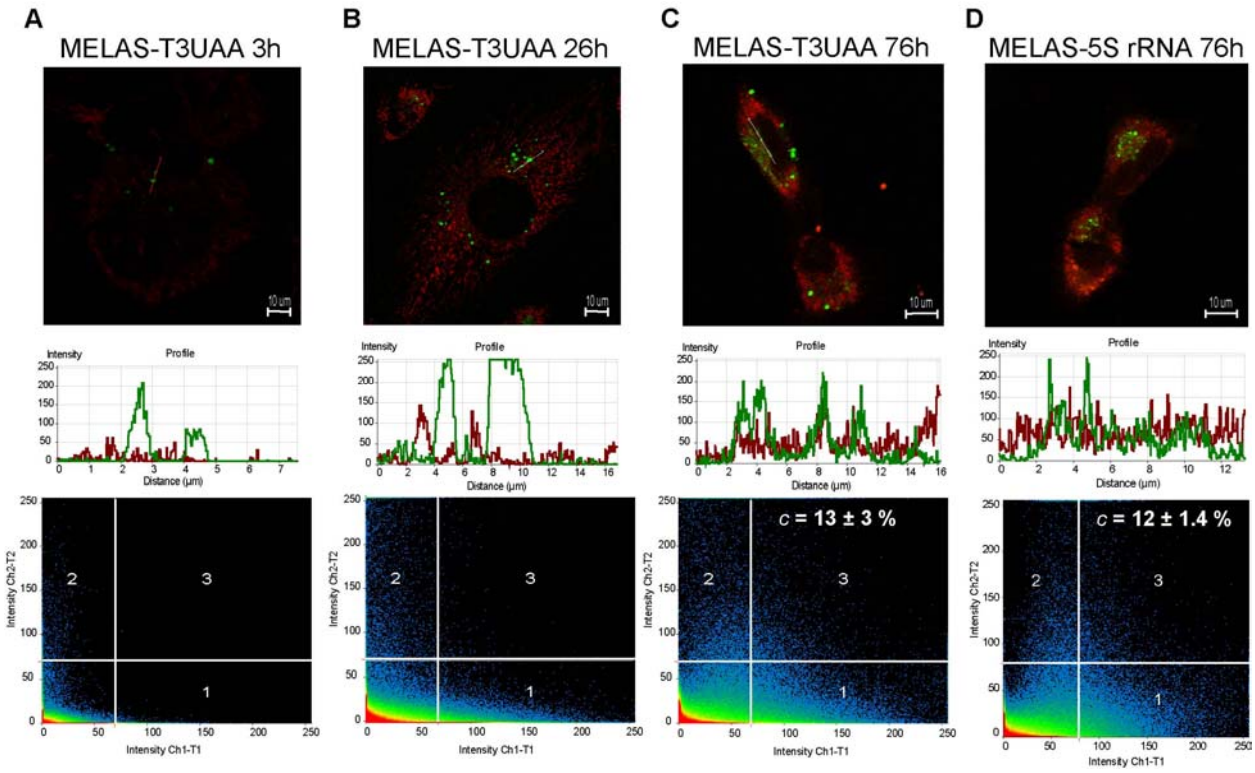

Fig. 2.7. Co-localization of tRK3UAA and 5S rRNA with mitochondria in human cells

Panels A-C correspond to MELAS cells transfected with tRK3UAA and analyzed 3h, 26h and 76h after transfection, respectively; panel D corresponds to MELAS cells transfected with 5S rRNA import control and analyzed 76h after transfection. In each of A-D panels top to bottom are as follows: merged microscopy images of the cell in green (RNA) and red (mitochondria) channels; the profile describing the intensity of both signals within the line of a given distance (shown on the photograph); and the scatter diagram, where the 3rd region corresponds to pixels having the same positions in both images and thus considered as co-localized. Microscopy images and data are presented as typical ones for each of the RNAs, while the co-localization coefficients (*c*) were calculated using the data from minimum 10 individual cells.

The efficiency of import was calculated as a ratio of a specific hybridization signals in mitochondrial RNAs *versus* total RNA, and was relative to the efficiency of import of tRK1 or tRK3 transcript taken as 100% in each case. All the synthetic tRNAs tested were found to be imported into mitochondria of MELAS cybrid cells *in vivo*. On the other hand, different T7-transcripts were imported into mitochondria with various efficiencies (Fig. 2.8B). Thereby, mutations introduced in the anticodon region of tRK3UAA (U35:A35, U36:A36) and tRK3CAA (U34:C34, U35:A35, U36:A36) did not significantly reduce efficiency of their import, while substitutions made in tRK1UUA (U73:A73, C34:U34, U35:A35, U36:A36) and tRK1CAA (U73:A73, U35:A35, U36:A36) decrease their import 3 and 5 times, respectively, compared to tRK1 without mutations.

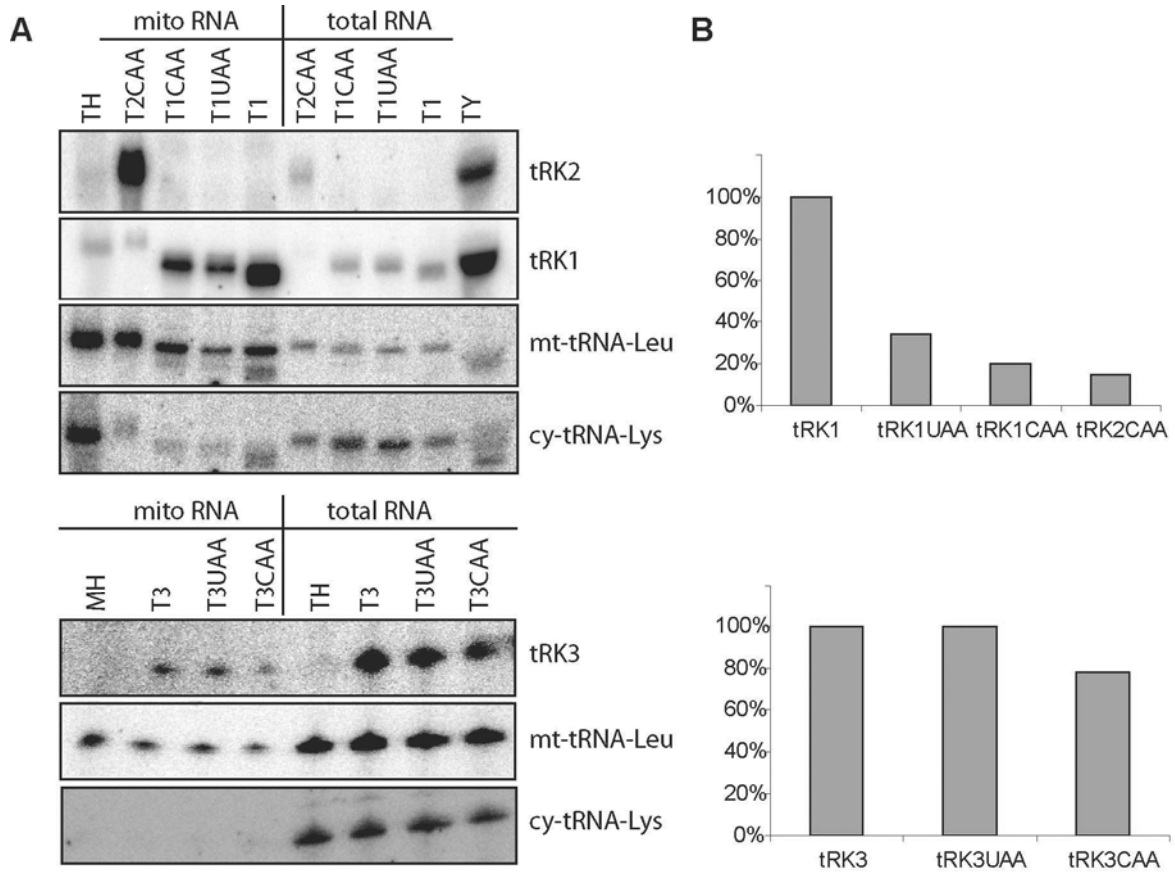

Fig. 2.8. Analysis of *in vivo* import of recombinant tRNAs in mitochondria of MELAS cybrid cells

(A) Northern-hybridization of total and mitochondrial RNA isolated from MELAS cybrid cells transfected with T7-transcripts. Specific [^{32}P]-oligonucleotide probes to tRK1, tRK2, and tRK3 were used to check for mitochondrial import, mt-tRNA-Leu probe - to control the absence of degradation of mitochondrial tRNAs and to cy-tRNA-Lys probe to control the absence of its contamination by cytosolic tRNAs (the probes are indicated at the right of the panels). Minor bands visible with tRK1 probe in tRK2-transfectants and with cy-tRNA-Lys probe in tRK1 and tRK2 ones represent the unwashed traces of previous hybridizations of the same membrane and are unspecific (they do not migrate as the cognate tRNAs and therefore do not alter any interpretation of the specific signals). TH and MH are total and mitochondrial RNAs from non-transfected MELAS cells, TY is total yeast RNA preparation used as the control of hybridization specificity. T1 and T3 are the transcripts of yeast tRK1 and tRK3 without mutations used to estimate the import efficiency of recombinant tRNAs (B) The import efficiency of the T7-transcripts, calculated as a ratio of a specific hybridization signal in mitochondrial RNAs to the total RNAs and relative to the efficiency of import of tRK1 or tRK3 taken as 100% in both cases.

Import efficiency of tRK2CAA was here estimated comparing to tRK1 transcript, since the wild-type tRK2 is not imported into mitochondria, and its efficiency of import was approximately 7 times lower compared to tRK1.

To verify if recombinant tRNAs were aminoacylated *in vivo*, the analysis using the acid gel method was performed (Varshney et al., 1991). Aminoacylation state of tRK1, tRK1UAA and tRK1CAA transcripts was analyzed both in total and mitochondrial RNA

preparations (Fig. 2.9A), while analysis of tRK3, tRK3UAA and tRK3CAA aminoacylation was done using only total cellular RNA fraction (Fig. 2.9B). In any case, T7-transcripts of tRK1, tRK3 and their recombinant leucine versions tRK1UAA/CAA and tRK3UAA/CAA were found to migrate in the gel as single bands corresponding to deacylated forms of yeast tRK1 and tRK3, respectively. In contrast, native cytoplasmic ($tRNA^{Lys}$) and/or mitochondrial ($tRNA^{Leu}$) tRNAs from the same preparations were found to be correctly aminoacylated.

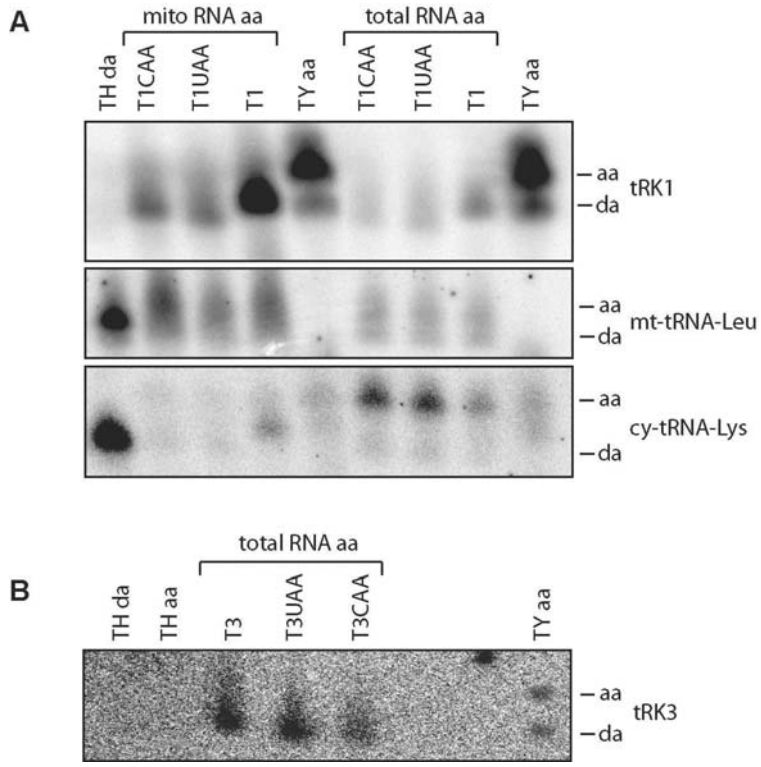

Fig. 2.9. Analysis of aminoacylation of synthetic recombinant tRNAs *in vivo* in transfected MELAS cybrid cells

Northern-hybridization of mitochondrial and/or total RNA isolated in acid conditions preserving aminoacylation of tRNAs (RNA aa) from MELAS cybrid cells transfected with recombinant T7-transcripts of tRK1 (A) and tRK3 (B). T1 and T3 are the transcripts of yeast tRK1 and tRK3 without mutations. Total yeast acid RNA (TY aa) and total human acid (TH aa) and deacylated RNA (TH da) preparations are used to control the migration level of aminoacylated and deacylated forms of tRNAs (shown as "aa" or "da" at the right of the panels). Use of specific [^{32}P]-oligonucleotide probes indicated at the right of the panels is described above, in the legend to Fig. 2.7. Minor bands visible with cy-tRNA-Lys probe in T1 and TY aa lanes represent the unwashed traces of previous hybridizations of the same membrane and are unspecific (they do not migrate as the cognate tRNAs and therefore do not alter any interpretation of the specific signals).

Thus, one can conclude that tRNAs obtained by T7-transcription, including wild-type tRK1 and tRK3, are present in the human cells essentially in deacylated form. On the other hand, yeast wild-type lysine tRK1 and tRK3 are expected to be at some extent aminoacylated in

human cells, taking into account described similarities of yeast and human lysinylation systems and the flexibility of the last one regarding cytoplasmic and mitochondrial tRNA substrates (Francin and Mirande, 2006; Kolesnikova et al., 2002; Sissler et al., 2004). One could explain the absence of aminoacylation of T7-transcripts by the fact that tRNAs synthesized *in vitro* lack post-transcriptional modifications needed for proper aminoacylation *in vivo* and/or are disadvantaged in competition with native tRNAs for cognate aaRS. In order to counter this problem, we decided to express recombinant tRNAs from mutated genes in MELAS cybrid cells.

To resume this section, we constructed a set of recombinant tRNAs on the basis of yeast $tRNAs^{Lys}$ where identity elements for hmtLeuRS were introduced, and we demonstrated that these tRNAs retained their capacity be imported into human mitochondria, *in vitro* and *in vivo*.

II.1.2 Characterization of the used MELAS cybrid cell lines

In this section I describe our attempts to use the recombinant tRNAs for their curative effect on the consequences of m.3243A>G MELAS mutation. As a first step, it was important to choose a proper cellular model and reliable parameters of mitochondrial function.

*Trans*mitochondrial cybrid cell lines are widely used cellular models of human disorders caused by mutations in mitochondrial DNA. They are obtained by the fusing mtDNA depleted (rho^0) immortalized cells (usually osteosarcoma 143B or HeLa cells) with cytoplasts (enucleated cells bearing mitochondria with mutant mtDNA) from patients (King and Attardi, 1989).

To examine the possible rescue of mitochondrial functions of MELAS cells by recombinant leucine tRNAs, we used two 143B-based cybrid cell lines bearing 85 ± 5% and 90 ± 5% of m.3243A>G MELAS mutation in the mt-$tRNA^{Leu(UUR)}$ gene (*MT-TL1*). Taking into account that there is no clear and generally accepted picture of molecular mechanisms of MELAS mutation, and cybrid cells properties vary depending on cell line studied, I performed an exhaustive analysis of these two lines. Namely, I have tested mt-$tRNA^{Leu(UUR)}$ stability and aminoacylation level, mitochondrial translation, steady state level of several mtDNA-encoded protein subunits of respiratory chain, enzymatic activities of Complex I and Complex IV, cell respiration and mitochondrial membrane potential. Both MELAS cell lines analysed revealed

similar defects in the above-listed parameters comparing to 143B cells bearing 0% of m.3243A>G MELAS mutation.

II.1.2.1 Mt-tRNA$^{Leu(UUR)}$ steady-state and aminoacylation levels

The Northern analysis of total RNA revealed that steady-state level of mt-tRNA$^{Leu(UUR)}$ in MELAS cybrid cells was 35 ± 10 % of its control level in 143B cells (Fig. 2.10A, B), whereas steady state level of other mitochondrial tRNAs (for example, mt-tRNA^{Val}) was not affected.

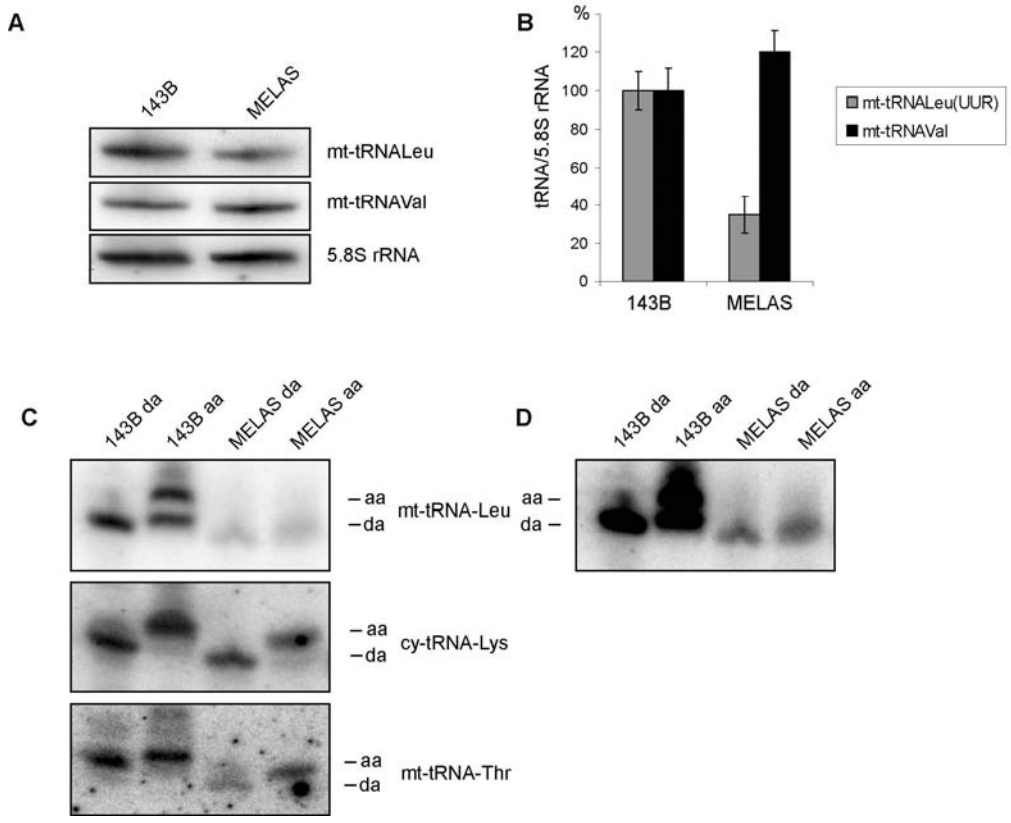

Fig. 2.10. Analysis of steady-state and aminoacylation levels of mt-tRNA$^{Leu(UUR)}$ in MELAS cells

(A) Northern-hybridization of total RNA isolated from MELAS cybrid cells and 143B control cells with specific [^{32}P]-oligonucleotide probes to mt-tRNA$^{Leu(UUR)}$, mt-tRNA^{Val} and 5.8S rRNA as indicated at the right of the panels. (B) Levels of mt-tRNA$^{Leu(UUR)}$ and mt-tRNA^{Val} normalized *versus* 5.8S rRNA in MELAS cybrid cells and in 143B control cells (taken as 100%) (C) Northern-hybridization of total RNA isolated from MELAS cybrid cells and 143B cells in acid conditions preserving aminoacylation of tRNAs (143B aa and MELAS aa) and after deacylation (143B da and MELAS da). Specific [^{32}P]-oligonucleotide probes used, aminoacylated (aa) and deacylated (da) forms of corresponding tRNAs are indicated at the right of the panel. The autoradiograph in (D) represents the longer exposure of the corresponding filter in (C).

Further analysis of tRNA aminoacylation *in vivo* by PAAG electrophoresis at acid pH has shown that mt-tRNA$^{Leu(UUR)}$ was almost completely deacylated in MELAS cells, while other mitochondrial (mt-tRNA^{Thr}) and cytoplasmic (tRNA^{Lys}) tRNAs were aminoacylated normally (Fig. 2.10C, D). The results obtained are in agreement with the data published previously on MELAS cybrid cells (Chomyn et al., 2000; El Meziane et al., 1998; King et al., 1992; Park et al., 2003) as well as on tissue samples of MELAS patients (Borner et al., 2000), and indicate on implication of mt-tRNA$^{Leu(UUR)}$ aminoacylation defect in molecular consequences of m.3243A>G mutation in cybrid cells used in this study. Although the decrease of mt-tRNA$^{Leu(UUR)}$ steady-state and aminoacylation levels was reproducible in MELAS cells, it was unlikely to change upon the presence of recombinant leucine tRNAs, and therefore could not be used to evaluate possible curative effect of the last ones.

II.1.2.2 Analysis of mitochondrial translation

I have further tested MELAS cells for translation of mtDNA-encoded proteins by a pulse-chase assay in the presence of [^{35}S]-methionine and upon conditions inhibiting cytosolic protein synthesis (Fig. 2.11).

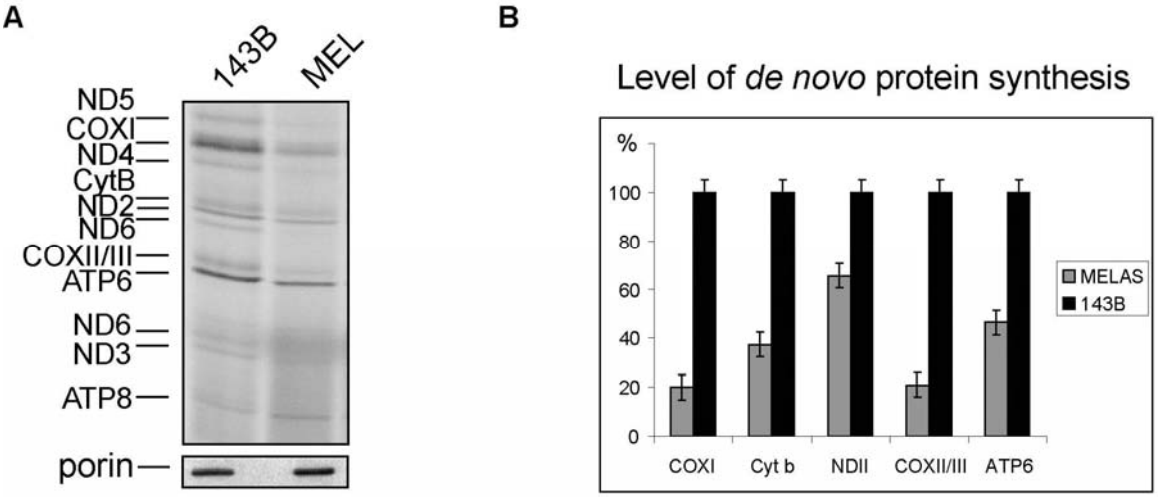

Fig. 2.11. Mitochondrial translation analysis in MELAS and 143B cells

(A) Pulse-chase analysis of mitochondrial translation in MELAS cybrid cells comparing to 143B cells. Radioautograph of [^{35}S]-labelled mitochondrial translation products separated by SDS-PAGE is presented. The bands corresponding to individual translation products are indicated according to a standard pattern (Enriquez et al., 2000). Equal amounts of proteins were loaded in each case, which was controlled by porin Western analysis (below the main panel). (B) The diagram shows the levels of individual translation products in MELAS cells relative to corresponding levels in 143B cells (taken as 100%).

In agreement with previous reports (Chomyn et al., 1992; King et al., 1992), MELAS cybrid cells showed reproducibly decreased mitochondrial protein synthesis, with COXI, II and III polypeptides being particularly affected, but the qualitative pattern of mitochondrial translation products was unchanged, *i.e*. no specific abnormally migrating bands were detected. Furthermore, no correlation was observed between the number of UUR codons and translation level of particular polypeptides. In any case, the difference in mitochondrial translation efficiency between MELAS and 143B cells was evident and, thus, the effect of recombinant leucine tRNAs on its level could be used as a criterion of complementation.

II.1.2.3 Analysis of steady-state levels of mtDNA-encoded proteins

In order to verify if mitochondrial translation defect had any consequences on steady-state levels of mitochondrial proteins, I performed immunocytochemical analysis of MELAS cells with antibodies against two mtDNA-encoded subunits, NDI and COXII, of Complex I and Complex IV, respectively. The majority of MELAS cybrid cells were found to be COXII (~90%) and NDI (~99%) negative, comparing to 143B cells (Fig. 2.12).

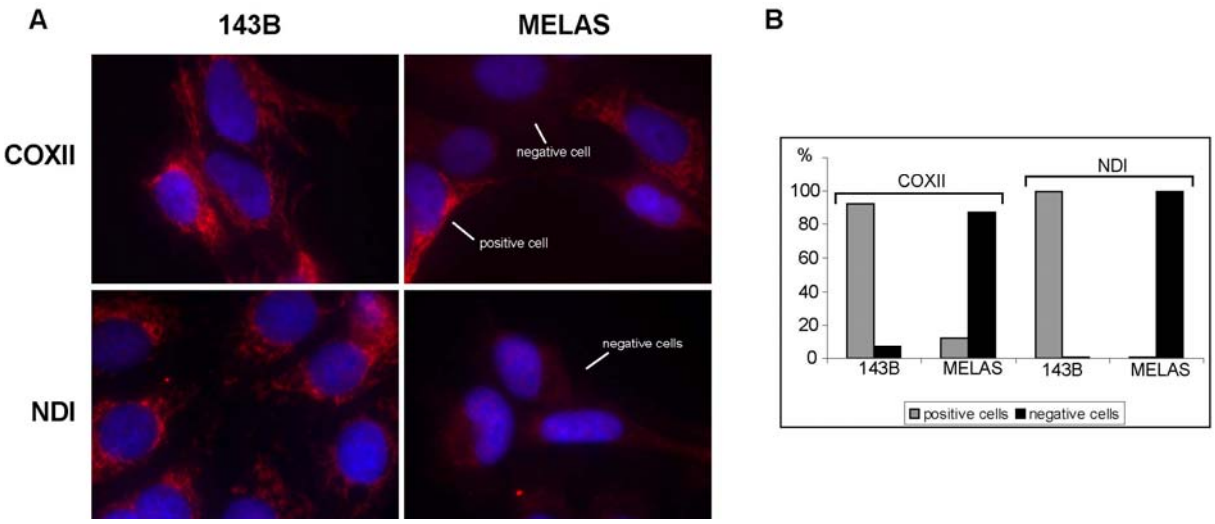

Fig. 2.12. Immunocytochemical detection of COXII and NDI in MELAS and 143B cells

Typical microscopy images of 143B and MELAS cells coloured with antibodies against COXII or NDI are shown in panel (A), where red signal corresponds to mitochondrial COXII or NDI proteins as indicated, and blue signal corresponds to cell nuclei coloured with DAPI. Quantification of COXII/NDI positive and negative cells (B) was performed by counting at least 100 cells of each type. MELAS cybrid cells with the same intensity of red fluorescence as that in 143B cells were considered as positive, and those with lower unspecific fluorescence – as negative.

This was also confirmed by Western blot analysis (Fig. 2.13). Steady-state levels of NDI and COXII proteins in MELAS cells represented 16% and 20%, respectively, of their levels in control 143B cells. In addition, COXI protein was almost undetectable in MELAS cybrid cells.

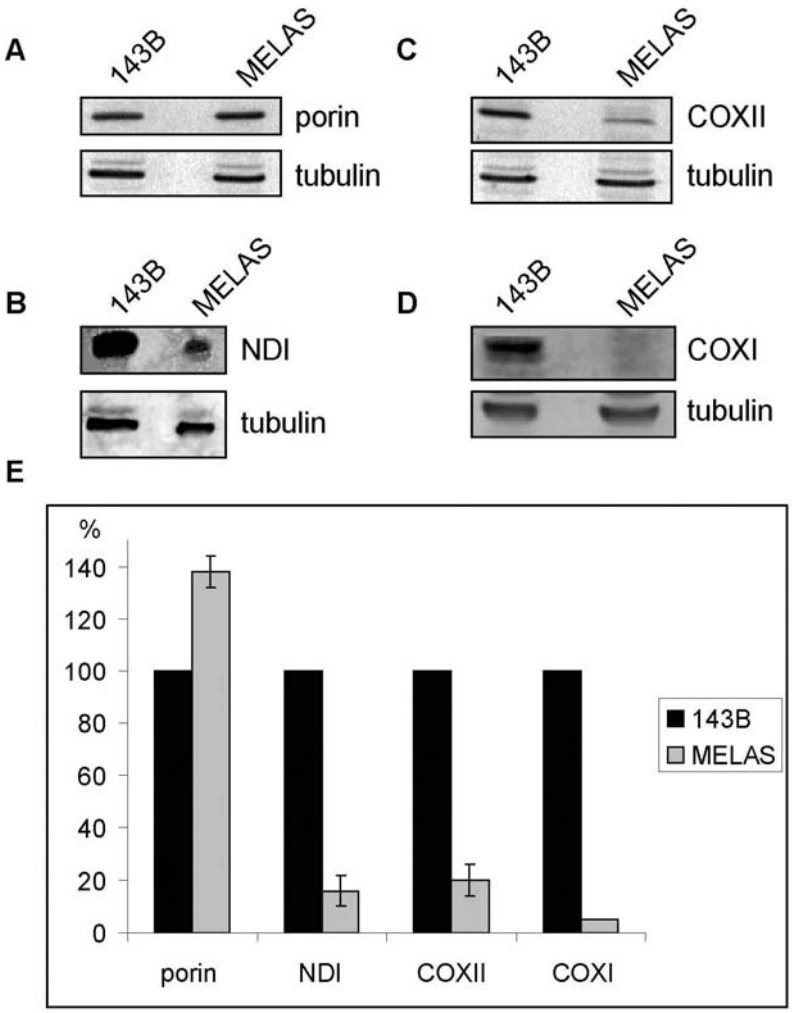

Fig. 2.13. Analysis of steady-state levels of mtDNA encoded proteins in MELAS and 143B cells

Western analysis of protein extracts from MELAS cybrid cells and 143B cells for porin (A), NDI (B), COXII (C) and COXI (D); anti-tubulin antibodies were used as a quantification reference. The diagram (E) shows the steady-state levels of porin, NDI COXII and COXI in MELAS cells, normalized to tubulin, and relative to level of corresponding proteins in 143B parental cells, taken as 100%. Error bars correspond to the results of 2 to 5 independent experiments.

II.1.2.4 Analysis of enzymatic activity of respiratory chain complexes

The extent of the drop in activity of mitochondrial complexes reported previously was variable and, moreover, not always correlated with heteroplasmy level (Obermaier-Kusser et al., 1991). In cybrid cells with more than 70% of m.3243A>G mutation the Complex I activity was reported in the range of 20-70%, and Complex IV activity - in the range of 2-70% of that of control cells (Dunbar et al., 1996; Flierl et al., 1997; van den Ouweland et al., 1999).

Therefore, I analyzed the used MELAS cybrid cells for the enzymatic activities of Complex I and Complex IV, respectively. First, MELAS cells and control 143B cells were compared using double cytochemical staining for mtDNA-dependent COX activity and mtDNA-independent SDH activity (succinate dehydrogenase or Complex II). MELAS cell population was found to be very heterogeneous, containing both COX positive brown cells and COX negative cells, in which blue coloration for SDH activity became visible. The population of 143B cells was COX positive at 100% (Fig. 2.14). As a matter of fact, the

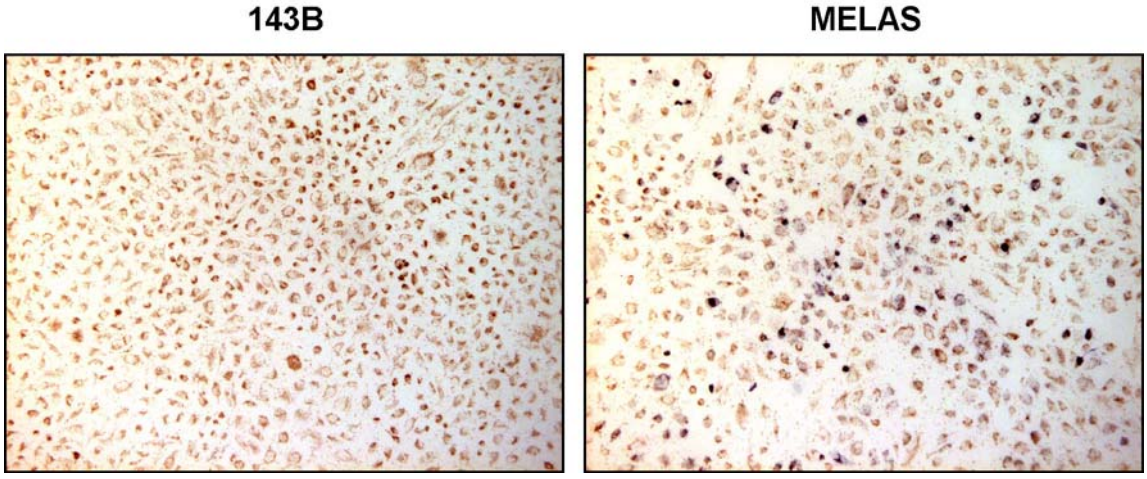

Fig. 2.14. COX/SDH staining in MELAS and 143B cells

Typical microscopy images of 143B and MELAS cells coloured for COX (brown) and SDH (blue) activities. COX-positive cells are brown, COX-negative cells are blue.

heterogeneity of MELAS population for certain parameters of mitochondrial function, including Complex IV activity, was already reported previously (Bakker et al., 2000). Moreover, it was in agreement with the results of immunocytochemical analysis (see II.1.2.3) showing that 10% of MELAS cell population was positive for COXII protein subunit. Although, both approaches gave similar results, they had mostly qualitative character. Therefore, it was interesting to evaluate the activity of respiratory chain complexes in a more

quantitative manner by spectrophotometry. Thus, I compared MELAS cells and 143B cells for Complex I, Complex IV and citrate synthase (CS) activities as described in Materials and Methods (Table 2.2). The experiments showed that activity of Complex IV was strongly decreased (to 13%) in MELAS cybrid cells, comparing to 143B cells. Complex I activity was decreased less, but still significantly (to 70%).

Table 2.2. Mitochondrial enzyme activities in MELAS and 143B cells

Enzyme	143B	MELAS
Citrate synthase (CS)	14.7	13.2
Complex I	13.1	7.5 (70%)
Complex IV	52.5	5.4 (13%)

The activities of mitochondrial enzymes are expressed as mU (nmol/min) per mg of total cell protein, and in parentheses as a percentage of activities in 143B cells, normalized against CS activity.

II.1.2.5 Analysis of cell respiration

Substrate dependent respiration in MELAS and 143B cells was compared. Digitonin was used to remove the permeability barrier of the plasma membrane and to open access to mitochondria for different respiratory substrates. The mixture of pyruvate and malate was used to induce electron transport by respiratory chain from NADH to O_2 through Complex I; succinate was used to donate electrons at Complex II, bypassing Complex I; and reduced cytochrome *c* – to donate electrons directly at Complex IV level. Maximal respiration rates were measured in the presence of FCCP uncoupler and oligomycin to obtain information on the ability of cells to regulate respiratory chain in response to a decrease of H^+ gradient, condition simulating high ATP production by ATP synthase. As expected, MELAS and 143B cells have similar succinate-supported respiration rates on both coupled and uncoupled mitochondria (Table 2.3). In a contrast, there was a substantial decrease in both coupled and uncoupled respiration rates through complexes I and IV. Therefore, the defects in the respiratory chain complexes bearing mtDNA-encoded subunits dramatically affected the respiratory chain in MELAS cybrid cells, which was in agreement with previously published data (James et al., 1996). The degree of mitochondrial coupling, measured as a ratio of uncoupled to coupled respiration rates (respiration control), was decreased when pyruvate/malate was used, which was not the case for cytochrome *c*–supported respiration.

This can be explained by the fact that Complex I is normally the main entry of electron-transport chain in human mitochondria, and therefore its malfunctioning has more impact on cell respiration than that of Complex IV.

Table 2.3. Substrate dependent respiration rate in digitonin-permeabilized MELAS cybrid cells comparing to 143B cells.

Cell type	Complex	Respiratory substrate	Coupled respiration rate (nmol O_2/min per mg of cell protein)	Uncoupled respiration rate (nmol O_2/min per mg of cell protein)	Uncoupled/coupled ratio
143B	I	pyr/mal	5.3 ± 1.2	11.2 ± 3.0	2.1 ± 0.1
MELAS	I	pyr/mal	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
143B	II	suc	2.9 ± 0.3	5.7 ± 0.3	2.0 ± 0.8
MELAS	II	suc	2.7 ± 0.9	5.6 ± 1.6	2.1 ± 0.1
143B	IV	cyt *c* red	4.2 ± 0.1	6.6 ± 0.1	1.6 ± 0.1
MELAS	IV	cyt *c* red	1.4 ± 0.6	2.9 ± 1.1	2.2 ± 0.2

Mitochondrial oxygen consumption was measured on digitonin-permeabilized cells using pyruvate/malate (pyr/mal), succinate (suc) or reduced cytochrome *c* (cyt *c* red). Uncoupled and coupled respiration rates, and their ratios are presented as the means ± SD of three independent experiments.

II.1.2.6 Analysis of mitochondrial membrane potential

Electrochemical potential on mitochondrial inner membrane (ΔΨ) is formed by H^+ gradient ($\Delta\mu H^+$) generated by electron transport chain (complexes I-IV) and ΔpH between mitochondrial matrix and cytosol. Among other roles, ΔΨ is required for ATP production by ATP synthase. To determine whether the respiration defect in MELAS cells affect mitochondrial energization, I have used two different types of mitochondria-targeted fluorescent dyes, ΔΨ-dependent TMRM (tetramethyl rhodamine methyl ester) and ΔΨ-independent Mitotracker Green. Stained MELAS and 143B cells were further analyzed both by confocal fluorescent microscopy and fluorescence activated cell sorting (FACS) (Fig.2.15). The cell populations of both types revealed some heterogeneity with the three types of cells observed: one with intensive red colour, one with less intensive yellow/orange colour, and one with green colour. The cells of last type were observed only in MELAS cybrid population, while the majority of cells were of yellow/orange colour and few cells were bright red. In 143B population most of cells were intensively red and no green cells were detected. Further analysis of MELAS cells by FACS has shown that 47.3% of cell

population had energized mitochondrial membranes binding TMRM, while 52.3% of cells were coloured solely by Mitotracker Green, and remaining 0.4% had no staining.

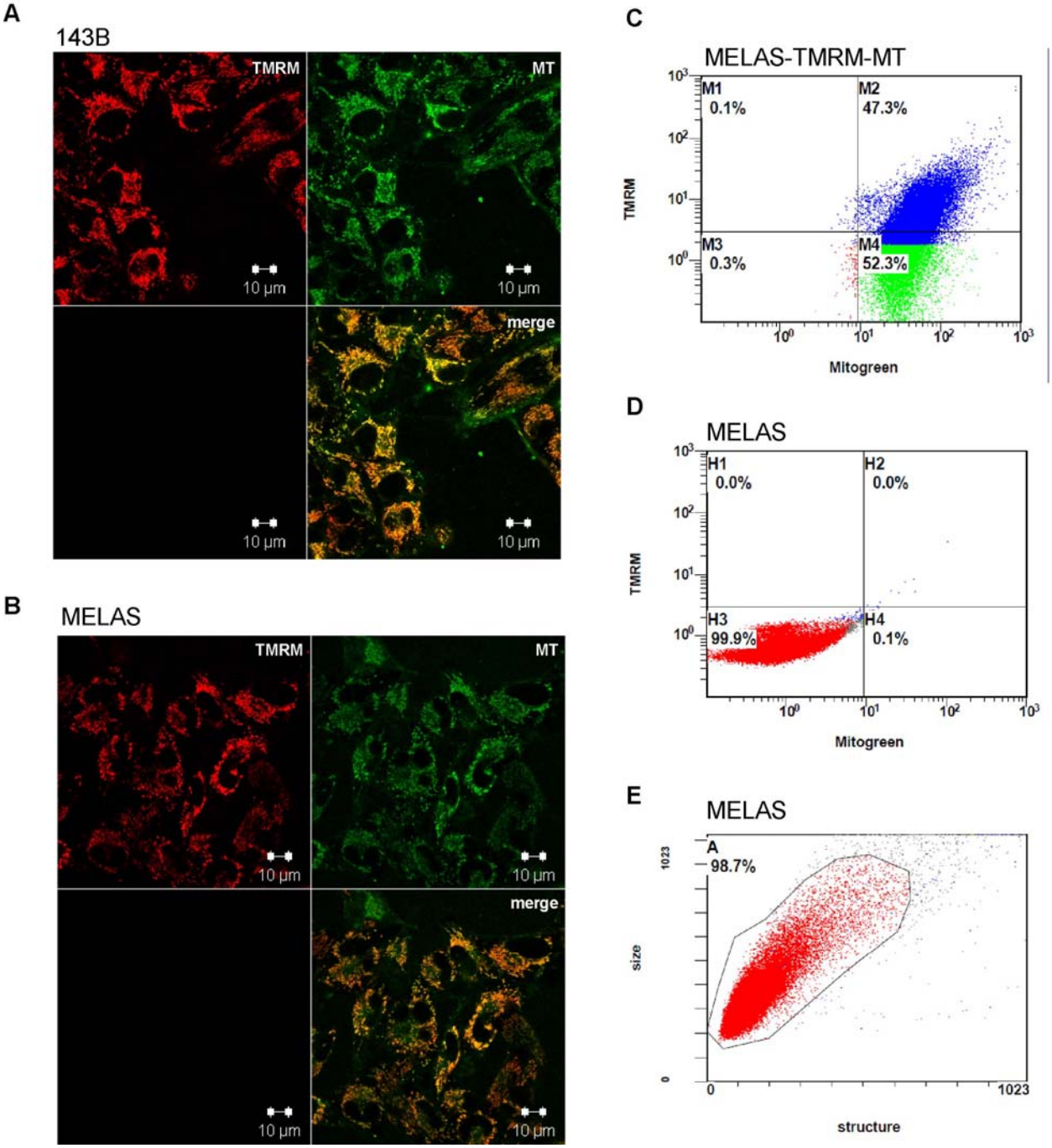

Fig. 2.15. Analysis of mitochondrial membrane potential (ΔΨ) in MELAS and 143B cells

Confocal microscopy images of 143B (A) and MELAS cells (B) coloured with ΔΨ-sensitive tetramethyl rhodamine methyl ester (TMRM, red signal) and Mitotracker Green FM (MT, green signal). FACS analysis of TMRM and MT fluorescence in coloured MELAS-TMRM-MT cells (C) comparing to uncoloured MELAS cells (D), where intensity of fluorescence is shown in relative values. FACS analysis of control MELAS cell population by size and structure (E).

II.1.2.7 Summary of comparative analysis of MELAS cybrid cells and parental 143B cells

Taken together (Table 2.4), our analysis of mitochondrial function of MELAS cybrid cells support one of the existing models of molecular mechanism of m.3243A>G MELAS mutation.

Table 2.4. Summary of the mitochondrial features of MELAS cybrid cells comparing to143B control cells.

	MELAS cybrids cells [a]
mt-tRNA$^{Leu(UUR)}$ steady-state level [b]	35 ± 10%
mt-tRNA$^{Leu(UUR)}$ aminoacylation state	deacylated
<u>Mitochondrial translation level</u> COXI Cyt *b* NDII COXII/III ATP6	 20 ± 6% 37 ± 4% 65 ± 7% 21 ± 4% 46 ± 4%
<u>Steady-state level of mtDNA-encoded proteins</u> NDI COXII COXI	 16% 20% undetectable
<u>Activity of respiratory chain complexes</u> Complex I [c] Complex IV [d]	 61% 13%
<u>Respiration</u> Complex I Complex II Complex IV	 deficient normal deficient
<u>Mitochondrial membrane potential (ΔΨ)</u>	affected ~50% of energized mitochondria

[a] compared to corresponding parameters in 143B control cells, whose values were taken as 100%; [b] normalized to 5.8S rRNA; [c] specific rotenone sensitive activity of Complex I normalized to citrate synthase (CS) activity; [d] normalized to CS activity.

Decrease in steady-state and aminoacylation levels of mt-tRNA$^{Leu(UUR)}$ might lead to a general mitochondrial translation defect, resulting in decrease of the mtDNA-encoded components of respiratory chain complexes, which, in turn, affects their enzymatic activity, and, finally, cell respiration rate and mitochondrial membrane potential.

Furthermore, the data obtained permitted to choose several the most reliable parameters of mitochondrial function in order to test the possible curative effect of recombinant leucine tRNAs, namely mitochondrial translation level, steady-state level of mtDNA-encoded proteins and respiration rate.

II.1.3 Restoration of mitochondrial function in MELAS cybrid cells by tRNA import into mitochondria

Once MELAS cybrid cell line has been characterized and mitochondrial functions affected by m.3242A>G mutation have been identified, we performed a number of experiments in order to test possible curative effect of mitochondrially imported recombinant tRNAs. To analyze mature modified recombinant tRNAs, corresponding mutated genes were expressed in MELAS cybrid cells. First, the effect of different leucine versions of tRK1/2/3 was compared in a transient transfection system, where the 2-3 times increase of COXII mitochondrial protein was observed, depending on tRK-version expressed. In this case, the restoration of COXII level in MELAS cells was only temporal, as one could expect upon transient expression of tRNA transgenes. The lentiviral transfection system was further successfully used to stabilize the curative effect. Stable expression of tRK1CAA in MELAS cybrid cells resulted in a partial restoration of their mitochondrial function, including increase in mitochondrial translation, steady-state level of mtDNA-encoded proteins and cell respiration. The strategy, results, and conclusions of these experiments are described in details in **Publication 1**.

Publication 1

(Submitted)

Correction of the consequences of mitochondrial 3243A>G mutation in the *MT-TL1* gene causing the MELAS syndrome by tRNA import into mitochondria

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Correction of the consequences of mitochondrial 3243A>G mutation in the *MT-TL1* gene causing the MELAS syndrome by tRNA import into mitochondria

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ABSTRACT

Mutations in human mitochondrial DNA are often associated with incurable human neuromuscular diseases. Among these mutations, an important number have been identified in tRNA genes, including 29 in the gene *MT-TL1* coding for the $tRNA^{Leu(UUR)}$. The m.3243A>G mutation was described as the major cause of the MELAS syndrome (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes). This mutation was reported to reduce $tRNA^{Leu(UUR)}$ aminoacylation and modification of its anticodon wobble position, which results in a defective mitochondrial protein synthesis and reduced activities of respiratory chain complexes. In the present study, we have tested whether the expression of recombinant tRNAs bearing the identity elements for human mitochondrial leucyl-tRNA synthetase can rescue the phenotype caused by MELAS mutation in human *trans*mitochondrial cybrid cells. We demonstrate that expression and mitochondrial targeting of specifically designed transgenic tRNAs results in an improvement of mitochondrial translation, increased levels of mitochondrial DNA-encoded NDI and COXII subunits, and significant rescue of respiration. These findings prove the possibility to direct tRNAs with changed aminoacylation specificities into mitochondria, thus extending the potential therapeutic strategy of allotopic expression to address mitochondrial disorders.

INTRODUCTION

The m.3243A>G (MIM 590050) mutation in the mitochondrial DNA (mtDNA) *MT-TL1* gene coding for mitochondrial $tRNA^{Leu(UUR)}$ (mt- $tRNA^{Leu(UUR)}$) was first identified as a genetic cause of mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS, MIM 5400000) (1). It is one of the most common mitochondrial pathogenic mutations with a carrier frequency estimated in the range between 0.95-18.4/100 000 in northern European populations (2-4). Like many mutations affecting mitochondrial respiratory chain, the m.3243A>G mutation is associated not only with MELAS, but also with other clinical phenotypes, including CPEO (Chronic Progressive External Ophthalmoplegia), DMDF (Diabetes Mellitus and DeaFness) *etc.* (5). In all cases, the m.3243A>G mutation was present in a heteroplasmic state, which means the coexistence of mutant and wild-type mtDNA molecules in one cell. The proportion of mutant mtDNA molecules that leads to the manifestation of the disease varied strongly in different tissues (6). Patients with m.3243A>G mutation often show severe respiratory chain deficiency with complex I affected in a more pronounced manner than complexes IV and V (7, 8), but the precise mechanism connecting the mutation with clinical phenotypes is still not fully understood. Accumulated data, mostly obtained on *trans*mitochondrial cybrid cells, suggest a deficiency of aminoacylation of mutant mt-$tRNA^{Leu(UUR)}$ (9-13) and hypomodification of its anticodon wobble position affecting recognition of UUG codons (14-17) to be the origin of a mitochondrial translation defect, which, in turn, leads to a decrease of respiratory chain complexes steady-state levels (7, 18) and affected respiration rate (11, 19, 20). The mitochondrial translation defect observed varied from moderate in some cell lines to severe in others. Moreover, different groups reported qualitatively different patterns of mitochondrial translation in cells bearing the m.3243A>G mutation. In association with the mutation, it has been observed that there was a specific

decrease of polypeptides rich in UUG codons (for instance, ND6) and appearance of additional bands corresponding to abortive translation products (18, 20). In other reports, no qualitative differences or specific correlation between number of leucine UUR codons and level of synthesis of particular mitochondrial proteins were found (11, 21). The data on amino acid misincorporation at UUR codons are also controversial (18, 22, 23).

Up to now, no efficient therapy neither for MELAS, nor for other mitochondrial diseases has been demonstrated. Antioxidants and vitamins have been used, but there have been no consistent successes reported (24). Spindle transfer, where the nuclear DNA is transferred to another healthy egg cell leaving the defective mtDNA behind, is a potential treatment procedure that has been successfully carried out on monkeys (25). Using a similar pronuclear transfer technique, healthy DNA in human eggs from women with mitochondrial disease was successfully transplanted into the eggs of women donors who were unaffected (26). Embryonic mitochondrial transplant and protofection have been proposed as a possible treatment for inherited mitochondrial disease, and allotopic expression of mitochondrial proteins (*i.e.* expression of mtDNA-encoded mitochondrial proteins in the nucleus) as a radical treatment for mtDNA mutation load. Promising results were obtained with MELAS cybrid cells overexpressing the mitochondrial leucyl-tRNA synthetase (mt-LeuRS) (27, 28). Authors observed an increase in steady-state level of aminoacylated mt-tRNA$^{Leu(UUR)}$, partial restoration of COXI, COXII and NDI steady-state levels and increase of respiration rate. Interestingly, the rate of mitochondrial protein synthesis was almost the same as that in parental cells bearing MELAS mutation. Authors suggested that mutation suppression occurred *via* a mechanism that increased protein stability rather than translation rate. In an independent study, overexpression of mitochondrial translation factors EFTu and EFG2 in myoblasts derived from a MELAS patient partially restored mitochondrial translation, steady-state levels of certain respiratory chain subunits, assembly and activity of the OXPHOS

complexes (18). A similar approach was also formerly and successfully modelled in yeast (29).

In addition to proteins, human mitochondria also import from the cytosol small RNAs such as 5S rRNA (30-32) or $tRNA^{Gln}$ (33). Moreover, we previously demonstrated that yeast importable $tRNA^{Lys}$ derivatives and some other small artificial RNA substrates could be imported into mitochondria after their expression in human cells (34, 35). We have shown that yeast $tRNA^{Lys}$ derivatives targeted to mitochondria of cybrid cells and patient fibroblasts with the m.8344A>G mutation in the mtDNA *MT-TK* gene coding for mt-$tRNA^{Lys}$ (commonly associated with the MERRF syndrome) partially restored their mitochondrial translation, activity of respiratory complexes, electrochemical potential across the mitochondrial inner membrane and respiration rate (36). In order to enlarge the spectrum of mtDNA mutations compensated we investigated here the possibility to rescue the MELAS mutation by allotopic expression of recombinant and importable tRNAs whose aminoacylation identity had been changed from lysine to leucine.

RESULTS

Construction of importable tRNAs with leucine aminoacylation identity

The major identity elements required for recognition of $tRNA^{Leu(UUR)}$ by mitochondrial LeuRS are "discriminator" base A73 and the A14 base (affected by m.3243A>G mutation) (Figure 1) (37, 38). Among yeast tRNA derivatives importable *in vivo* in human cells we have previously characterized three lysine isoacceptor tRNAs (tRKs): two cytosolic, tRK1 and recombinant tRK2 (G1-C72; G73; U34), and one mitochondrial: tRK3 (Figure 1) (36). We introduced in these three tRNAs the discriminator base A73 and leucine anticodons, either

UAA or CAA, the first one with the expectation that the U in the wobble position would be correctly modified, the last one with the purpose to decode UUG codons even if the anticodon will be not modified. Thereby, 6 different versions of potentially therapeutic tRNAs: tRK1UAA, tRK1CAA, tRK2UAA, tRK2CAA, tRK3UAA and tRK3CAA were designed and further used for *in vitro* and *in vivo* assays (Figure 1). Several of these *in vitro* synthesized tRNAs (tRK1 and tRK3 versions) were shown to be aminoacylated by purified recombinant mitochondrial LeuRS with the same efficiency as the human mt-tRNA$^{Leu(UUR)}$ transcript (data not shown).

We tested then whether recombinant tRNAs with altered aminoacylation identity preserved their ability to be imported into mitochondria of human cells after introduction of the mutations. To this end, MELAS cybrid cells were transfected with corresponding T7-transcripts, total and mitochondrial RNAs from cells were isolated 24h after transfection and analyzed by Northern-hybridization with ^{32}P-5'-end labelled oligonucleotide probes (Figure 2A). The efficiency of import was calculated as a ratio of a specific hybridization signal in mitochondrial RNA to that in total RNA and was relative to the efficiency of import of tRK1 or tRK3 transcript taken as 100% in each series. All synthetic tRNAs tested were found to be imported into mitochondria of MELAS cybrid cells *in vivo* with various efficiencies (Figure 2B). Mutations introduced in the anticodon region of tRK3UAA (U35:A35, U36:A36) and tRK3CAA (U34:C34, U35:A35, U36:A36) did not significantly reduce efficiency of their import, while substitutions made in tRK1UUA (U73:A73, C34:U34, U35:A35, U36:A36) and tRK1CAA (U73:A73, U35:A35, U36:A36) decreased their import 3 and 5 fold, respectively, compared to tRK1 without mutations. Import efficiency of tRK2CAA was estimated comparing to tRK1 transcript, since wild-type tRK2 is not imported into mitochondria, and its efficiency of import was approximately 7 times lower compared to tRK1.

Using the same approach, we found that T7-transcripts were stable in transfected cells at least 48h after transfection. Despite efficient targeting into mitochondria of all recombinant tRNAs, we were not able to observe any effect on mitochondrial translation (data not shown). Moreover, the analysis of aminoacylation state of these transcripts using the acid gel method (39) showed that they were mostly present in deacylated form, a finding which could be explained by the absence of posttranscriptional modifications in T7-transcripts.

Analysis of transient expression of recombinant tRNAs in MELAS cybrid cells

In order to express functional recombinant tRNAs in cybrid cells, we performed transfection with DNA constructs containing corresponding mutated genes. To this end, we first used mammalian expression vector pBK-CMV and cloned recombinant tRNA genes in an opposite direction with respect to the CMV promoter in order to favour their transcription from their internal promoter for RNA polymerase III (RpoIII) and their further correct maturation. MELAS cybrid cells were transiently transfected with pBK-CMV-tRK plasmids with the efficiency close to 90% (as revealed by FACS analysis of the transfection control with a GFP-expressing plasmid). Total cellular RNA was then analyzed by Northern hybridization 24h, 48h and 72h after transfection to check expression and stability of recombinant tRNAs. tRK1 and tRK2- based versions were stable for 24h., while their amount strongly decreased at day 2 (Figure 3A). Expression of tRK3 versions was not detected, which can be explained by non-optimal sequence of internal promoter for RpoIII in these transgenes as compared to tRK1/tRK2 versions. In order to increase the period during which transgenic tRNAs are present in transfected cells, we performed successive transfections, with a second transfection on the 3rd day after the first one. MELAS cybrid cell line used has defective steady-state level of the mtDNA-encoded COXII subunit and decreased cytochrome *c* oxidase

(COX) enzymatic activity, which is in agreement with previously published data (40). We analyzed steady-state level of COX II protein during 6 days after transfection by immunoblotting of total cell protein extracts (Figure 3B). In several cases, reproducible increase of COXII was observed with the most pronounced effect detected after transfection with tRK2CAA, where COXII level was increased approximately three times as compared to non-transfected cybrid cells with its maximum being at the fourth day (one day after the second transfection). tRK1UAA version also caused a two-fold increase of COXII on the fifth day (two days after second transfection), while tRK1CAA induced only a slight increase (1.5 times) one day after first transfection. The increase of COXII level was however temporary.

Partial suppression of mitochondrial deficiency by stable expression of recombinant tRNAs in MELAS cybrid cells

To stabilize the curative effect of mitochondrial import of recombinant tRNAs, we used the lentiviral transfection system. tRNA genes were cloned in the vector pLKO.1 under the control of an external U6 RpoIII promoter. Before infection, pLKO.1-tRK constructs were verified in a transient transfection system for tRNA expression, which was detected for tRK1UAA/CAA and tRK2UAA/CAA versions. MELAS cybrid cells were analyzed 72h or 96h after infection for transgene expression, and tRK1CAA was found to be the only one stably expressed in cybrid cells with a high efficiency (Figure 4A). The other constructions, giving no sufficient levels of expression (although the presence of the transgene was confirmed by PCR), were not further analyzed. The MELAS-pLKO.1- tRK1CAA cell line was therefore analyzed in depth.

In agreement with previous reports (11, 21), the original MELAS cybrid cells showed strongly decreased mitochondrial protein synthesis, with large polypeptides ND2, COXI, II

and III being particularly affected, but the qualitative pattern of mitochondrial translation products was unchanged. In MELAS-pLKO.1- tRK1CAA cells amounts of mitochondrial translation products were found to be increased to 30%-50% in a generalized manner, compared to control cells transfected with an empty vector (MELAS-pLKO.1) (Figure 4B). Western analysis of mitochondrial DNA-encoded respiratory subunits revealed a reproducible two-fold increase of COXII and slight increase of NDI (Figure 4A). MELAS-pLKO.1 reference cybrid cells demonstrated strongly decreased respiration with the ratio of uncoupled to coupled respiration (respiratory control) approximately two times lower than for 143B cells, which was in agreement with previously reported data (19). In MELAS-pLKO.1-tRK1CAA cells the rates of coupled and uncoupled oxygen consumption were increased about two times in comparison with the respective rates in MELAS-pLKO.1 cells, while respiration control was also improved (Figure 4C). Since the heteroplasmy level in MELAS-pLKO.1- tRK1CAA was unchanged upon transfection (~90%), one can conclude that the observed functional improvement of mitochondrial functions was due to expression and mitochondrial import of the recombinant tRNA.

DISCUSSION

This study demonstrates that nuclear expression and subsequent mitochondrial import of specifically designed tRNAs of yeast origin with artificially changed identity (cytoplasmic lysine to mitochondrial leucine) partially rescues various negative effects of the mutation m.3243A>G in the mitochondrial $tRNA^{Leu}$ gene underlying the MELAS syndrome in cultured human cells. This rescue was observed at the levels of mitochondrial translation, steady-state of mitochondrial DNA-encoded subunits of the respiratory chain and respiration.

The mitochondrial RNA import identified in protozoan, fungi, animals and plants now is considered as quasi-universal process, although its mechanism and number of imported tRNAs vary strongly among different species (41). The flexibility of this pathway turned out to be exploitable for transfer of the yeast tRNA import factor on human cells (35), which has a potential interest for development of allotopic expression of "therapeutic" tRNA genes to correct disorders caused by mutations in mitochondrial tRNA genes. Earlier, we have shown that MERRF syndrome (Myoclonic Epilepsy with Ragged-Red Fibres) caused by m.8344>G mutation in mitochondrial $tRNA^{Lys}$ gene could be partially rescued by targeting yeast lysine tRNAs into mitochondria of affected cells (36). These promising results encouraged us to further develop this strategy by broaden the spectrum of tRNA species concerned, which is of an undoubtable interest, since more than 170 mutations in other tRNA genes were shown to be associated with human disorders (5).

In yeast, several cytoplasmic tRNAs were reported to be addressed into mitochondria - one of two lysine isoacceptor tRNAs - tRK1 (42) and two $tRNA^{Gln}$ (43). Additionally, the second lysine isoacceptor tRNA (tRK2) provided with appropriate import determinants (G1-C72, G73; U34) as well as mitochondrial lysine tRNA (tRK3) could be addressed both in yeast and human mitochondria (31, 36, 44). Furthermore, yeast tRK1 with altered aminoacylation identity (Lysine > Methionine) was shown to be imported in both yeast and human isolated mitochondria (35). Therefore, it appeared to us possible to alter the aminoacylation specificity of importable tRNAs to fit the MELAS - causing mutation m.3242A>G localized within mitochondrial $tRNA^{Leu(UUR)}$ gene (*MT-TL1*), known to be the hot spot for mutations (29 of 170 mt-tRNA gene mutations characterized to date are located therein). Furthermore, the MELAS m.A3243>G mutation is one of the most frequently described in patients with mitochondrial disorders. Despite the numerous assays and promising tracks mentioned above, this condition still awaits an efficient treatment.

The increase of COXII steady-state level that we observed in MELAS cells in transient transfection system varied in strength and duration depending on tRNA version expressed (see Fig. 3). This could indicate on possible differences in behaviour and action of different recombinant tRNAs in the cell. Thus, lower import efficiency of tRK1CAA comparing to tRK1UAA (see Fig. 2) can partially explain its moderate effect on COXII level. On the other hand, the less efficient import substrate tRK2CAA lead to the best improvement of COXII level, probably taking advantage of its CAA anticodon, which did not need post-transcriptional modification to decode leucine UUG codons. Obviously, the improvement of mitochondrial function depended not only on the efficiency of a particular tRNA but on the time it was present in cell. For example, the effect of tRK1CAA on COXII steady-state level was more pronounced once it has been expressed in cybrid cells in a stable manner.

Meanwhile, MELAS-pLKO.1-tRK1CAA cells clearly demonstrated a significant rescue of mitochondrial functions at all levels analyzed (see Fig. 4). Thus, synthesis of certain polypeptides reached (for CytB) or even exceeded (for ND2 and ATP6) its level in 143B wild type cells, while synthesis of others was improved up to 30% (for COXI) - 50% (for COXII/III) of its wild type level, indicating that tRK1CAA participated in mitochondrial translation. However, no clear correlation between number of leucine codons (neither UUG, nor UUR) and translation level of particular mitochondrial proteins was observed in MELAS cybrid cell before or after transfection, which is corroborated by previously reported data (11, 21). The partial nature of the effect could be explained by insufficient level of expression of the recombinant tRNA gene(s), defective post-transcriptional modifications of recombinant tRNAs, ineffective export of tRNAs from nucleus and targeting into mitochondria or, finally, low efficiency of re-aminoacylation inside the organelle. Furthermore, one could also imagine that the growth conditions used (high glucose and pyruvate) do not favour the switch from glycolysis to oxidative phosphorylation in transfected cybrid cells energy production. In any

case, the observed increase of mitochondrial translation level and subsequent augmentation of COXII and NDI steady-states was sufficient to improve the rate of oxygen consumption in MELAS-pLKO.1- tRK1CAA cells about two fold. Such an increase may prove sufficient for further therapeutic approaches.

In conclusion, the recombinant leucine tRNAs validated in the present work could be tested in modelling therapeutic approaches to a number of other disorders caused by dysfunction of mt-tRNA$^{Leu(UUR)}$. As an important issue of the study, it clearly appears that the mitochondrial translation system in human cells possesses a significant extent of flexibility, accepting not only cytosolic - type tRNAs, but also tRNAs of yeast origin, which are evolutionary very distinct from the human ones. This observation, together with previously published data (35, 36), strongly support the approach of allotopic expression of mitochondrially-imported RNAs as a powerful tool for different kinds of disorders caused by mutations in mitochondrial tRNA-coding genes.

MATERIALS AND METHODS

Cell culture

The MELAS cybrid cell line used in this study was kindly provided by E.A. Shoubridge (Montreal Neurologic Institute, Quebec, Canada). It carried 90 ± 5% of m.3243A>G mutation and was characterized previously (40) MELAS cybrid cells were obtained by fusing rho0 cells from osteosarcoma cell line 143B.TK- with cytoplasts from clonal primary myoblasts established from a patient carrying the m.3243A>G point mutation in *MT-TL1* gene (MELAS mutation) as described elsewhere (45). Cybrid cells were cultivated in DMEM medium with high glucose (4.5 g/l), sodium pyruvate (110 mg/l) and L-glutamine (2 mM) from Sigma, supplemented with 10% (w : v) fetal calf serum (FCS), 50 mg/ml uridine, standard

concentrations of antibiotics (penicillin, streptomycin and fungizone) and, for stable transfectants, 2 μg/ml of puromycin. 143Brho+ cells were used as healthy cell control and were cultivated in the same conditions as MELAS cybrid cells. HEK-293T cells were used for production of lentiviral particles and were cultivated in standard DMEM medium with 1g/l glucose. All cell lines were cultivated at 37°C and 5% of CO_2.

Cell transfection

Transfection of MELAS cybrid cells with tRNA transcripts was performed using Lipofectamine2000 (Invitrogen) as described previously (32) with minor modifications: 1 μg of transcript and 12.5 μl Lipofectamine2000 were used per 2×10^6 cells. Transient transfection was performed with a mix of 4 μg of pBK-CMV-tRK plasmid and 12 μl of Lipofectamine2000 per 600×10^3 cells according to manufacturer protocol. Efficiency of transfection was estimated by FACS analysis of GFP expression from pmax-GFP plasmid transfected in parallel. MELAS cybrid cells stably expressing recombinant tRNAs were obtained by lentiviral transfection. Production of lentiviral particles was performed in HEK-293T cells using FuGENE6 transfection reagent (Roche Applied Sciences), 3 μg of pLKO.1-tRK (Addgene), 1.5 μg of pLP1, 0.75 μg of pLP2 and 0.75 μg pLP-VSGV packaging plasmids (Invitrogen) according to manufacturer protocol. Infection of MELAS cybrid cells was performed with virus-containing medium from HEK-293T cells during 2-3 days. Cells containing transgenes were selected in the presence of 2μg/ml of puromycin during 2-3 days.

Construction of recombinant tRNA genes and plasmids

Expression of recombinant hmtLeuRS was performed from pET3a (Amp^r) plasmid containing hmtLeuRS gene without mitochondria-targeting sequence, cloned from cDNA that was purchased from the RIKEN Collection.

Cloning of yeast tRK1, tRK2 (G1-C72; G73; U34) and tRK3 genes was performed previously (46). tRNA gene coding sequence was placed under control of T7 promoter in pUC19 (Amp^r) (Invitrogen), *Bst*NI site was introduced at its 3'-terminus to further gave rise to the CCA-3' sequence in tRNA. Mutations aimed to change tRNA aminoacylation identity (Lys > Leu) were introduced by several steps of PCR-mutagenesis. Discriminator base A73 - using oligonucleotides: tRK1-T7 GGGATCCATAATACGACTCACTATA GCCTTGTTGGCG, tRK1-A73-BstNI GGGATCCTGGTGCCCTGTAGGGGGCTCG, tRK2-G1-T7 GGGATCCATAATACGACTCACTATAGCCTTGTTAGCTCAG, tRK2-C72A73-BstNI GGGATCCTGGTGCCTCATAGGGGGCTCG. Change of anticodon was performed by "Quick Change Site-Directed Mutagenesis Kit" (Stratagene) according to manufacturer protocol. The following pairs of oligonucleotides were used ("As" for forward and "Br" for reverse): tRK1UAAAs GACTTAAAATCATAAGG, tRK1UAABr TATGATTTTAAGTCATACGC, tRK1CAAAs GACTCAAAATCATAAGG, tRK1CAABr TATGATTTTGAGTCATACGC, tRK2UAAAs GTTCGGCTTAAAACCG, tRK2UAABr CATTTCGGTTTTAAGCCG, tRK2CAAAs GTTCGGCTCAAAACCG, tRK2CAABr CATTTCGGTTTTGAGCCG, tRK3UAAAs GTCTTAAAAGCAACCC, tRK3UAABr GCTTTTAAGACAAC, tRK3CAAAs CAGTTGTCTCAAAAGCAACCC, tRK3CAABr GGGTTGCTTTTGAGACAACTG.

For transient expression in MELAS cybrid cells, tRK1UAA/CAA, tRK2UAA/CAA, tRK3UAA/CAA genes we re-cloned in pBK-CMV (Kan^r) vector (Stratagene) in *Bgl*II/*Bam*HI sites using oligonucleotides: TRK1/F1-Aviv GGCAAGATCTGGTCAGATTTCCAATAA CAGAATATCCTTGTTAGCCTTGTTGGCG, TRK1 /F1-A73-Bviv GGCAAGATCTGTC ATCGTGTTTTAAAAAAAAAAAGAATGCCCTGTAGGGGGCTC, TRK2 /F1-G1-Aviv GGCAAGATCTGGTCAGATTTCCAATAACAGAATATCCTTGTTAGCCTTGTTA GCTCAG, TRK2 /F1-C72A73-Bviv GGCAAGATCTGTCAT CGTGTTTTAAAAAAAA

AAAGAATGCCTCATAGGGGGCTCG, TRK3/F1-Aviv CCCAAGAGATCTGGTCAGA TTTCCAATAACAGAATAGAGAATATTGTTTAATG, TRK3 /F1-Bviv CCCAAGAGA TCTGTCATCGTGTTTTAAAAAAAAAAAGAATGAGAATAGCTGGAGTTG. tRNA genes were flanked by non-coding flanking regions of one of the well expressed tRK1 copies and were cloned in an opposite direction with respect to the CMV promoter in order to favour their transcription from internal promoter by RNA polymerase III (RpoIII) and further correct maturation.

For stable transfection tRK1UAA/CAA, tRK2UAA/CAA genes were cloned in plKO.1 (Amp^r) lentiviral vector (Addgene) (47) in *Age*I/*Eco*RI sites under the control of external U6-promoter without any flanking regions using oligonucleotides: trk1plkoAs GGCAACCGGTGCCTTGTTGGCG, trk1plkoBr GGCAGAATTCAAAAATG CCCTGTA GGG, trk2plkoAs GGCAACCGGTGCCTTGTTAGCTCAG, trk2plkoBr GGCAGAATT CAAAAATGCCTCATAGGGGG.

Purification of hmtLeuRS and *in vitro* aminoacylation assay

His-tagged hmtLeuRS was purified from BL21 CodonPlus (DE3)-RIL *E. coli* strain through nickel affinity chromatography, followed by protein concentration through Nanosep 30K (Pall) columns, and stored as 40% glycerol solution at -20°C. Activities of different enzyme fractions were tested on commercially available preparation of *E. coli* tRNA. Aminoacylation of tRNA T7-transcripts was done according to the described procedure (37). Final conditions were: 50 mM HEPES-NaOH (pH 7.6), 25 mM NaCl, 12 mM $MgCl_2$, 2.5 mM ATP, 0.2 mg/ml BSA, 0.8 μM [^{3}H]-Leu and adapted concentrations of tRNA and enzyme. Aminoacylation plateaus were estimated over 30 minute incubation time, using 1μM of -transcripts. Aminoacylation efficiency recombinant tRNA transcripts was compared to that of wild-type human mt-$tRNA^{Leu(UUR)}$ transcript

Isolation and analysis of DNA

Total cellular DNA was isolated by standard procedures and the m.3243A>G mutation level was tested systematically by *Apa*I restriction analysis (48). Briefly, mutation containing mtDNA region was PCR-amplified using oligonucleotide primers: hp3081 GTAATCCAGGTCGGTTTCT and hp3380 CGTTCGGTAAGCATTAGG, PCR-products were digested by *Apa*I for 2h and analyzed by gel-electrophoresis.

Isolation and analysis of total and mitochondrial RNAs

Total and mitochondrial RNAs were isolated by standard TRIzol-extraction (Invitrogen) from cells and purified mitochondria, respectively. Mitochondria were isolated from cells as described previously (49). RNA preparations were analyzed by Northern-hybridization with [^{32}P]-5'-end labelled oligonucleotide probes. To detect tRK1 versions we used oligonucleotide probe anti tRK1(1-34): GAGTCATACGCGCTACCGATTGCGCCAA CAAGGC, for tRK2 versions, the probe anti tRK2(2-32): GCCGAACGCTCTACCAACT CAGCTAACAAGG, for tRK3 versions, the probe anti tRK3 (1-39): CTTAAAAGACAACTGTTTTACCATTAAACAAATATTCTC, the probe anti mt-tRNA^{Leu}: GAACCTCTGACTCTAAAG and the probe anti mt-tRNA^{Thr}: CATCTCCGGTTTACAAG were used to control the quality of RNA, and the probe anti cy-tRNA^{Lys}: CTTGAACCCTGGACC to control the absence contamination of mitochondrial RNA by cytosolic tRNAs.

Analysis of aminoacylation *in vivo*

Analysis of aminoacylation levels of recombinant tRNAs in cells was performed through PAGE in acid conditions and subsequent Northern-hybridization analysis as

described elsewhere (39). Briefly, RNAs from cells were isolated with TRIzol-reagent (Invitrogen), precipitated on ice with 50% iso-propanol and dissolved in 10 mM NaAc pH4.5, 1mM EDTA. Deacylated controls were prepared by 10 min incubation at 75°C in 0.25M TrisHCl pH8.5, 0.25M $MgCl_2$ followed by RNA precipitation. RNAs in a loading buffer with 0.1 M NaAC pH5.0 were run in a cold room through denaturing acid 6.5% PAAG with 0.1M NaAc pH5.0 and analyzed by Northern-hybridization with [^{32}P]-5'-end-labelled oligonucleotide probes.

Immunoblotting

For immunoblotting, whole cells were solubilized in a Laemmli's buffer (50) in the way to have SDS: protein ratio ~25-30 (w/w), sonicated for 5 sec to break cellular DNA; incubated for 10 min at 60°C, and 30 μg of protein were run on a 12.5% SDS-PAGE, and subsequently transferred to a nitrocellulose membrane. For immunodetection following antibodies were used: polyclonal antibodies against COXII, NDI and commercially available monoclonal antibodies against, porin (Calbiochem 529538), α-tubulin (Sigma T6074). Detection was done using ECL™ horseradish peroxidase linked secondary antibodies, "ECL Plus Western Blotting Detection Reagent" on "Typhoon" from GE Healthcare. Signal quantification was performed by and ImageQuantTL programme from the same manufacturer.

***In vivo* mitochondrial translation**

The analysis of mitochondrial protein synthesis was performed as previously described (36) with minor modifications. Briefly, 600×10^3 cells were incubated for 10 min in DMEM w/o methionine (Sigma) in the presence of 100 μg/ml of emetine to inhibit cytoplasmic translation, followed by 30 min with 200 μCi/ml [^{35}S]-methionine, and, finally, 10 min chase in the normal growth medium. Cells were solubilized in a Laemmli's buffer (50), sonicated

for 5 sec to break cellular DNA; incubated for 10 min at 37°C, and 100 μg of protein were run on a 10-20% gradient SDS-PAGE. Protein amounts loaded were before normalized by anti-porin immunoblotting of the same preparations. Visualization and quantification were performed using "Typhoon" and ImageQuantTL software from GE Healthcare.

Measurement of oxygen consumption

The rates of oxygen consumption were measured using Hansatech Oxygraph on 1×10^6 intact cells in PBS in the presence of 5mM glucose. 1 μg/ml of oligomycin, FCCP in the range of 10-500 nM and 1 mM of KCN were sequentially added to measure coupled, uncoupled and non-mitochondrial oxygen consumption, respectively.

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Legends to Figures

Figure 1. Cloverleaf structures of tRNAs used in this study.

From left to right: native human mitochondrial $tRNA^{Leu(UUR)}$, major identity elements of recognition by mtLeuRS are in blue filled circles and MELAS m.3243A>G mutation is indicated by the red arrow; three yeast lysine tRNAs, tRK1, tRK2; tRK3 and their recombinant versions, tRK1UAA/CAA, tRK2UAA/CAA, tRK3UAA/CAA, with determinants of mitochondrial import indicated in green filled circles, identity elements for human mtLeuRS are in blue filled circles, mutations and regions where they were introduced are indicated by arrows and enclosed in blue for leucine aminoacylation identity elements, in green - for import determinants and in red - for leucine anticodons. Post-transcriptional modifications: 5-taurinomethyluridine (τm^5U), 5-methylcarboxymethyl-2-thiouridine (mcm^5s^2U), 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$), 1-methylguanosine (m^1G), 2-methylguanosine (m^2G), 7-methylguanosine (m^7G), 1-methyladenosine (m^1A), 5-methylcytidine (m^5C), pseudouridine (Ψ), dihydrouridine (D), 5-methyluridine (T) and N6-threonylcarbamoyladenosine (t^6A).

Figure 2. Analysis of *in vivo* import of synthetic recombinant tRNAs in mitochondria of MELAS cybrid cells.

(A) Northern-hybridization of total and mitochondrial RNAs isolated from MELAS cybrid cells transfected with T7-transcripts. Specific [^{32}P]-oligonucleotide probes to tRK1; tRK2, tRK3 were used to check for mitochondrial import, mt-tRNA-Leu probe - to control the absence of degradation of mitochondrial tRNAs and cy-tRNA-Lys probe to control the absence of its contamination by cytosolic tRNAs (the probes are indicated at the right of the panels). Minor bands visible with tRK1 probe in tRK2-transfectants and with cy-tRNA-Lys

probe in tRK1 and tRK2 ones represent the unwashed traces of previous hybridizations of the same membrane and are unspecific (they do not migrate as the cognate tRNAs and therefore do not alter any interpretation of the specific signals). TH and MH are total and mitochondrial RNAs from non-transfected MELAS cells, TY stands for total yeast RNA used as the control of hybridization specificity. T1 and T3 are the transcripts of yeast tRK1 and tRK3 without mutations used to estimate the import efficiency of recombinant tRNAs (B) The import efficiency of the T7-transcripts, calculated as a ratio of a specific hybridization signal in mitochondrial RNAs to the total RNAs and relative to the efficiency of import of tRK1 or tRK3 taken as 100% in both cases.

Figure 3. Effect of transient expression of recombinant tRNAs on COXII level in MELAS cybrid cells.

(A) Northern hybridization analysis of total RNAs isolated from pBK-CMV-tRK transfected MELAS cybrid cells. Cells were transfected twice, second transfection was performed on the third day after the first one, and RNAs were isolated 1 to 6 days after the first transfection. Specific [^{32}P]-oligonucleotide probes are indicated on both sides of the autoradiographs (tRK1, tRK2 or cy-Lys - for the control cytoplasmic $tRNA^{Lys}$ used as the quantification reference). (B) Western analysis of protein extracts from the transfected cells with anti-COXII antibodies. Anti-tubulin antibodies were used as the quantification reference. The steady-state levels of COXII before and after transfection, normalized to tubulin, are shown as diagrams for each tRNA-version in the lower panel of the figure. Values are presented relative to COXII level in 143B parental cells, taken as 1. Error bars correspond to the results of 2 to 5 independent assays.

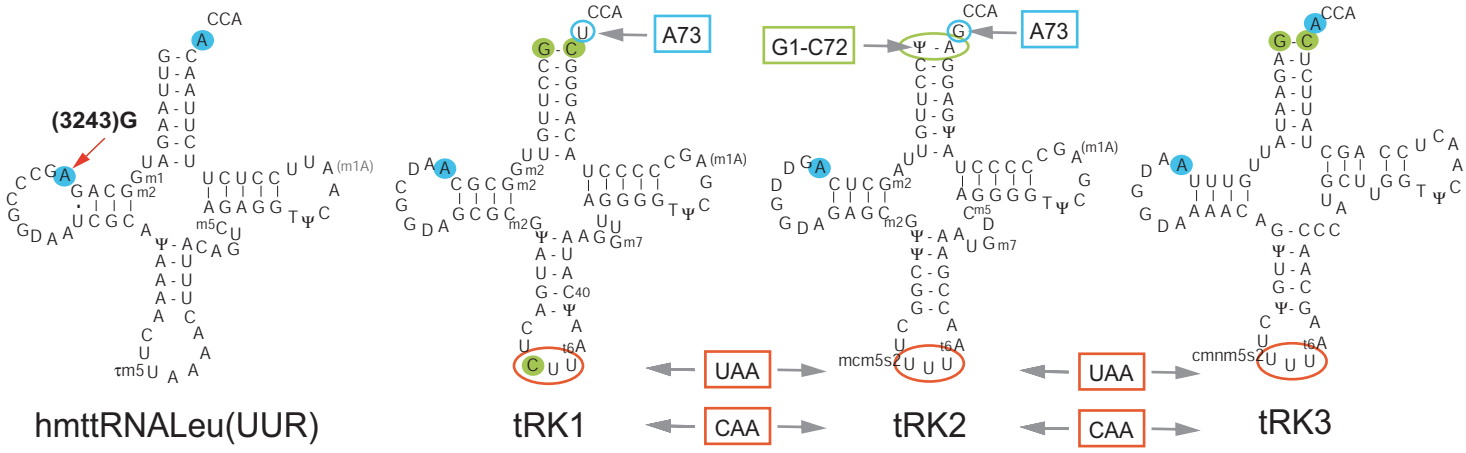
Figure 4. Effect of stable expression of tRK1CAA in MELAS cybrid cells on their mitochondrial functions.

(A) Northern hybridization of total RNAs isolated from MELAS cells expressing tRK1CAA (Mel-T1CAA) and cells transfected with an empty vector (Mel-pLKO) with tRK1 and mt-tRNAThr - specific [^{32}P]-labelled oligonucleotide probes (TY is total yeast RNA preparation used as the control of hybridization specificity). (B) Western analysis of protein extracts from 143B, Mel-pLKO and Mel-T1CAA cell lines with antibodies against mitochondrial COXII, NDI, and tubulin used as the quantification reference. (C) COXII and NDI steady-state levels in each cell line, normalized to tubulin and relative to the steady-state level of the corresponding protein in parental 143B cells (taken as 1). (D) Pulse-chase analysis of mitochondrial translation. Radioautograph of [^{35}S]-labelled mitochondrial translation products separated by SDS-PAGE is presented. The lines used are indicated at the top: 143B, Mel-pLKO and Mel-T1CAA, the bands corresponding to individual translation products are indicated according to standard pattern (51). Equal amounts of proteins were loaded in each case, which was controlled by Western analysis of porin in the same samples performed in parallel (below the main panel). (E) The diagram shows the levels of individual translation products in each cell line relative to those in 143B cells (taken as 1). (F) The rates of coupled and uncoupled oxygen consumption (the mean values ±SD, n=3) in 143B, Mel-pLKO and Mel-T1CAA cell lines in nmol $O_2/10^6$ cell*min. (G) Box and whisker plots (52) represent the ratios of uncoupled to coupled respiration rates in 143B, Mel-pLKO and Mel-T1CAA cell lines. The overall data range is represented as a vertical line, while boxes represent the interquartile range containing central 50% data.

ABBREVIATIONS

MELAS, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; MERRF myoclonic epilepsy with ref ragged fibres; mtDNA, mitochondrial DNA; ND, NADH-dehydrogenase; COX, cytochrome *c* oxydase; mtLeuRS, human mitochondrial leucyl-tRNA synthetase; DMEM, Dulbecco modified Eagle Medium; FACS, Fluorescence Activated Cell Sorting; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; CMV, cytomegalovirus.

Karicheva et al., Fig. 1



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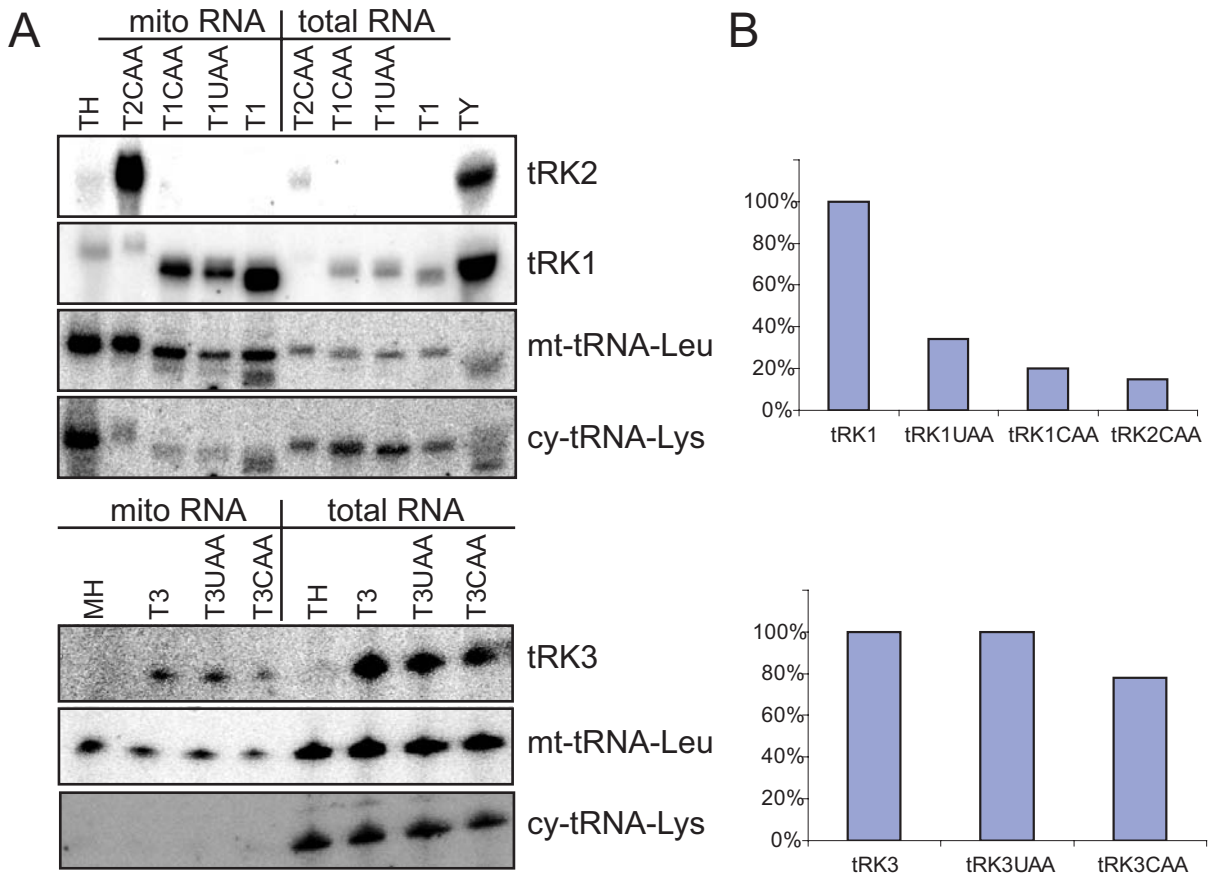

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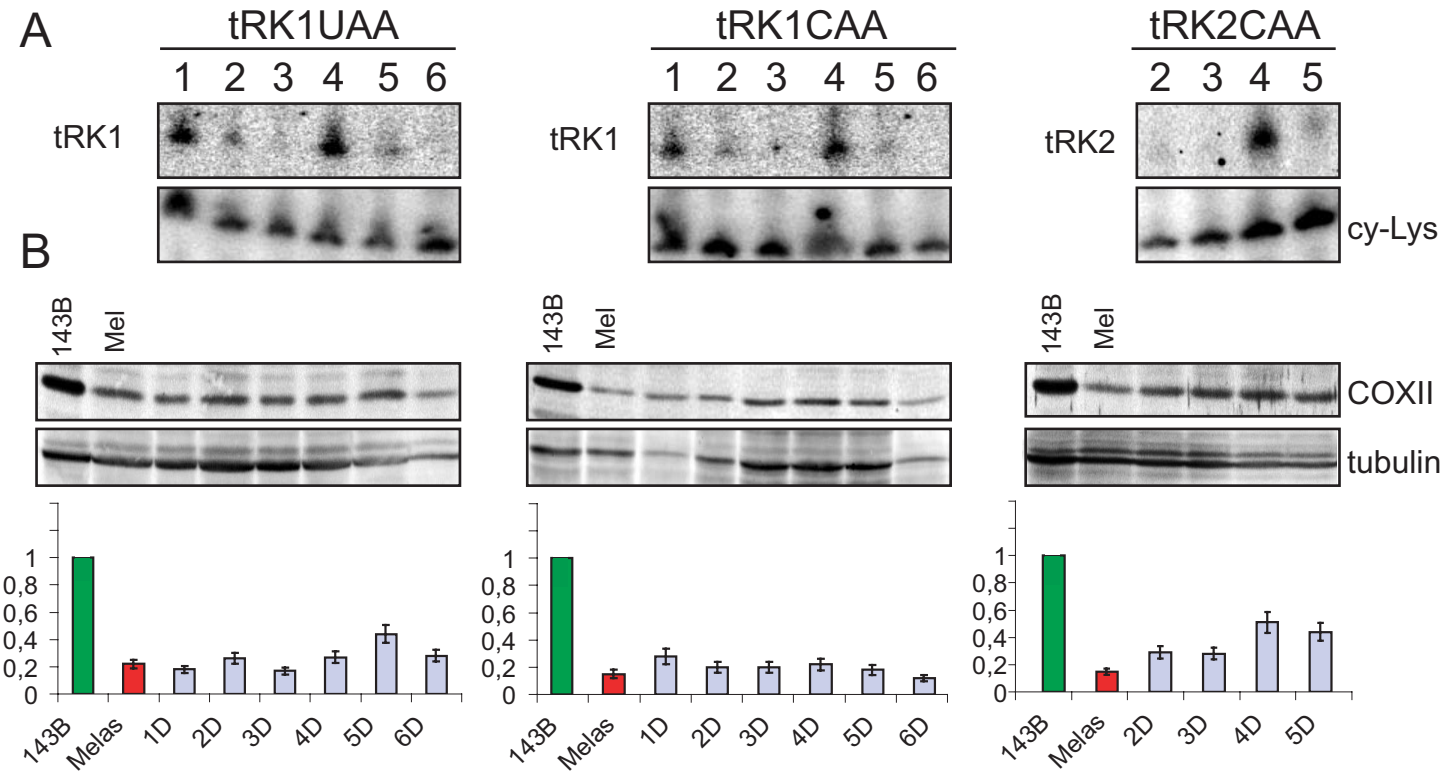
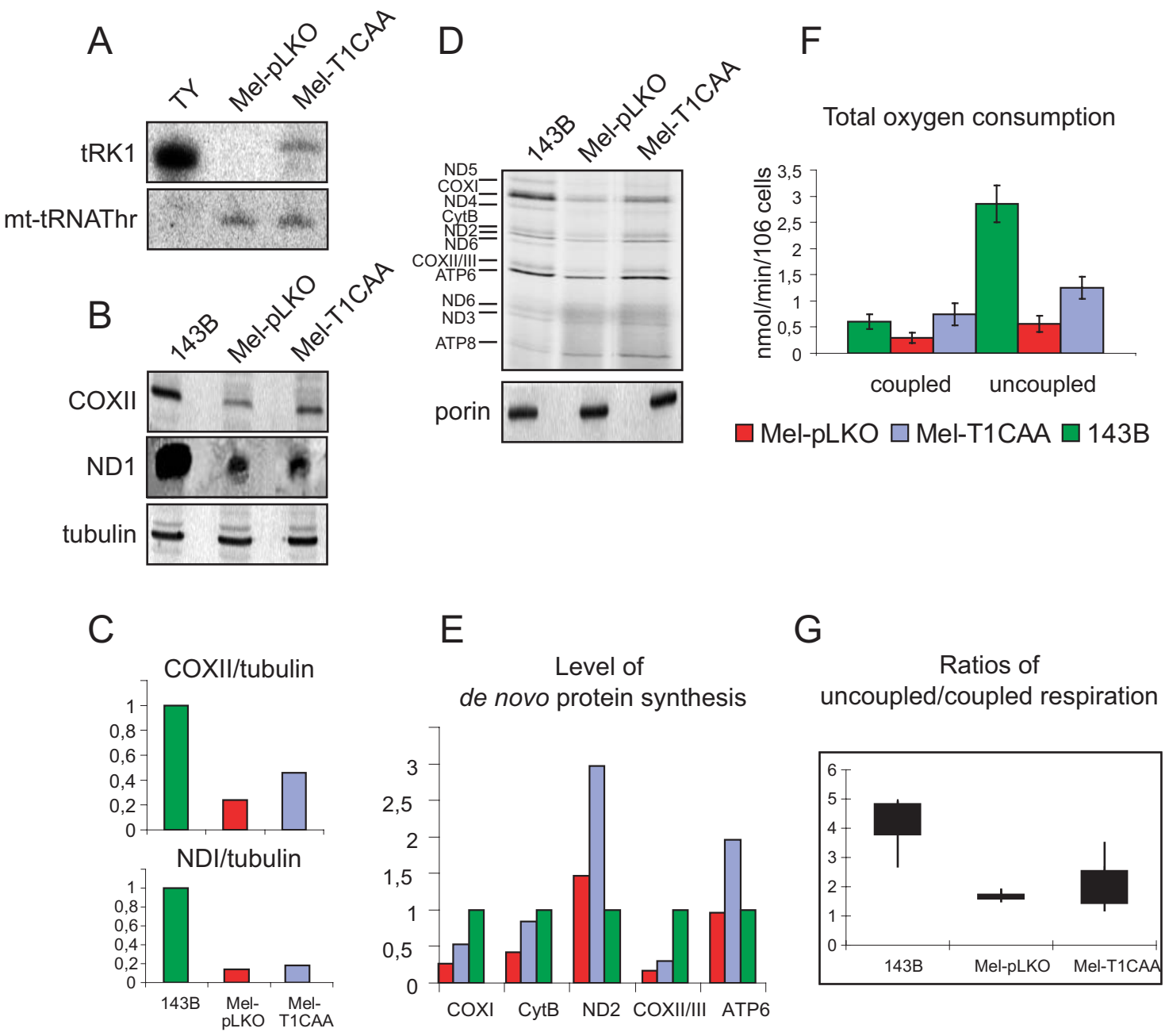


Karicheva et al., Fig. 4



CONCLUSIONS AND PERSPECTIVES

Based on the results reported in this work, the following goals have been achieved:

1. We have created a set of recombinant "therapeutic" tRNAs able to be imported into human mitochondria both *in vitro* and *in vivo* and be aminoacylated with leucine by recombinant hmtLeuRS;
2. We have shown that m.3243A>G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene (*MT-TL1*) results in the decrease of mitochondrial translation, steady-state of mitochondrial DNA-encoded subunits of the respiratory chain and respiration rates in MELAS cybrid cells used in this study;
3. We have demonstrated that nuclear expression and subsequent mitochondrial import of recombinant leucine tRNAs in MELAS cybrid cells partially rescues the negative effects of m.3243A>G mutation.

The results of this study clearly demonstrate that one can alter aminoacylation identity of a tRNA preserving its ability to be imported into human mitochondria and participate in mitochondrial translation. The recombinant leucine tRNAs validated in the present work have been shown to partially rescue the molecular consequences of one of the most frequent mt-tRNA gene mutations, m.3243A>G mutation underlying the MELAS syndrome. Although the observed partial increase of oxygen consumption upon tRNA import may prove to be sufficient for further therapeutic approach (taking into account the heteroplasmic character of m.3243A>G mutation in most MELAS patients), it will be useful to optimize the level of expression of the recombinant tRNA gene(s) and import of the corresponding tRNAs into mitochondria of human cells. On the other hand, additional mutations could be introduced in order to improve the efficiency of aminoacylation of the recombinant tRNAs, their post-transcriptional modification, and/or nucleus export in human cells. Next, it will be important to verify the efficiency of this therapeutic approach on primary cells derived from MELAS patients, as well as on animal models, once the lasts will be available.

Moreover, the validated recombinant leucine tRNAs could be tested in modelling therapeutic approaches to a number of other disorders caused by dysfunction of mt-

$tRNA^{Leu(UUR)}$, whose gene is known to be the hot spot for mutations (29 of 170 mt-tRNA gene mutations characterized to date are located therein, (MITOMAP, 2010)).

Finally, it will be extremely interesting to study if RNA mitochondrial import pathway in human cells can be exploited to deliver in mitochondria functional RNAs able to specifically inhibit replication of mtDNA copies carrying point mutations (anti-replicative approach).

In conclusion, together with previously published data (Kolesnikova et al., 2004; Kolesnikova et al., 2000), the results of this study strongly support the approach of allotopic expression of mitochondrially-imported RNAs as a powerful tool for different kinds of disorders caused by mutations in mitochondrial tRNA-coding genes.

II.2 Other studies

II.2.1 Investigation of mitochondrial diseases related to mt-tRNA modification defect

In a parallel with the main theme of my thesis, "Development of the gene therapy model for MELAS disease", I have also participated in a collaborative study devoted to investigation of mitochondrial diseases related to mt-tRNA post-transcriptional modification defect. This study was run with the team of Orly Elpeleg (Hadassah, The Hebrew University Medical Centre, Jerusalem, Israel).

Previously a life-threatening syndrome of acute infantile liver failure, has been associated with the mutations in three nuclear genes coding for deoxyguanosine kinase (DGUOK) (Mandel et al., 2001), mtDNA polymerase γ (POLG) (Naviaux and Nguyen, 2004) and MPV17 inner mitochondrial membrane protein with yet unknown function (Spinazzola et al., 2006; Viscomi et al., 2009), and was accompanied by mtDNA depletion. Nevertheless, there were a number of infants who presented with acute liver failure with normal mtDNA content. Four died during the acute episodes, and the survivors never had a recurrence. Therefore the existence of a distinct mechanism was suggested and studied. Our collaborators from Israel have identified in these patients 9 different mutations in *TRMU* gene coding for mitochondria-specific tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase. TRMU is responsible for 2-thiolation of the wobble base in mt-tRNA^{Lys}, mt-tRNA^{Gln}, mt-tRNA^{Glu}. This modification is essential for codon-anticodon interaction and confers to tRNA an efficient ribosome binding. I have participated in the analysis of molecular consequences of the *TRMU* mutation. It was shown that amount of the thio-modified mt-tRNAs in the fibroblasts of patients at time of acute episodes has been indeed severely reduced and accompanied by two-times reduction of total mitochondrial translation level. Therefore, it was proposed that reduced modification of several mt-tRNAs resulted in mitochondrial translation defect was in the origin of the disease. The timing of the clinical presentation, usually 2-4 months of age and the lack of recurrence in survived patients could be explained by TRMU dependence on cysteine limited in neonatal periods. The strategy, the results and important conclusion from these experiments are provided in the following **Publication 2**.

Publication 2

Acute infantile liver failure due to mutations in the *TRMU* gene

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Am J Hum Genet, 2009, **85**, 401-407.

Acute Infantile Liver Failure Due to Mutations in the *TRMU* Gene

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Acute liver failure in infancy accompanied by lactic acidemia was previously shown to result from mtDNA depletion. We report on 13 unrelated infants who presented with acute liver failure and lactic acidemia with normal mtDNA content. Four died during the acute episodes, and the survivors never had a recurrence. The longest follow-up period was 14 years. Using homozygosity mapping, we identified mutations in the *TRMU* gene, which encodes a mitochondria-specific tRNA-modifying enzyme, tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase. Accordingly, the 2-thiouridylation levels of the mitochondrial tRNAs were markedly reduced. Given that sulfur is a TRMU substrate and its availability is limited during the neonatal period, we propose that there is a window of time whereby patients with *TRMU* mutations are at increased risk of developing liver failure.

Acute liver failure in infancy is a life-threatening condition manifested by poor feeding, vomiting, jaundice, distended abdomen, hemorrhagic diathesis, irritability, and hypoactivity. Routine laboratory investigations reveal elevated liver transaminases, hypoglycemia, coagulopathy, hyperammonemia, and direct hyperbilirubinemia. The differential diagnosis includes viral infections, intoxications, and inborn errors of metabolism. The finding of hyperlactatemia directs the diagnosis toward mitochondrial respiratory chain disorders, and in about half of the patients there is a defect in the mtDNA synthesis machinery, resulting in mtDNA depletion (MIM 251880). This was heretofore attributed to mutations in three genes: *DGUOK* (MIM 601465), *POLG* (MIM 174763), and *MPV17* (MIM 137960).[1–3]

In the past 14 years, we have encountered eight patients in seven unrelated families of Yemenite Jewish origin, who presented in infancy with acute liver failure. All were born at term, had birth weights appropriate for gestational age, and had physiologic hyperbilirubinemia that resolved in a normal manner. All were reportedly healthy during the early neonatal period but were admitted at 2–4 months because of irritability, poor feeding, and vomiting. On physical examination, all were found to be well-nourished but lethargic, with pale-gray skin color, jaundiced sclerae, distended abdomen, and hepatomegaly. All of the patients required intensive care for several weeks, with supportive nutrition and blood products given as compensation for coagulopathy and active GIT bleeding. Liver transplantation was considered but was not performed in any of the patients.

Laboratory investigation disclosed acute liver failure (clinical and biochemical data presented in Table 1) with severe coagulopathy that included low factor 5 and 11 and was not corrected by vitamin K supplementation, low albumin, direct hyperbilirubinemia, metabolic acidosis, hyperlactatemia, and high alpha-fetoprotein. Blood ammonia level was normal or slightly elevated, and plasma amino acid profile was noted for high phenylalanine, tyrosine, methionine, glutamine, and alanine. Urinary organic acid analysis revealed massive excretion of lactate, phenylalanine and tyrosine metabolites, and ketotic dicarboxylic and 3-hydroxydicarboxylic aciduria. Serology for hepatitis viruses and body fluid cultures failed to detect an infectious etiology. Abdominal ultrasound disclosed enlarged homogenous liver with normal diameter of the bile ducts and the portal vein.

Clinical and biochemical improvement started after 2–3 weeks, and liver functions returned to normal within 3-4 months. Nonetheless, liver size had normalized only after 3 months to 3 years. Seven patients survived the acute episode, were observed on a long term follow-up (the oldest currently 14 years of age) to be developing normally, and never experienced a similar episode. One patient (2859) died of intractable lactic acidosis and multiple organ failure. During the acute phase, there was usually no indication of extrahepatic involvement, as evidenced by normal electrolytes, creatinine and renal function, blood count, bone marrow aspiration, creatine phosphokinase (CPK), electromyography (EMG), echocardiogram, ophthalmologic examination, brain magnetic resonance imaging (MRI), electroencephalogram (EEG), and nerve

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Table 1. Clinical and Biochemical Data of the Patients

Patient	Origin	Age at Presentation	Outcome	Peak Values				
				ALT (IU/L)	GGT (IU/L)	INR	T-Bil (mg%)	Lactate (mM)
2624	Y-J	6 mo	A&W at 2 yrs	367	356	2.6	3.3	5.5
3032	Y-J	4 mo	A&W at 9 mo	169	621		5.7	4.5
1432	Y-J	2 mo	A&W at 10 yrs	1150		3.4	10	20
1116	Y-J	3 mo	A&W at 10 yrs	293	139		9.7	6.6
111	Y-J	4 mo	A&W at 8 yrs	417		3.0		7.0
421	Y-J	4 mo	A&W at 14 yrs	430		3.0	4.3	20
2859	Y-J	3 mo	death at 4 mo	400	157	7.0	24.0	30
2375	Y-J	6 mo	A&W at 2 yrs	532	305	3.6	7.5	3.2
2006	Arab	1 mo	death at 2 mo	1193	77	3.4	14.4	19
3015	Arab	6 mo	A&W at 2 yrs					
1910	Ashk.	1 day	A&W at 5 yrs	1146	270	2.3	0.1	20
Akh	Alger	1 day	death at 3 mo	93			13.2	7.0
Aza	Alger	2 days	death at 4 mo	229			6.3	10.0
control				<52	<142	<1.0	<0.4	<2

Abbreviations are as follows: ALT, alanine aminotransferase; GGT, gamma glutamyl transpeptidase; T-Bil, total bilirubin; INR, international normalized ratio; Y-J, Yemenite Jewish; Ashk, Ashkenazi-Jewish; Alger, Algerian; A&W, alive and well.

conduction velocity (NCV). An exception was patient 1116, who suffered from dilated cardiomyopathy with impaired myocardial contractility and from nephromegaly with massive proteinuria that resolved only after several months.

During the acute phase, liver biopsy, performed in two patients, revealed minimal chronic inflammation and mild focal proliferation of bile ductules with variable portal and sinusoidal fibrosis. In the parenchyma, extensive oncocytic change in the hepatocytes was noted, as well as focal macrovesicular steatosis and focal ballooning of their cytoplasm (Figure 1A). Iron stain revealed slight accumulation of pigment, primarily within the hepatocytes. In the liver sample of patient 3015, obtained when the patient was 9 months of age, during which time the patient was still symptomatic, the liver architecture was markedly disrupted by micronodule formation separated by delicate fibrous septae. The nodules were composed of enlarged hepatocytes, with thickening of the liver plates and hepatocanalicular cholestasis (Figure 1B). The pathological and histochemical examinations of muscle tissue obtained from three patients were invariably normal.

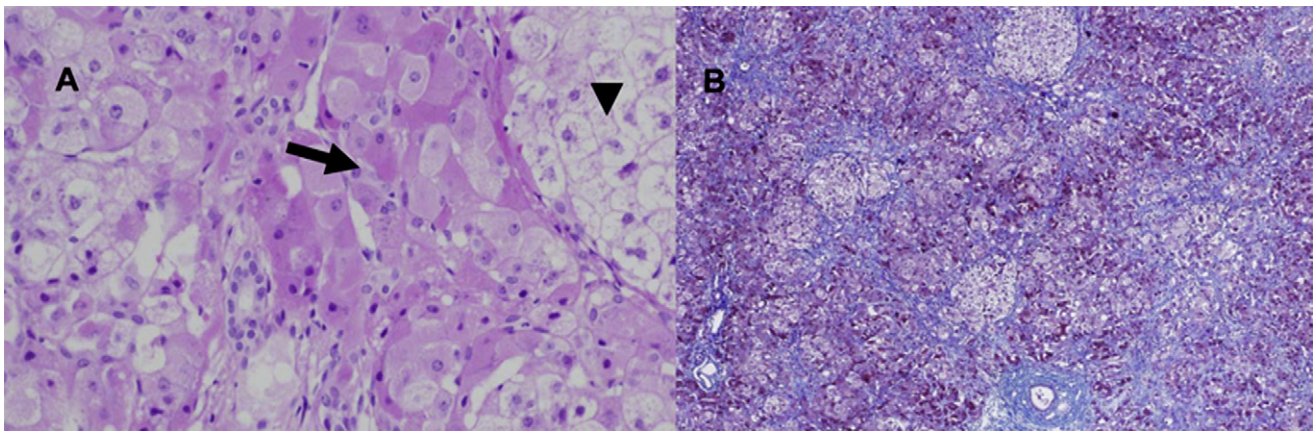

Figure 1. Histopathological Findings in Liver Tissue
(A) Liver tissue showing marked oncocytic change in the hepatocytes (arrow) and focal ballooning degeneration of hepatocytes (arrowhead) (H&E).
(B) Hepatic tissue with markedly disrupted architecture characterized by nodule formation with prominent sinusoidal fibrosis (Masson Trichrome stain).

Table 2. Mitochondrial Enzymatic Activities, mtDNA Content, and *TRMU* Genotype of the Patients

Patient	Tissue	Citrate Synthase	Complex I	Complex II	Complex II+III	Complex IV	mtDNA Content	*TRMU* Genotype
2624	L	270%	29%	66%	43%	15%		Y77H/Y77H
	M	21%	71%	95%	76%	47%		
3032	L	238%	7%	51%	8%	22%	143%	Y77H/Y77H
1432	L	211%		75%	34%	10%	78%	Y77H/[a]
	M	38%		132%	108%	86%		
1116	L*	65%		260%	141%	103%		Y77H/Y77H
	M	64%		75%	63%	60%		
111	N.A.							Y77H/Y77H
421	N.A.							Y77H/Y77H
2859	L	208%	11%	65%	12%	16%	380%	Y77H/c.706-1G>A[b]
2375	N.A.							L233F/A10S
2006	L	148%	25%	70%	17%		250%	V279M/c.500-510del
3015	L	302%	8%	80%	39%	14%	104%	G272D/G272D
1910	M	75%	42%	97%	89%	29%	107%	G14S/[c]
Akh	M		12%		44%	17%		M1K/M1K
Aza	M	68%	14%		47%	22%	38%	M1K/M1K

Tissue samples (L, liver; M, muscle) were obtained during the acute phase, with the exception of patient 1116, whose liver (L*) was obtained 6 mo after the acute episode. N.A. denotes not available. All enzymatic activities are given as a percentage of the control mean and are normalized for citrate synthase activity. The citrate synthase activity and mtDNA content are given as a percentage of the control mean.
[a] A second mutation was not identified in the 11 exons of the *TRMU* gene, and cDNA of this patient was not available.
[b] This mutation resulted in exon 3 skipping (107 bp).
[c] The patient was heterozygous for the G14S mutation, but the patient's cDNA consisted of only the paternal allele carrying this mutation.

The enzymatic activities of the mitochondrial respiratory chain complexes I–IV in liver homogenate and in mitochondria isolated from the patients' muscles were determined by standard spectrophotometric methods.[4] In liver obtained during the acute phase, the activities of complexes I, III, and IV normalized to citrate synthase activity were markedly reduced; only complex II activity was relatively preserved (Table 2). The mitochondrial respiratory chain activities were normal in homogenate of the liver tissue obtained six months after the onset of the acute episode in patient 1116. In mitochondria isolated from the acute phase muscle tissue, only complex IV activity was slightly reduced.

The markedly reduced activities of complexes I, III, and IV in liver homogenate and the relatively normal activity of complex II—the only complex that is encoded solely by the nuclear genome—suggested a defect in the synthesis of the mtDNA-encoded proteins. The normal ratio of mtDNA to nuclear DNA in the patients' liver, as determined by real-time PCR (Table 2), ruled out mtDNA depletion. The mtDNA transcription was investigated in patient 2859 fibroblasts by determination of the abundance of the 12S and 16S rRNA transcripts and of the COX2 mRNA. The normal results of these analyses (data not shown) not only indicated intact transcription but have also excluded a defect in the mitochondrial ribosomal assembly, which would lead to a severe reduction of the rRNA transcripts.[5] Assuming a defect in mitochondrial translation, we determined the sequence of the 22 mitochondrial *tRNA* genes and the two *rRNA* genes in patient 2859 liver but did not identify any mutation, suggesting a defect in a nuclear-encoded mitochondrial translation factor. We next quantified mitochondrial translation by pulse-chase incorporation of ^{35}S-methionine into mitochondrially synthesized polypeptides in fibroblasts of three patients, in the presence of 0.5 mg/ml of emetine for inhibition of cytoplasmic translation, as previously described.[6] To assure correct quantification, we performed immunoblotting of tubulin in the same samples. In all three patients' fibroblasts, the overall mitochondrial translation level was reproducibly twice lower than that in control cells (Figure 2).

In order to localize the mutated gene, we performed homozygosity mapping with the DNA of patients 3032 and 2624, using the GeneChip Human Mapping 250K Nsp Array of Affymetrix, as previously described.[8] All experiments involving DNA of the patients, their relatives, healthy controls, and patients' cells were approved by the Hadassah Ethical Review Committee. This analysis disclosed two nonoverlapping homozygous regions > 5 Mb in each sample. The only genomic region of identical homozygous markers was a 3.06 Mb region on chromosome 22, from 43.49 Mb to 46.55 Mb, which included 223 SNP markers (from rs5765930 to rs7292036). Within this

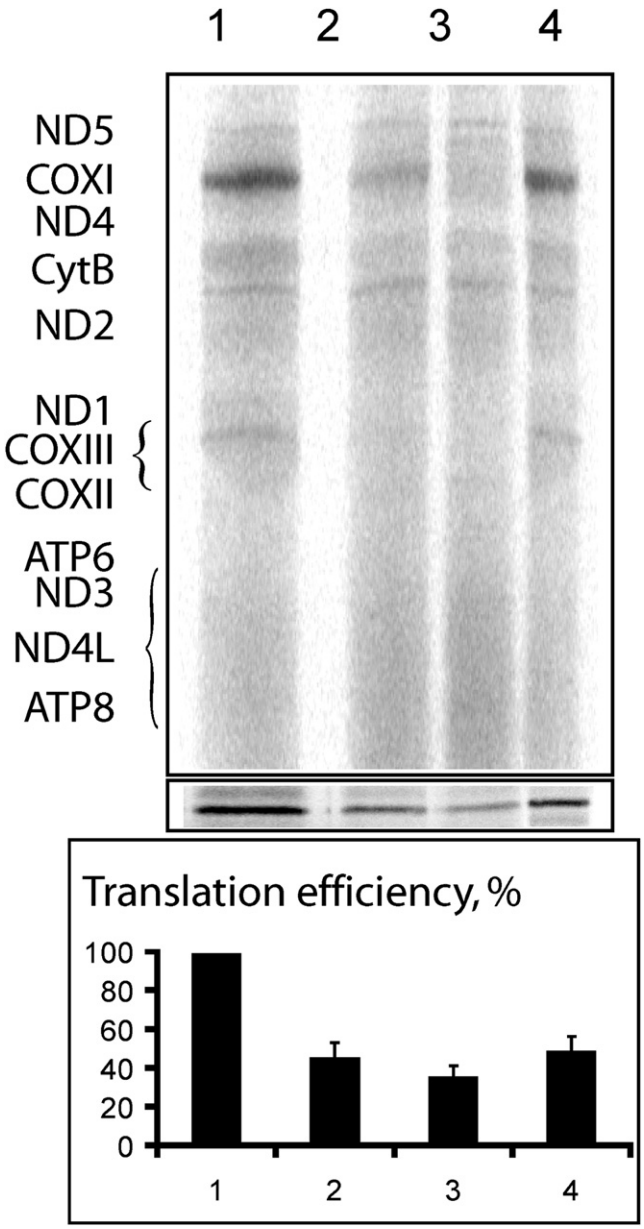

Figure 2. Analysis of Mitochondrial Translation in the Patients' Fibroblasts

The mitochondrial translation products on SDS-PAAG are indicated according to a standard pattern.[7] Assays were performed in the fibroblasts of a control (lane 1) and three patients (lanes 2–4 for patients 2624, 2859, and 1910, respectively). The relative values were normalized to tubulin (panel below the autoradiographs) and are presented as a diagram. Error bars represent the results of two independent experiments.

region, there were 27 open reading frames, including *TRMU* (MIM 610230), which encodes the mitochondria-specific tRNA-modifying enzyme, tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase. Sequence determination of the 11 exons of *TRMU* and their flanking intronic regions identified a homozygous mutation, c.232T>C, which changes the highly conserved Tyr77 to His (Y77H). Five patients were homozygous for the mutation and two were heterozygous. Because the mutation created an MslI restriction site, we used this enzyme for the screening of 120 anonymous individuals of Yemenite Jewish origin and identified three carriers. Patient 2859, who was heterozygous for the Y77H mutation on the maternal allele, carried a second mutation, c.706-1G>A, on her paternal allele, which resulted in skipping of exon 3. The only Yemenite Jewish patient who did not carry the Y77H mutation, patient 2375, was compound heterozygous for c.697C>T (L233F) and c.28G>T (A10S), both changing highly conserved residues. Because the patient cDNA and parental DNA were not available, we could not assign the phase of the mutations. We then screened the *TRMU* gene for mutations in DNA of patients of non-Yemenite-Jewish origin who presented with infantile liver failure and a similar pattern of respiratory chain defects and identified five additional mutations in five unrelated patients (Table 1 and Table 2). Four mutations, c.2T>A (M1K), c.40G>A (G14S), c.835G>A (V279M), and c.815G>A (G272D), changed highly conserved residues, and the fifth, c.500-510del, was a frame-shift mutation. Patient 1910 carried the G14S mutation on his paternal allele, but homozygosity for this mutation was present in cDNA produced from his fibroblasts, suggesting a nonexpressing maternal allele. No mutation was detected in the promoter region and at the ~1100 nucleotide, which separates *TRMU* from the neighboring 5′ gene. The M1K mutation was identified in two Algerian patients, the G14S mutation was found in an Ashkenazi Jewish patient, and the rest of the mutations were detected in patients of Arabic ethnicity. We did not detect any carrier for the M1K mutation among 106 individuals of North African origin. Altogether, we identified nine mutations in 13 patients who presented with acute liver failure during infancy (Figure 3). Of note, no mutations were detected in the *TRMU* gene of 17 unrelated patients of North African, Jewish, and Arabic origin having a similar pattern of enzymatic defects and presenting with isolated mitochondrial liver disease immediately after birth, nor in three patients with chronic extrahepatic involvement, indicating that mutations in the *TRMU* gene primarily affect the liver at a specific window of time.

The human *TRMU* gene encodes 421-aa-long protein that participates in the modification of mitochondrial tRNAs and is therefore important for mitochondrial translation. Specifically, it is responsible for the 2-thiolation of the wobble position of the mitochondrial tRNA-Lys, tRNA-Gln, and tRNA-Glu. We therefore studied the 2-thiouridylation at the wobble nucleotide of these three tRNAs in patients 2624, 2859, and 1910. This was tested by retardation in an electrophoretic system consisting of a 10% PAAG with 7 M urea, tris-borate buffer polymerized in the presence of 50 μg/ml of (N-)Acroylamino-phenyl-mercuric chloride) (APM), which was synthesized by the procedure described by Igloi.[9] Total cellular RNA was isolated with Trizol-reagent (Invitrogen). RNA hybridization was performed as described by Shigi et al.,[10] with the following [32P]-5′-end-labeled oligonucleotide probes: mt-tRNA-Lys, GGTTCTCTTAATCTTTAAC; mt-tRNA-Glu, CCACGACCA

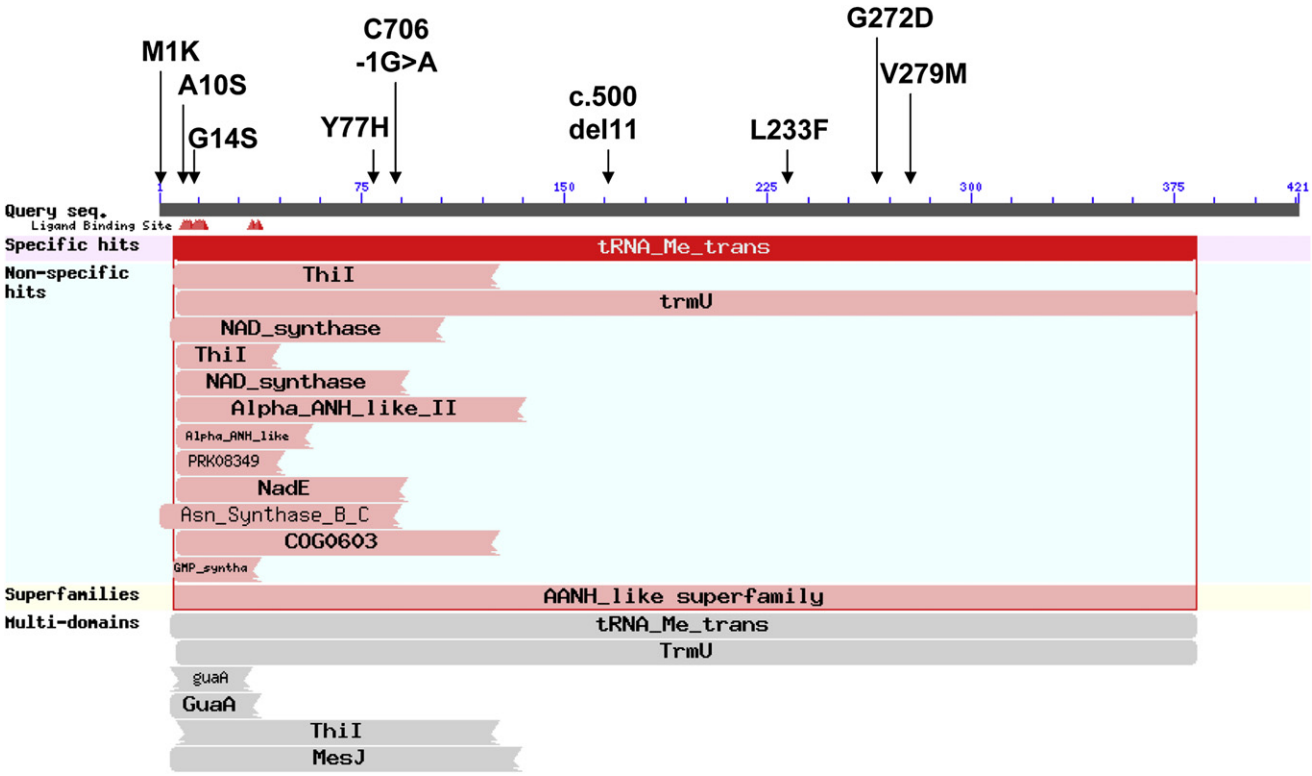

Figure 3. The Mutations Identified in the *TRMU* Gene
The mutations identified in the *TRMU* gene of patients with acute liver failure, depicted on a schematic representation of the conserved domains (NCBI conserved domains website). G14 is one of six residues (red arrowheads) that form the P loop motif (SGGXDS), which is an ATP-binding motif commonly found in enzymes responsible for RNA modifications.[14]

ATGATATG; mt-tRNA-Gln, CGAACCCATCCCTGAG, and cy-tRNA-Lys, ACTTGAACCCTGGACC. In this system, the thiolated tRNAs are covalently retained by Hg-groups incorporated in the polyacrylamide gel and have lower mobility than nonthiolated ones. For the purpose of quantification, hybridizations were performed in parallel after separation of the same samples on gels without APM. The results of this analysis clearly disclosed that the amount of the thio-modified mitochondrial tRNAs is severely reduced in all three patients, whereas the pattern of hybridization obtained for the cytosolic tRNA (cy-tRNA-Lys) modified by another enzyme was similar in control and patient cells (Figure 4). Finally, the pattern of hybridization obtained for the mitochondrial tRNA-Leu, which is not subjected to thio-modification, was similar in control and patient cells (data not shown).

To study the effect of the hypomodification on tRNA stability, we performed RNA hybridization of total RNA extracted from the patients' fibroblasts. This analysis disclosed slightly lower levels of several tRNAs, which was nonspecific for the thio-modified tRNAs (Figure 5). We therefore conclude that the *TRMU* mutations did not affect either the transcription level or the stability of the hypomodified tRNAs to a significant extent.

In view of these findings, we propose that the mitochondrial translation defect in our patients is the result of reduced modification of several mitochondrial tRNAs. In *E. coli*, the 2-thiouridylation stabilizes the codon-anticodon interaction and confers the tRNA an efficient ribosome binding.[11,12] Until now, only one mutation in the human *TRMU* gene, A10S, had been reported. Homozygosity for this mutation had aggravated the deafness phenotype of patients who harbored the homoplasmic A1555G mutation in the mitochondrial gene encoding the 12S rRNA, *MTRNR1* (MIM 561000). The combination of *TRMU* and *MTRNR1* mutations was associated with reduced 2-thiouridylation and low content of the mitochondrial tRNAs, which led to impaired mitochondrial protein synthesis.[13]

The TRMU protein requires sulfur for its activity; cysteine desulfurase, which transfers sulfur from cysteine to the TRMU ortholog, has been shown to be essential for the thio-modification of bacterial tRNAs.[14] The availability of cysteine in the neonatal period is limited because its endogenous synthesis from methionine by the transsulfuration pathway is markedly attenuated. The activity of the rate-limiting enzyme in the pathway, cystathionase, is very low at birth and increases slowly during the first few months of life.[15] For this reason, cysteine is considered a conditionally essential amino acid, at least in preterm infants. Furthermore, metallothionein, a source of cysteine, is at its peak at birth and declines rapidly during the first month of life.[16] We propose that there is a window of time, during 1–4 months of age, whereby patients with TRMU mutations are at an increased risk of developing liver

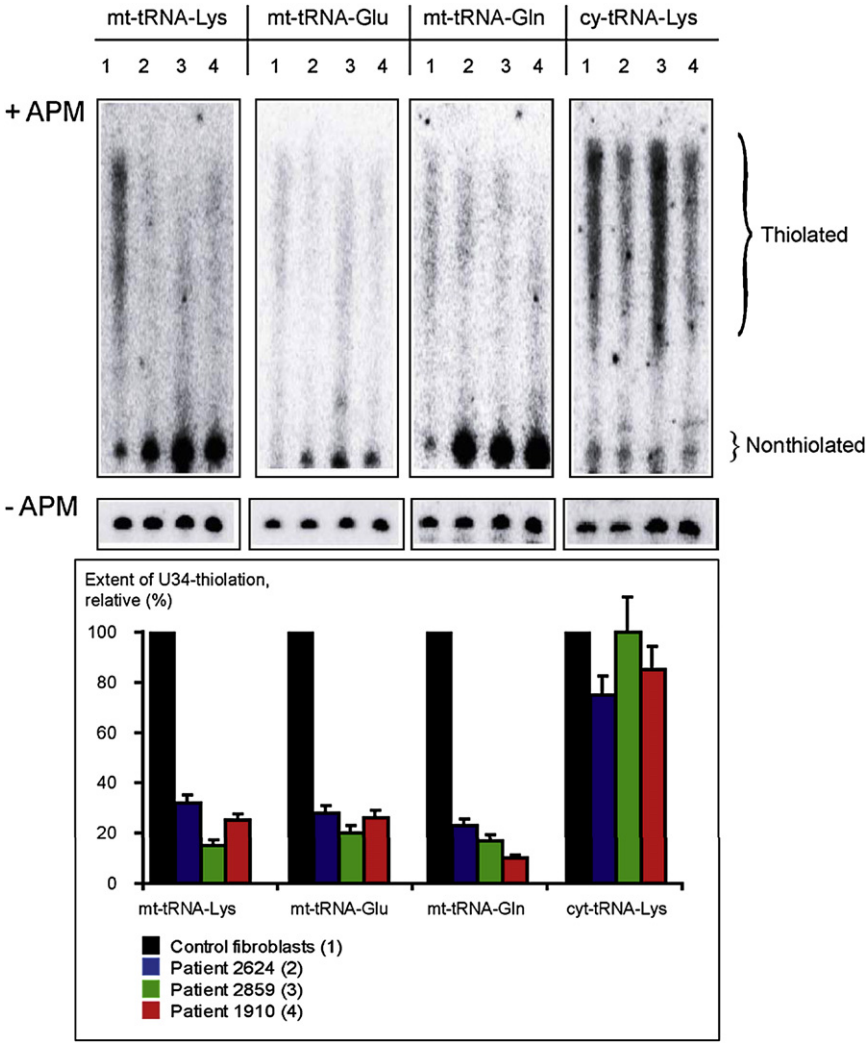

Figure 4. Thio-Modification in Mitochondrial tRNAs

Analysis of thio-modification at position 2 of the wobble uridine via RNA hybridization of mitochondrial (mt-tRNA-Lys, mt-tRNA-Glu, and mt-tRNA-Gln) and cytoplasmic (cy-tRNA-Lys) tRNAs separated in APM-containing gels (+APM, upper panel). For quantification, the same amount of RNA obtained from patient and control fibroblasts was separated in gels without APM (-APM, middle panel). The retarded diffused zones correspond to the thiolated and nonthiolated versions of each tRNA (Thiolated and Nonthiolated, respectively). The hybridization probes and the numbers of the RNA samples are indicated at the top of the autoradiographs; the numbers correspond to the samples described under the diagram at the bottom. The quantification of the modification is presented at the bottom panel and is expressed as a percentage of the thiolated signal from the thiolated + nonthiolated signals (as presented in the -APM gel at the middle panel), normalized against the control fibroblasts. The deviations are indicated as a result of two to three independent measures (for the control fibroblasts, the deviation was quasi null and is therefore not indicated).

failure. Dietary- and metallothionein-derived cysteine may provide some protection during the first month of life, and the rising activity of cystathionase serves a similar purpose after 3–4 months of age. Nonetheless, an intercurrent illness combined with reduced dietary (cysteine) intake at 1–4 months of age may further compromise TRMU activity in these patients. This may account for the timing of the clinical presentation, mostly at 2–4 months of age, and the lack of recurrence in patients who survive the neonatal episode. Sequence determination of the *TRMU* gene is warranted in patients with acute liver failure in the first year of life, predominantly when the onset is at 1–4 months of age.

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Web Resources

The URLs for data presented herein are as follows:

NCBI Conserved Domains, http://www.ncbi.nlm.nih.gov/sites/entrez?db=cdd
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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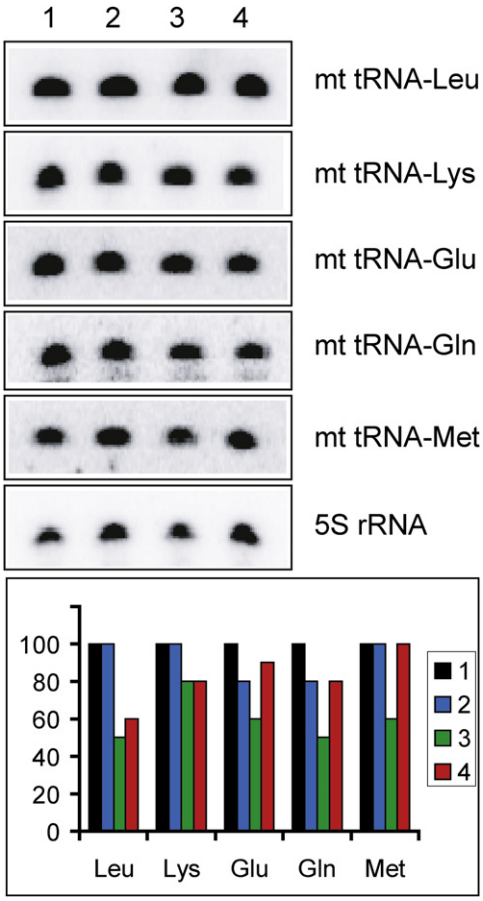

Figure 5. Quantification of Mitochondrial tRNAs by RNA Hybridization

RNA was isolated from the fibroblasts of a control (1) and three patients (2–4 for patients 2624, 2859, and 1910, respectively). Relative values normalized to the 5S rRNA signal are presented in the diagram below the autoradiographs (the various tRNAs are indicated on the x axis only by their respective amino acid abbreviation; thus, Leu stands for mitochondrial tRNA-Leu transcript). Average values of two to three independent experiments are presented. The error was never higher than 10%.

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II.2.2 Studying the mitochondrial tRNA import in yeast *Saccharomyces cerevisiae*

In a parallel with the main theme of my thesis, I have also participated in other studies running in our laboratory and concerning the mechanism of tRNA import in yeast *Saccharomyces cerevisiae*. Namely, I have participated in the identification of one of the tRK1 import factors, glycolytic enzyme enolase-2. The mechanism and function of tRNA import in yeast, as well as its possible application to modelling of gene therapy for human mitochondrial disorders are reviewed in **Publication 3**.

Publication 3

Import of nuclear DNA-encoded RNAs into mitochondria and mitochondrial translation

Tarassov, I., Kamenski, P., Kolesnikova, O., Karicheva, O., Martin, R.P., Krasheninnikov, I.A. and Entelis, N.

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Review

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

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KEY WORDS

mitochondria, translation, aminoacylation, base modification, RNA import, aminoacyl-tRNA synthetase, *Saccharomyces cerevisiae*, *Ashbya gossypii*

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ABSTRACT

Targeting nuclear DNA-encoded tRNA into mitochondria 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 it 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 proteins.[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), in 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 tRK3, 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 of 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 perturbations 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 localized in the introns, and only three—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 exploits 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 is altered 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

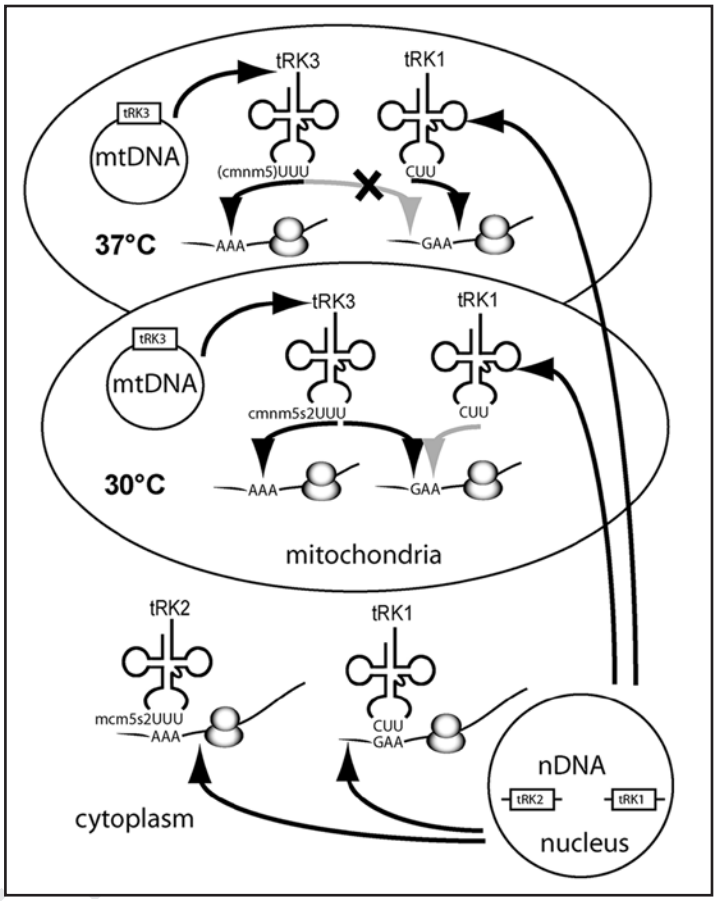

Figure 1. Hypothesis explaining the function of imported tRK1 in yeast mitochondria. mtDNA, mitochondrial DNA; nDNA, nuclear DNA. Solid arrows indicate either the RNA targeting direction, or the effective use of the given tRNA in translation. Gray arrows indicate optional or inhibited functions of tRNAs.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

of the modification group (before of after the modified nucleotide, 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 would not be 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.

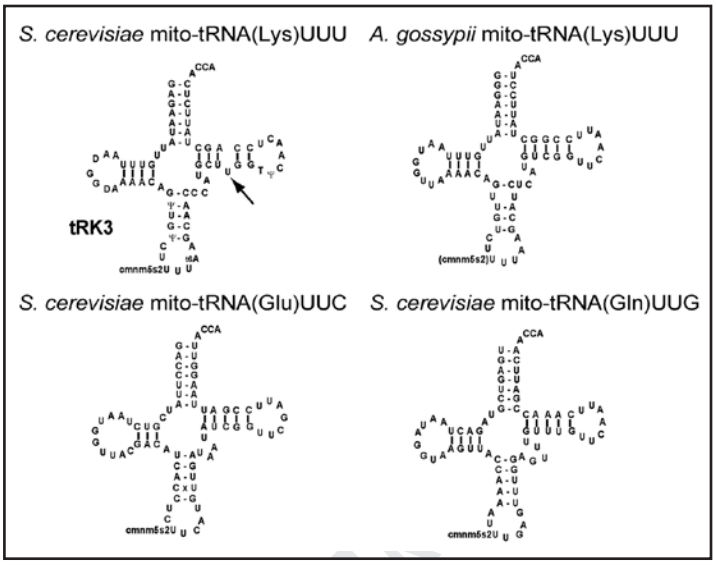

Figure 2. Comparison of secondary structures of *S. cerevisiae* and *A. gossypii* mitochondrial tRNAs bearing the cmnm5s2-modification. Cloverleaf structures were generated by the tRNAscan SE software available at http://rna.wustl.edu/tRNAscan-SE. The cmnm5s2-modification in *A. gossypii* mitochondrial $tRNA^{Lys}$ 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 $tRNAs^{Lys}$). The arrow indicates the position of a bulged U in the TΨC-arm of tRK3.

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 was 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 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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III MATERIALS AND METHODS

III.1 MATERIALS

III.1.1 Cell lines

III.1.1.1 *Escherichia coli* cell strains

For cloning and plasmid production, we used:

DH5α F- *end*A1 *gln*V44 *thi*-1 *rec*A1 *rel*A1 *gyr*A96 *deo*R *nup*G φ80d*lac*ZΔM15 Δ*(lac*ZYA-*arg*F*)* U169*, hsd*R17(rK- mK+)*,* λ–;

XL1 Blue *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17(rK- mK+) *sup*E44 *rel*A1 *lac* [F′ *pro*AB *lac*I^{q}ZΔM15 Tn*10* (Tet^{r})] (Stratagen);

E. cloni 10G F- *mcr*A Δ*(mrr-hsd*RMS-*mcr*BC*) end*A1 *rec*A1 φ80d*lac*ZΔM15 Δ*lac*X74 *ara*D139 Δ*(ara,leu)*7697 *gal*U *gal*K *rps*L *nup*Gλ *ton*A (Lucigen);

SURE e14^{-}(McrA^{-}) Δ*(mcr*CB-*hsd*SMR-*mrr)*171 *end*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *lac rec*B *rec*J *sbc*C *umu*C*::*Tn*5* (Kan^{r}) *uvr*C [F′ *pro*AB *lac*I^{q}ZΔM15 Tn*10* (Tet^{r})] (Stratagen).

For PCR site-directed mutagenesis, we used:

XL10-Gold *end*A1 *gln*V44 *rec*A1 *thi*-1 *gyr*A96 *rel*A1 *lac Hte* Δ*(mcr*A*)*183 Δ*(mcr*CB-*hsd*SMR-*mrr)*173 Tet^{r} [F′ *pro*AB *lac*I^{q}ZΔM15 Tn*10* (Tet^{r} Amy Cam^{r})] (Stratagen).

For expression of recombinant proteins, we used:

BL21 CodonPlus (DE3)-RIL F– *omp*T *hsd*S(rB- mB+) *dcm*$^{+}$ Tet^{r} *gal* λ(DE3) *end*A Hte [*arg*U *ile*Y *leu*W Cam^{r}] (Stratagen).

III.1.1.2 Human cell lines

HepG2 hepatocellular carcinoma cells (tissue origin: liver) (provided by IGBMC) were used for isolation of mitochondria and import directing proteins (IDPs) for *in vitro* assays; for testing of transfection efficiency and preliminary transfection tests with pBK-CMV-tRK plasmids.

HEK-293T cells (tissue origin: kidneys) (provided by IGBMC) were used to produce viral particles for lentiviral transfection. These cells are isolated from human embryonic kidneys (HEK). The 293T cells are transformed with the large T antigen.

143B.TK- human bone osteosarcoma cells (tissue origin: bone marrow) (kindly provided by E.A. Shoubridge, Montreal Neurologic Institute, Quebec, Canada) were used as a wild-type control cells for MELAS cybrid cells.

Two *trans*mitochondrial MELAS cybrid cell lines were used in this study. Both were obtained by fusing rho0 cells from osteosarcoma cell line 143B.TK- with cytoplasts from clonal primary myoblasts established from a patient carrying the A3243G mtDNA tRNALeu(UUR) point mutation (MELAS mutation) as described elsewhere (King and Attardi, 1989). MELAS cybrid cells bearing 85±5% of m.3243A>G MELAS mutation (MELAS-1) were kindly provided by R. Lightowelrs (The University of Newcastle, UK). MELAS cybrid cells bearing 90±5% of m.3243A>G MELAS mutation (MELAS-2) were kindly provided by E.A. Shoubridge.

III.1.2 Growth media and conditions

III.1.2.1 Media for bacterial cell lines

E. coli cells were routinely grown in LB (Lysogeny Broth, Luria-Bertrani) medium (bacto-trypton 10g/l, yest extract 5g/l, NaCl 5g/l) at 37°C. Selection of transformants was performed in the presence of ampicillin (100 μg/ml), kanamycine (30 μg/ml) or hygromycin (20 μg/ml) depending on plasmid. White-blue selection for cloning purpose was performed in the presence of 80 μg/ml of X-Gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside) and 0.2 mM of IPTG (isopropyl β-D-1-thiogalactopyranoside).

E. coli BL21 were grown in LB or SOC (Super-Optimal broth with Catabolite repression) medium (bacto-trypton 20g/l, yest extract 5g/l, NaCl 5g/l, 0.01 M $MgCl_2$, 0.01 M $MgSO_4$, 0.02 M of glucose) at 37°C. Selection of transformants was performed in the presence of ampicillin (100 μg/ml), chloramphenicol (34 μg/ml) and tetracycline (12.5 μg/ml).

"Recovery media" from Lucigen was used to improve transformation efficiency with ligation mix. Plates with "Fast-Media Hygro X-Gal" available from InvivoGen were used for growing *E. coli* psiRNA-h7SK (Hyg^r) cells.

III.1.2.2 Media for human cell lines

MELAS-1 cybrid cells were grown in Ham's F14 medium with 6 mg/ml glucose and 1 mg/ml ATP (VWR International) supplemented with 20% (w : v) fetal calf serum (FCS) and 50 mg/ml uridine.

MELAS-2 cybrid cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) with high glucose (4.5 g/l), sodium pyruvate (110 mg/l) and L-glutamine (2 mM) from Sigma, and supplemented with 10% (w : v) fetal calf serum (FCS) and 50 mg/ml uridine.

HepG2 and HEK-293T cells were grown in DMEM (Sigma), containing 1g/l of glucose, 110 mg/l sodium pyruvate and supplemented with 10% (w : v) fetal calf serum (FCS).

Penicillin/streptomycin and fungison were currently added in all medium at standart concentrations, to prevent bacterial and fungal contamination.

For selection of transfected cells we used G418 (200 μg/ml), Hygromycin (200 μg/ml) or Puromycin (2 μg/ml).

143B control cells were grown in the same conditions as MELAS cybrid cells.

Cell transfection was performed either in OptiMEM (Sigma) or in DMEM without serum and antibiotics.

III.1.3 Plasmids

The maps of the plasmids used in this study are shown in Fig. 3.1.

III.1.3.1 Plasmids for *in vitro* T7-transcription of tRNA genes

pUC18/pUC19 (Amp^r) plasmids were used for initial clonning of yeast tRNA genes and their mutagenesis. pUC19 is a small, high-copy number *E. coli* plasmid cloning vector. pUC18 is identical to pUC19 except that the multiple cloning site is inverted.

III.1.3.2 Plasmid for protein gene expression in bacteria

For production of recombinant hmtLeuRS we used pET3a (Amp^r) expression plasmid containing the T7 RNA polymerase promoter, that provided expression of protein gene in BL21 CodonPlus (DE3)-RIL *E. coli* strain expressing under IPTG induction the RNA polymerase of T7 bactreiophage. We cloned in pET3a the coding sequence of human mitochondrial leucyl-tRNA synthase (hmtLeuRS) without mitochondrial import signal derived from cDNA in pBlueScript II SK^+ (Amp^r) vector provided by Japanese cDNA RIKEN Collection.

III.1.3.3 Plasmids for gene expression in human cells

pBKE (Kan^r) mammalian expression vector is derived from commercially available vector **pBK-CMV** (Stratagen) by excision of 202 base pairs between *SpeI* (1098) and *NheI* (1300) sites. The pBK-CMV phagemid vector is a cloning vector derived from a high-copy-number pUC-based plasmid. This vector allows expression in both eukaryotic and prokaryotic systems. Normally, the cytomegalovirus (CMV) immediate early promoter for RNA polymerase II drives gene expression in eukaryotic cells. However, in order to express tRNA genes from their internal promoter for RNA polymerase III (POLR III) and provide their further correct maturation, they were cloned in pBK-CMV vector in an opposite direction with the respect to the CMV promoter.

psiRNA-h7SK (Hyg^r) vector belongs to a family of expression vectors designed to generate shRNA from the human 7SK small nuclear RNA POLR III promoter. The *hph* gene from *E. coli* confers resistance to Hygromycin B. The *hph* gene is situated under the control of the CMV enhancer/promoter in tandem with the bacterial EM7 promoter allowing selection in both mammalian cells and *E. coli*.

pLKO.1-TRC cloning vector (Amp^r) (Addgene) is a replication-incompetent lentiviral vector (was kindly provided by I. Davidson, IGBMC) (Moffat et al., 2006). pLKO.1 can be introduced into cells *via* direct transfection, or can be converted into lentiviral particles for subsequent infection of a target cell line. Once introduced, the puromycin resistance marker encoded in pLKO.1 allows for convenient stable selection.

To produce lentiviral particules we used together with pLKO.1 three packaging plasmids from Invitrogen:

- **pLP1** (Amp^r), containes HIV *gag* encoding for the viral core proteins required for forming of the lentivirus and HIV *pol* encoding for the viral replication enzymes required for replication and integration of lentivirus. Expression of *gag* and *pol* depends on CMV promoter;

- **pLP2** (Amp^r), encodes the Rev protein that induce Gag and Pol expression and promotes the nuclear export of viral RNA for packaging into viral particles;

- **pLP/VSVG** (Amp^r), encodes the envelope G glycoprotein from Vesicular Stomatitis Virus to allow production of virus.

To estimate the efficiency of transfection of human cells we used either GFP-coding **pmaxGFP** plasmid from Amaxa, or **PGL3-Control** vector from Promega constitutively expressing firefly luciferase.

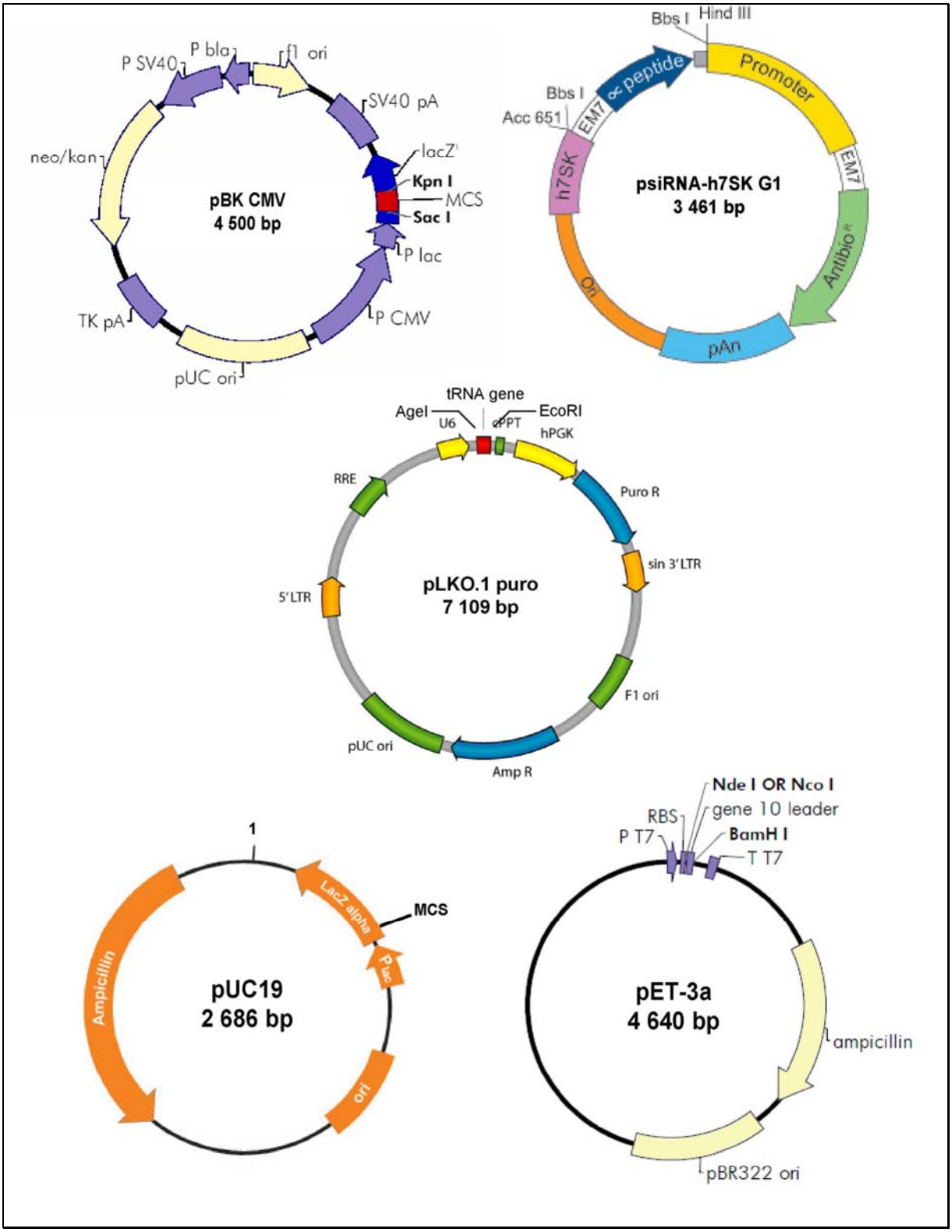

Fig. 3.1. The maps of cloning vectors used in this study (see the text for details)

III.2 METHODS

Basic molecular biology methods (*i.e.* cloning, sequencing, agarose and polyacrylamide gel electrophoresis of nucleic acids, *etc*.) used are as described in a laboratory manual "Molecular Cloning" (Sambrook, 2001).

III.2.1 Construction and cloning of recombinant tRNA genes

III.2.1.1 pUC18/pUC19 constructs

The genes of yeast *Sachoromices cerevisiae* lysine tRNAs (tRKs), tRK1, tRK2 (G1-C72; G73; U34) and tRK3, were cloned previously in pUC18/pUC19 plasmids either in *BamHI*, or in *HindIII/EcoRI* sites. T7 promoter sequence at 5'–end and *BstNI* site at 3'–end were introduced by PCR to further gave rise to properly processed tRNA with the CCA at 3'–end. Mutations aimed to change tRNA aminoacylation identity (Lys > Leu) were introduced in pUC-tRK constructs by several steps of PCR-mutagenesis.

PCR Site-Directed Mutagenesis

"Quick Change Site-Directed Mutagenesis Kit" (Stratagene) was used to change anticodon nucleotides (underlined) following manufacturer protocol. tRK sequences were PCR amplified by *PfuTurbo* DNA-polymerase from specific primers carrying desired mutation in anticodon nucleotides (underlined):

trk1UAAAs 5' GACTTAAAATCATAAGG 3'
trk1UAABr 5' TATGATTTTAAGTCATACGC 3'
trk1CAAAs 5' GACTCAAAATCATAAGG 3'
trk1CAABr 5' TATGATTTTGAGTCATACGC 3'
trk2UAAAs 5' GTTCGGCTTAAAACCG 3'
trk2UAABr 5' CATTTCGGTTTTAAGCCG 3'
trk2CAAAs 5' GTTCGGCTCAAAACCG 3'
trk2CAABr 5' CATTTCGGTTTTGAGCCG 3'
trk3UAAAs 5' GTCTTAAAAGCAACCC 3'
trk3UAABr 5' GCTTTTAAGACAAC 3'
trk3CAAAs 5' CAGTTGTCTCAAAAGCAACCC 3'
trk3CAABr 5' GGGTTGCTTTTGAGACAACTG 3'

PCR mix was treated by methylation sensitive *DpnI* restriction endonuclease (10 u/rxn) at 37°C for 1 min to digest the initial pUC19-tRK plasmid. 1 µl of *DpnI*-treated PCR mix was used to transform *E. coli* XL-10 Gold cells (Stratagen). Transformation was performed on ice for 30 min, followed by heat shock 45 sec at 42 ^{o}C and ice-cooling for 2 min. Further 0.5 ml of LB medium warmed at 42 ^{o}C was added to cells, followed by 1 h incubation upon shacking at 37^{o}C and seeding on LB agar plates with ampicillin.

PCR Mutagenesis

The discriminator base A73 for human mt-tRNA$^{Leu(UUR)}$ was introduced in tRK genes by PCR using oligonucleotides (mutations introduced – in bold):

trk1-T7 5'G*GGATCC*ATAATACGACTCACTATA-GCCTTGTTGGCG 3'
BamH1 T7 promoter tRK1

trk1-A73-*BstNI* 5' GGGAT*CCTGG*-**T**GCCCTGTAGGGGGCTCG 3'
BstN1 tRK1

trk2-G1-T7 5'G*GGATCC*ATAATACGACTCACTATA-**G**CCTTGTTAGCTCAG 3'
BamH1 T7 promoter tRK2

trk2-C72A73-BstNI 5'GGGAT*CCTGG*-**TG**CCTCATAGGGGGCTCG 3'
BstN1 tRK2

After amplification, PCR products were purified either from native 10% PAAG-TAE, or from 2% low melting agarose gel. Elution from PAAG gel was performed overnight at room temperature in the elution buffer (EB) containing 0,5 M NH_4Ac, 10 mM MgAc, 0,1% SDS, 0,1 mM EDTA (pH 8,0). Elution from agarose gel was done using "Qiaquick Gel Extraction Kit" (Qiagen). Purified PCR products were cut with *BamHI* restriction exonuclease and cloned into *BamHI* site of pUC19 vector.

III.2.1.2 pBKE constructs

For expression in MELAS cybrid cells tRK1UAA/CAA, tRK2UAA/CAA, tRK3UAA/CAA genes we re-cloned in pBKE (Kan^{r}) vector in the context of non-coding flanking regions of one of the natively well expressed tRK1 copies. For this, we used the following oligonucleotides:

trk1/F1-Aviv

5’GGCAAGATCTGGTCAGATTTCCAATAACAGAATATCCTTGTTAGCCTTGTTGGCG 3’
BglII *tRK flanking sequence* tRK1

trk1 /F1-A73-Bviv

5’GCAAGATCTGTCATCGTGTTTTAAAAAAAAAAAGAA**T**GCCCTGTAGGGGGCTC 3’
BglII *tRK flanking sequence* tRK1

trk2 /F1-G1-Aviv

5’GGCAAGATCTGGTCAGATTTCCAATAACAGAATATCCTTGTTA**G**CCTTGTTAGCTCAG 3’
BglII *tRK flanking sequence* tRK2

trk2 /F1-C72A73-Bviv

5’GGCAAGATCTGTCATCGTGTTTTAAAAAAAAAAAGAA**TG**CCTCATAGGGGGCTCG 3’
BglII *tRK flanking sequence* tRK2

trk3/F1-Aviv

5’CCCAAGAGATCTGGTCAGATTTCCAATAACAGAATAGAGAATATTGTTTAATG 3’
BglII *tRK flanking sequence* tRK3

trk3 /F1-Bviv

5’CCCAAGAGATCTGTCATCGTGTTTTAAAAAAAAAAAGAATGAGAATAGCTGGAGTTG 3’
BglII *tRK flanking sequence* tRK3

Purified PCR products were cut with *BglII* restriction exonuclease and cloned into *BamHI* site of pBKE vector, since these two enzymes produce compatible sticky ends.

III.2.1.3 psi-RNA constructs

To express tRK3UAA/CAA and human mt-tRNA$^{Leu(UUR)}$ genes in MELAS cybrid cells from the external promoter, we cloned them in psiRNA-h7SK (Hyg^{r}) vector under control of human 7SK small nuclear RNA POLR III promoter. tRNA genes were cloned in *Acc651/HindIII* sites using oligonucleotides:

psi-trk3-As 5'CGCGGTACCTC-$\overset{+1}{G}$AGAATATTGTTTAATGG 3'
Acc651

psi-trk3-Br 5'ACGAAGCTTTTCCAAAAA-TGAGAATAGCTGGAGTTG 3'
HindIII terminator

psi-tLeuUUR-As 5'CGCGGTACCTC-$\overset{+1}{G}$TTAAGATGGCAGAGCCC 3'
Acc651

psi-tLeuUUR-Br 5'ACGAAGCTTTTCCAAAAA-TGTTAAGAAGAGGAATTGAAC 3'
HindIII terminator

Oligonucleotides were designed according to manufacturer instructions to produce the correct strart (+1 G) of transcription and poly U termination signal for RNA-polymerase III.

III.2.1.4 pLKO.1 constructs

For stable lentiviral transfection tRK1UAA/CAA and tRK2UAA/CAA genes were cloned in pLKO.1 (Amp^r) lentiviral vector in *AgeI/EcoRI* sites under the control of external U6-promoter without any flanking regions using oligonucleotides:

trk1plkoAs 5' GGCAACCGGT-GCCTTGTTGGCG 3'
AgeI tRK1

trk1plkoBr 5' GGCAGAATTCAAAAA-**T**GCCCTGTAGGG 3'
EcoRI terminator tRK1

trk2plkoAs 5' GGCAACCGGT-**G**CCTTGTTAGCTCAG 3'
AgeI tRK2

trk2plkoBr 5' GGCAGAATTCAAAAA-**TG**CCTCATAGGGGG 3'
EcoRI terminator tRK2

III.2.2 Cloning of recombinant hmtLeuRS gene

HmtLeuRS gene lacking a sequence coding for the predicted mitochondrial signal peptide (186-302 nt coding for the first 39 amino acids) was cloned using PCR with specific primers:

start
LeuRSmtF 5'GGAATTCCATATGATCTACAGTGCCACGGGAAAGTG 3'
NdeI hmtLeuRS-N-ter

LeuRSmtR 5'CGGGATCCTCAGTGGTGGTGGTGGTGGTGATCTTGTACCAGG
BamHI 6-His tag hmtLeuRS-C-ter
AAGTTGATGAGGGCAG 3'

LeuRSmtR primer carried a sequence coding six histidine tag (6-His) for further purification. PCR amplification was performed using a mix of *Taq* (Amersham) and *Vent* (Pharmacia) polymerases in ratio 5/1 units (Barnes, 1994). After amplification, 2535 bp PCR product was purified from 1% agarose gel using "Qiaquick Gel Extraction Kit" (Qiagen), cut with *NdeI* and *BamHI* restriction exonucleases and cloned into pET3a vector under T7-promoter.

III.2.3 Transformation of *E.coli* cells

III.2.3.1 Chemical transformation

E. coli DH5α competent cells were prepared by a standard procedure (Sambrook, 2001) and stored at -80°C. Cells with 1-5 μg of plasmid DNA were incubated on ice for 20 min, followed by 30 sec heat shock at 42 ^{o}C and ice cooling for 2 min. 1 ml of LB medium was then added to cells, followed by 1h incubation upon shacking at 37^{o}C and seeding on LB agar plates with appropriate antibiotic.

III.2.3.2 Electroporation

Electroporation of commercial competent *Ecloni* cells was used for transformation with the ligation mixes during cloning procedure. Other bacterial strains were also transformed by electroporation in alternance with chemical transformation. Electrocompetent cells were prepared in the laboratory by a standard procedure (Sambrook, 2001) and stored at -80°C. Plasmid DNA or ligation mix were mixed on ice with 50 μl of electrocompetent cells, transferred in pre-chilled 0.1 cm gap Gene pulse cuvette (Bio-Rad, Hercules, CA, USA) and subjected to the electric pulse in a "Bio-Rad Pulser" at 1.66 kV, usually resulting in a time constant of 4.5 -5 msec. Immediately after that 1 ml of LB or "Recovery media" (Lucigen) was added to cells, cell suspension was transferred in a new tube and incubated for 1h at 37 ^{o}C under shacking. Further, cells were seeded on solid LB medium containing antibiotic.

III.2.4 Plasmid DNA preparation

To multiply DNA plasmid, we either transformed chemically *E. coli* DH5α cells or used electroporation of *E. coli XL1 Blue* cells. To isolate plasmid DNA, we routinely used Qiagene Mini, Midi or MaxiPrep Kits, as well as "Jetstar Plasmid Maxiprep Kit" from Genomed and "PureYield Plasmid System" from Promega. To prepare plasmid DNA for transfection of human cells, "QiaFilter Kit" from Qiagen was used to obtain the best efficiency of transcfection. Quantity and quality of plasmid DNA was evaluated spectrophotometrically (Nanodrop).

III.2.5 T7-transcription of tRNA genes

III.2.5.1 Standard T7 transcription

To obtain T7-transcripts of tRNAs having CCA at the 3'-end, pUC19-tRK plasmids were digested with *BstNI* (1 u of enzyme per 1 μg of plasmid DNA) for 2h at 65°C. Effeciency of digestion was checked by electrophoresis in 1% agarose gel.

T7-transcription was performed using either T7-RNA-polymerase or "RiboMax™ Express Large Scale RNA Production Systems" from Promega. In the first case, the reaction was performed in 50 μl of solution containing 10-15 μg of *BstNI* treated plasmid DNA, 160 u/μl of T7-RNA polymerase, 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 20 mM DTT, 0.05% Tween-20, 1 mM of each NTP and RNasin for 4 h at 37°C. New portion of T7-RNA polymerase (40 u) was added each hour. When "RiboMax™" Kit was used 50 μl of reaction mixture contained 5 μg of *BstNI* treated plasmid DNA, 5 μl of enzyme mix (T7-RNA polymerase, RNasin, yeast pyrophosphatase and ribonuclease inhibitor) in "RiboMax™ Express T7" buffer containing NTP mix. Reaction was performed for 40 min at 37°C. At the end of the reaction in both cases, we added 1 u of DNase per 1 μg of plasmid and incubated reaction mixture for 15 min at 37°C. An equal volume of a loading buffer (0.05% bromphenol blue, 0.05% xylene cyanol, 0.5 mM EDTA in deionized formamide) was then added to the reaction mixture and incubated for 5 min at 90°C. Transcription products were separated by preparative denaturing 12% PAAG-TBE electophoresis. Bands corresponding to tRNAs were cut from the gel under UV and RNA was eluted at 4°C overnight uder shaking in 0.4 ml of EB with 50 μl of water satureted phenol. RNA from aqueous phase was ethanol precipitated, dissolved in water and quantified spectrophotometrically.

III.2.5.2 Synthesis of fluorescently labeled tRNA transcripts

Alexa Fluor 488-5-UTP (Molecular Probes) was incorporated in transcripts of tRNAs by a 2h T7-transcription. Reaction mixure of a total volume of 20 μl contained: 0.5 μg of DNA template, 80 u of T7-RNA polymerase (2 consecutive additions of 40 u), 0.5 mM of ATP, 0.5 mM CTP, 0.5 mM GTP, 0.37 mM UTP, 0.125 mM Alexa Fluor 488-5-UTP, 10 mM DTT, 40 u of RNaseOUT (Invitrogen). Fluorescent transcripts were gel-purified as described in III.2.5.1. To check the incorporation of the label, we compared the dye absorbance at 492 nm and the nitrous bases absorbance at 260 nm using NanoDrop Microarray Programme. The efficiency of labeling was calculated according to the following formula:

$$\text{Base: Dye} = (A_{base} \times \varepsilon_{dye}) / (A_{dye} \times \varepsilon_{base})$$

Where ε_{dye} is the extinction coefficient for the fluorescent dye, and is equal to 62000 $cm^{-1}M^{-1}$, and ε_{base} is the average extinction coefficient for a base in RNA, and is equal to 8250 $cm^{-1}M^{-1}$. A_{base} is calculated as

$$A_{base} = A_{260} - (A_{dye} \times CF_{260})$$

Where CF_{260} is a correction factor, and is equal to 0.3. The average efficiency of labeling obtained was 5 fluorophors per one tRNA molecule.

III.2.6 [^{32}P]-Labelling of tRNA T7-transcripts

Before labelling T7-transcripts were dephosphorylated in the buffer containing 50 мM Tris-HCl (pH 9,0), 1 мM $MgCl_2$, 0,1 мM $ZnCl_2$, 1 мM spermidine, 10 u of RNasin and 2 u of alkaline phosphotase from calf intestine. Probes were incubated for 30 min at 37^{o}C, deproteinated by phenol, RNA was ethanol precipitated and dissolved in the minimal volume of water.

Dephosphorylated transcripts were incubated for 2 min at 60^{o}C to unfold RNA, immediatly put on ice and supplemented with 10x buffer, containing 0,5 M Tris-HCl (pH 7.6), 0,1 M $MgCl_2$, 50 mM DTT, 1 mM of spermidine and 1 mM of EDTA. 100 μCi of [α-^{32}P]-ATP (3000 Ci/mmol) was added per 1 μg of transcript and phosphorylation was performed by T4 polynucleotidekinase (10 u/rxn) for 1h at 37^{o}C. Labelled transcripts were mixed with an equal volume of a loading buffer, denatured for 5 min at 90^{o}C and separated by a preparative denaturing 12% PAAG-TBE electrophoresis. Bands were cut after autography of the gel and RNA was eluted at 4^{o}C overnight under shaking in 0.4 ml of EB with 50 μl of water satureted phenol.

III.2.7 tRNA – protein interaction by gel shift assay

Recombinant C-terminus His-tagged pre-MSK was purified from *E. coli* inclusion bodies by Ni-affinity chromatography, as previously described (Kamenski et al., 2007).

[^{32}P] -labelled T7-transcripts (1-5 pmol) were incubated with recombinant pre-MSK in increasing concentration (from 0,05 to 0,4 μM) for 10 min at 30 ^{o}C in buffer, containing 50

mM sodium cacodylate (pH 7.5), 270 mM KCl, 20 mM $MgCl_2$, 0.02% BSA. Then probes were mixed with ½ volume of loading buffer, containing 30% glecerol, 0.25% bromophenol blue, 0.25% xylencyanol) and loaded on native 6% PAAG-0.5xTBE with 5% glycerol. tRNA-protein complexes were separated from free tRNA by eletrophoresis at 10 mA and 4°C. Then the gel was fixed in 10% acetic acid and 10% ethanol until bromophenol blue became yellow (~30 min), dryed, and exposed with a phosphorimager plate. Results were analysed using "Typhoon TRIO" (GE Healthcare) and ImageQuantTL software.

III.2.8 Isolation of mitochondria from cultured human cells

Depending on a purpose and available amount of cells, we have used two methods to disrupt cells and prepare mitochondria. Cells were deattached in PBS with 1mM of EDTA, washed with PBS and resuspendent in the cold breackage buffer (BB): 0,6 M sorbitol, 10 mM HEPES-NaOH (pH 8.0), 1 mM EDTA containing 0,3% BSA. Large amounts of cells (~50×10^6, 4×175 cm^2 flasks) were disrupted using laboratory warring blender. Cells in 10 ml of BB were transferred in pre-chilled blender recipient and disrupted 3 times for 15 sec, between cycles of disruption the recipient with cells was kept on ice for 1 min, after disruption the recipient was washed with 5 ml of BB. Disruption of small amounts of cells (min 2×10^6, 25 cm^2 flask) was done by passing cell suspension in 1 ml of BB through a needle (№16, 23G × 1'' 0.6 × 25 mm) using a syringe on ice by at least 20 piston strokes. All further manipulations, if other is not specified, were performed at 4°C. Suspension of disrupted cells was centrifuged (2000g, 4°C, 5 min) to precipitate undisrupted cells and debris, and the supernatant was transferred in fresh tubes. If a pellet of unsidisrupted cells was comparable with initial amount of cells, we proceeded to second round of disruption and low speed centrifugation. Supernatants were pooled and subjected to high speed centrifugation (15000g, 4°C, 30 min). Pelellets containing mitochondria were resuspended in BB without BSA, and an aliquote of suspension was taken for protein assay. Purification of mitochondria for *in vitro* import experiments was limited by this step, mitochondria were aliqouted in BB, frozen in liquid nitrogen and stored at -80°C. Mitochondria for RNA preparation were then treated with RNase A (10 µg per 1 mg of mitochondrial protein) for 15 min at 4°C. RNase was inhibited by 10 times dilution with a BB with 2 mM of EDTA, followed by centrifugation (15000g, 4°C, 20 min). Mitoplasts (mitochondria without outer membrane) were generated by incubation with digitonin for 15 min on ice (50 µg per 1 mg of mitochondrial protein), washed twice in BB,

and mitoplast pellet was either immediately used for RNA isolation either frozen in liquid nitrogen and stored at -80°C.

III.2.9 *In vitro* import assay

Import assay was performed according to protocol previously developped in the laboratory (Entelis et al., 2002).

Mitochondria were purified from human cultured cells as described in III.2.8. Before the import assay, we unfroze mitochondria at 37°C in pre-warmed BB with 5mM succinate. Mitochondria were pelleted by centrifugation (14 000 g, 4°C, 5 min) and resuspended in the import buffer, containing 0,44 M sorbitol, 20 mM HEPES-NaOH (pH 6.8), 20 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP, 5 mM DTT, 0.5 mM phosphoenol pyruvate, 0.5 mM PMSF and 0.5 mM DIFP. Import of tRNAs into human mitochondria was performed in the presence of recombinant yeast pre-MSK and human import directing protein (hmIDP) fraction obtained by DEAE cellulose chromatography of human cell extract, following fractionation by differential ammonium sulphate precipitation, as described in as described previously (Entelis et al., 2001a).

tRNA T7-transcripts were prepared and labeled with [^{32}P]-ATP at their 5'-end, as descrided in III.2.5.1 and III.2.6, respectively. Before import assay, transcripts were incubated for 10 min at 90°C, and then folded for 10 min on ice in the presence of 0.5 mM $MgCl_2$. As a positive control, we used native yeast tRK1, which was labeled, folded and aminoacylated by recombinant yeast LysRS for 5 min at 37°C in a buffer, containing 100 mM Tris-HCl (pH 7.5), 30 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 1 mM DTT and 0.1 mM of lysine. [^{32}P]-labeled T7-transcripts or tRK1 (3 pmol) were mixed with 50 µg of human import directing proteins (hmIDPs), 0.2 µg of pre-MSK and 2 u of pyruvate kinase in the import buffer. Leucin was added in certain reactions up to 0.2 mM. This mixture was incubated for 10 min at 30°C and centrifuged (10 000g, 4°C, 10 min) at to pellet possible unspecific protein agregates. Supernatant was transferred into new tubes and 50 µg of mitochondria were added to it. As a negative control, we performed the same incubation without mitochondria, in this case an equal volume of import buffer was added. Reaction of import was performed for 20 min at 30^{o}C. Then non-imported tRNA was subjected to RNase degradation during 5 min at room temperature by adding the equal volume of RNase mix, containing 50 µg / ml of RNase A, 5 u/ml of micrococcal nuclease, 2 mM $CaCl_2$ and 10 mM $MgCl_2$. Afterwords, control reactions without mitochondria were immediately supplemented with lysis-buffer containing 0,1 M

NaAc (pH 4.8), 1% SDS and 0,05% DEPC and incubated for 1 min at 100 °C. RNA was extracted with hot phenol by vortexing the tubes for 5 min at 60 °C and ethanol precipitated from aqueous phase (Mager-Heckel et al., 2007). To import reactions with mitochondria, we added 4 volumes of cold BB with 5 mM EDTA and 4 mM EGTA in order to inhibit RNases. Mitochondria were pelleted, resuspended in 100 µl of BB and treated with digitonin (100 µg per 1 mg of mitochondrial protein) for 15 min on ice in order to disrupt the outer mitochondrial membrane. Resulting mitoplasts were pelleted, washed twice with BB, resuspended in lysis buffer and subjected to hot phenol extraction. RNA containing aqueous phase was mixed with equal volume of loading buffer and RNA was separated on 12% denaturing PAAG-TBE. After electrophoresis, gels were fixed in 10% acetic acid and 10% ethanol until bromophenol blue became yellow (~30 min), dryed and exposed with a phosphorimager plate. Results were analysed using "Typhoon TRIO" (GE Healthcare) and ImageQuantTL software.

III.2.10 Protein Polyacrylomide Gel Electrophoresis (SDS - PAGE)

Prior loading protein samples (10-30 µg) were mixed with 2x Laemmli's protein loading buffer (Laemmli, 1970), containing 125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.004% bromophenol blue, and incubated either for 10 min at 100°C or for 15 min at 60°C. Proteins were concentrated in 4% PAAG with 0,375 M Tris-HCl (pH 6,8) and 0,1% SDS, and separated in 10-15% PAAG with 0,125 M Tris-HCl (pH 8,8) and 0,1% SDS. We used Tris-Glycine-SDS electrophoresis buffer (EuroMedex), containing 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS, pH 8.5. We used the following protein ladders: Bio-Rad "High Range" (212, 121, 96, 48 kDa), Fermentas "PageRuler Prestained Protein Ladder" (170, 130, 100, 72, 55, 40, 33, 24, 17, 11 kDa), and EuroMedex Prestained Protein Ladder (170, 130, 100, 70, 55, 40, 35, 25, 15, 10 kDa). Electrophoresis was performed at 5-10V/cm. To visualize proteins we coloured the gels with colloidal Coomassie fom Bio-Rad or used them for transfer and subsequent Western blot analysis.

III.2.11 Expression and purification of recombinant hmtLeuRS

III.2.11.1 Expression of hmtLeuRS

Construction of pET3a-hmtLeuRS expression vector is discribed in III.2.2. *E. coli* BL21 CodonPlus (DE3)-RIL cells were transformed with pET3a-hmtLeuRS plasmid; resulting colonies were used to prepare the standard overnight culture, which was then diluted 1:20 with

SOC media at grown for 2 hours to the logarithmic stage. Expression of hmtLeuRS was induced by growing cells for 2h in the presence of 1 mM IPTG either at 10°C, or at 30°C. For verification of induction, aliquots of cells before and after IPTG addition were lysed in a protein loading buffer and analysed by SDS-PAGE (III.2.10). After induction, cells were pelleted by centrifugation (3 000 g, 4°C, 5 min) and resuspended in 1 ml of buffer for native lysis, containing 50 mM NaP (pH 8.0), 300 mM NaCl, 0.1% Triton X-100, 20 mM β-mercaptoethanol, 2 mM $MgCl_2$, 0.5 mM of protease inhibitors PMSF and DIFP. To destroy bacterial cell wall, lysozyme was added to final concentration of 1 mg/ml and cells were incubated for 15 minutes on ice. Lysates were sonicated 4 times for 15 seconds on ice with VibraCell 72408 (Bioblock Scientific) sonicator (20kHz) and centrifuged (10 000 g, 4°C, 30 min). The supernatant containing soluble proteins was collected and used for further purification (see III.2.11.2). The pellet containing inclusion bodies was resuspended in the buffer for denaturing lysis (100 mM NaP (pH 8.0), 100 mM Tris-HCl (pH 8.0), 8 M urea and 0.5 mM PMSF and DIFP) and incubated at 20°C for 2h with vigorous shacking. After centrifugation (10 000 g, 20°C, 30 min) we collected the supernatant (denaturated lysate), which contained proteins from inclusion bodies. The content of two cell lysates was analysed by SDS-PAGE. Renaturation of proteins from denaturated lysate was performed by two step dialysis. The first dialysis was done against buffer I (20 mM HEPES-NaOH (pH 7.6), 50 mM NaCl, 1 mM $MgCl_2$, 10% glycerin and 0.5 mM PMSF and DIFP) overnight, then the dialysis bag was transferred to buffer II (20 mM HEPES-NaOH (pH 7.6), 150 mM NaCl, 2.5 mM $MgCl_2$, 40% glycerin and 0.5 mM PMSF and DIFP) for another night. Dialyzed protein was stored at -20°C and used for evaluation of hmtLeuRS activity (see III.2.12).

III.2.11.2 Purification of hmtLeuRS by affinity chromatography

For purification of recombinant hmtLeuRS from native lysate of *E. coli* we have performed nickel affinity chromatography on Ni-NTA Agarose from Qiagene. The resin suspension was added to the lysates in proportion 1 ml per 10 mg of protein and this suspension was incubated for 1h at 4°C with shaking. Then suspension was loaded into the column and washed with a 50 mM NaP buffer (pH 8.0), 300 mM NaCl containing 5 mM and 10 mM of imidazole, subsequently, in order to get rid of non-specifically bound proteins. Elution was performed in the same buffer with 50 mM of imidazole and then with 100 mM of imidazole. Concentration of protein was measured by Bradford method using “Protein Assay” reagent (Bio-Rad). Protein fractions were then analysed by SDS-PAGE to verify the presence

and the purity of hmtLeuRS. Selected fractions containing recombinant protein were pooled together, concentrated through Nanosep 30K Device (Pall) and measured for their enzymatic activity in an *in vitro* aminoacylation assay (see III.2.12). Active fractions of hmLeuRS were supplemented with glycerol to 40% and inhibitors of proteases PMSF and DIFP to final concentration 0.5 mM and stored at – 20°C.

III.2.12 *In vitro* aminoacylation by hmtLeuRS

HmtLeuRS activity was tested on commercially available preparation of *E. coli* tRNA, on total or mitochondrial human RNA prepared as described in III.2.16. Final aminoacylation mixture (10 µl) contained 50 mM HEPES-NaOH (pH 7.6), 25 mM NaCl, 3 mM $MgCl_2$, 2.5 mM ATP, 0.2 mg/ml BSA, 0.8 µM [^{3}H]-Leu (63 Ci/mmol), 5 µg of RNA, and appropriate amount of hmtLeuRS. Prior aminoacylation, RNA preparations were deaminoacylated for 30 min at 37 °C in 0.5 M Tris-HCl buffer (pH 9.0), ethanol precipitated and diluted in a minimal volume of water. They were then incubated for 1 min at 90°C and re-folded for 10 min at room temperature in aminoacylation buffer. Then, 0.8 µM [^{3}H]-Leu and appropriate amount of hmtLeuRS were added, and aminoacylation reaction was done for 15 min at 37 °C. Samples were then applied to Whatman 3MM filters, and [^{3}H]-Leu – tRNAs were precipitated with cold 10% TCA. Then filters were washed two times for 10 min with cold 10% TCA and two times for 10 min with 96% ethanol at room temperature. Dryed filters were put into liquid scintillator and analysed for radioactivity using Beckman Coulter Multi-Purpose scintillation counter.

Aminoacylation of tRNA T7-transcripts by hmtLeuRS was done following the same protocol, but at higher concentration of $MgCl_2$, 12mM instead of 3 mM (Sohm et al., 2003). Thus, final aminoacylation mixture (10 µl) contained 50 mM HEPES-NaOH (pH 7.6), 25 mM NaCl, 12 mM $MgCl_2$, 2.5 mM ATP, 0.2 mg/ml BSA, 0.8 µM [^{3}H]-Leu (63 Ci/mmol), 5 µM tRNA transcript and 100 nM hmtLeuRS. Prior aminoacylation, tRNA transcripts were incubated for 1 min at 90°C and then folded for 10 min at room temperature in aminoacylation buffer. Then we added 0.8 µM [^{3}H]-Leu and 100 nM of hmtLeuRS. Aminoacylation was estimated over 10 min incubation time at 37 °C. Aminoacylation efficiency of T7-transcripts of recombinant tRNAs was compared to those of T7-transcript of wild-type human mt-$tRNA^{Leu(UUR)}$.

III.2.13 Cell culture techniques

Cells were grown in CO_2-incubator at 37°C and 5% CO_2. They were inspected daily using an inverted microscope. The medium was changed every 2 days and cells were split once they became confluent.

Cell culture medium was purchaised from Sigma, foetal calf serum (FCS) and other supplements from Gibco BRL Life Technologies and plasticsware from Sarstedt. MELAS cybrids cells were grown exclusively on "Cell+" plastic (Sarstedt).

We used Dulbecco's Phosphate Buffered Saline (PBS) from Sigma, containing 0.04683 g/l $MgCl_2$, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 8g/l NaCl and 1.15 g/l Na_2HPO_4.

Sub-culturing of adherent cell lines

HepG2, MELAS cybrid cells and 143B control cells were usually split 1: 2 – 1: 4, while HEK-293T cells were split 1: 10. Cells were detached from the surface of flasks using two different techniques.

The first one was to add 1xPBS with 1 mM EDTA to cover cells and incubate them for 5-10 min at 37°C. Then we detached cells by flapping the flask or by sprutting cells down with a pipette. Cells then were pelleted from suspension by centrifugation (600g, 25°C, 5 min), resuspended in desired volume of growth medium, and plated in new flasks.

The second one was to add a minimal volume of 1xPBS with 0.5% trypsin to cover the cells. Then cells were incubated for a few minutes at 37°C. Once cells were detached, 9 volumes of growth medium were added to inhibit trypsin and cells were plated in new flasks.

Freezing, cryostorage and thawing

To freeze the cells, they were detached from the surface using PBS-EDTA and cell pellet was resuspended in 200 µl of FCS with 10% of DMSO in cryo-storage vials. These were then gradually brought to -80°C by leaving them overnight at -80°C in a Cryo Freezer Block (Fisher Bioblock Scientific). The vials were then stored in the vapor phase of a liquid nitrogen storage facility.

To thaw the cells, the vials were removed from liquid nitrogen, 1 ml of pre-warmed (37°C) medium was added to cells and the vials were brought as fast as possible into a 37°C water bath. Cells were then pelleted by centrifugation, resuspended in the medium and seeded.

Counting

To count cells, an aliquot of cell suspension in growth medium was transferred in a well of Kova®-slide (Hycor Biomedical) and counted according to manufacturer instructions. To estimate a number of viable cells an aliquot of cell suspension was first mixed with 0.4% Trypan blue in PBS.

Mycoplasma testing

All cell lines were regulary tested for infection with mycoplasma using either the method of DNA staining with DAPI (4', 6 – Diamidine-2'-phenylindole dihydrochloride) (Roche Applied Science), or by PCR-based detection method (Dussurget and Roulland-Dussoix, 1994). The last one consisted in PCR-amplification from concentrated growth medium of a fragment of 16S rRNA gene of *Mycoplasma sp* by using three genus-specific primers:

RNA5: 5' AGAGTTTGATCCTGGCTCAGGA 3'

RNA3: 5' ACGAGCTGACGACAACCATGCAC 3'

UNI-: 5' TAATCCTGTTTGCTCCCCAC 3'

These primers were obtained from three regions in 16S rRNA sequence of *Mycoplasma* species, the combination of three primers was used to increase the specificity and to detect only mycoplasmas and not other nonmycoplasma bacteria and even yeast possibly present in growth medium. In the case of genus-specific contamination by *Mycoplasma sp* a 772 bp band was observed in the gel in addition to unspecific 1055 bp band, which could be also present in the case of nonmycoplasma bacterial or yeast contamination.

III.2.14 Transfection of cultured human cells

III.2.14.1 Transfection with T7-transcripts

Transfection of MELAS cybrid cells and 143B control cells with T7-transcripts of tRNAs was performed using Lipofectamine2000 (Invitrogen), basing on previously described protocol (Smirnov et al., 2008). We used 1 μg of transcript T7-transcript and 12.5 μl of Lipofectamine2000 for transfection of 2×10^6 cells. Cells were seeded one day prior transfection in 25 cm^2 flasks in order to obtain approximately 80% of confluence at the moment of transfection. T7-transcripts and Lipofectamine2000 were diluted each in 625 μl of

OptiMEM, incubated for 5 min at room temperature, mixed, and then incubated for 20 min more. RNA-lipofectamin mix was then added to cells in 3.5 ml of their growth media. Cells were exposed to RNA-lipofectamine complexes for 5h, after which media was changed and cells were analysed 24-48h after transfection.

III.2.14.2 Transient transfection with plasmids

(pmaxGFP plasmid was a kind gift of Derrick Gibbings, IBMP, Strasbourg; FACS analysis was performed in collaboration with Fanny Monneaux and Frederic Gros, IBMC, Strasbourg)

Transient transfection of MELAS cybrid cells with genes of recombinant tRNAs was performed with Lipofectamine2000 (Invitrogen), using various tRK-coding plasmids based on pBKE, psiRNA-h7SK, pLKO.1 vectors. The manufacturer protocol was adjusted for cybrid cells using pmaxGFP vector (Amaxa). The efficiency of transfection was estimated by FACS analysis (FACSCalibur, BD Biosciences) of transfected cells for GFP fluorescence 48h after transfection. Optimised conditions were as follows: 4 μg of "QiaFilter" (Qiagen) purified circular plasmid and 12 μl of Lipofectamine2000 for $600x10^3$ cells in a well (10 cm^2) of a 6-well plate. DNA and Lipofectamine2000 were diluted in OptiMEM and incubated for 15 min at 25°C. Formation of complexes was performed for 30 min at 25°C, and cells were incubated with complexes for 5h in DMEM without serum and antibiotics. In these conditions transfection effeciency of MELAS cybrid cells reached 90%. In further experiments transfection with pmaxGFP vector was routingly performed in parallel to control the efficiency of transfection.

III.2.14.3 Stable transfection

Stable transfection of MELAS cybrid cells with genes of recombinant tRNAs was performed with Lipofectamine2000 (Invitrogen). For this, tRK-coding plasmids were linearized by *SnaBI* (pBKE-tRK), or *ApaLI* (psiRNA-h7SK-tRK), respectively. MELAS cells were seeded a day prior transfection in 55 cm^2 plates, and were transfected at approximately 80% confluence. Formation of complexes and transfection were performed following the same protocol as for transient transfection, but with linearized vectors. We used 12 μg of linearized plasmid and 36 μl of Lipofectamine2000 in 1.5 ml OptiMEM to transfect 4×10^6 cells in 4.5 ml of DMEM without serum and antibiotics. DNA-lipofectamine complexes were removed after 5h incubation. We started selection of stable transformants 48h after

transfection with previously adjusted concentrations of antibiotics: 200 μg/ml of G418 for pBKE-tRK transfections and 200 μg/ml of Hygromycin for psiRNA-h7SK-tRK transfectants. Selection was performed until non-transfected control cells were dead, 1-2 weeks. Afterwards, depending on the number of cells, we either isolated individual clones, or analysed total cell population for transgene expression and then cloned cells by the limiting dilution technique. Cells were detached from the plate by PBS with 1mM EDTA, pelleted by centrifugation (600g, 5 min), resuspended in growth medium and counted using Kova®-slide. Then cells were diluted and seeded in 96-well plate in order to have one cell per well.

III.2.14.4 Stable transfection with lentiviruses

(The lentiviral trasnfection was performed in a BL2 laboratory in IGBMC, Strasbourg in collaboration with the teams of Helene Puccio and Irwin Davidson.)

MELAS cybrid cells stably expressing recombinant tRNAs were obtained by lentiviral transfection. Production of lentiviral particles was performed in HEK-293T cells. For this 1×10^6 of HEK-293T cells were seeded 1 day prior transfection in 55 cm^2 plates. Transfection mix contained 18 μl of FuGENE6 transfection reagent (Roche Applied Sciences), 3 μg of pLKO.1-tRK plasmid, 1.5 μg of pLP1, 0.75 μg of pLP2 and 0.75 μg pLP-VSGV packaging plasmids (Invitrogen). Transfection was performed according to the manufacturer protocol. FuGENE6-DNA complexes were formed in OptiMEM for 20 min and added to HEK-293T cells in DMEM with 4.5 g/l of glucose (medium normally used for MELAS cells). The medium was changed 24h after transfection. The same day, MELAS cybrid cells were seeded in 6-well plates (400×10^3 cells/well). The next day, 48h after transfection, virus-containing medium from HEK-293T cells was filtered through 0.4 μm Millipore filters to remove floating cells and added to MELAS cells, while fresh DMEM was added to HEK-293T cells. This infection procedure was then repeated 72h and 96h after HEK-293T transfection. 12h-24h after third infection selection of cells containing transgenes was started in the presence of 2μg/ml of puromycin. After 2-3 days of puromycin selection cells were analysed for transgene expression by Nothern hybridization and for steady-state level of mitochondrial proteins by immunoblotting.

III.2.15 Isolation and analysis of DNA from cultured human cells

To isolate total DNA, cells were resuspended in TE buffer with 1% SDS and 0.8 μg/μl of proteinase K and incubated either 2h at 50°C, or overnight at 37°C. Then DNA was precipitated from the solution by addition of NaCl (to 0.4 M), iso-propanol (to 50%), and incubation for 1h at -20°C.

The m.3243A>G mutation level in MELAS cybrid cells was tested systematically by *ApaI* restriction analysis of PCR-amplified fragment of mtDNA. For this, the 299 bp region of mtDNA (3081-3380) containing the *ApaI* restriction site introduced by m.3243A>G mutation was PCR-amplified using oligonucleotide primers: hp3081 GTAATCCAGGTCGGT TTCT and hp3380 CGTTCGGTAAGCATTAGG. Resulted PCR-products were digested by *ApaI* for 2h, and restriction fragments were then analyzed by native 10% PAAG-TAE electrophoresis. BET colored gel was scanned using "G:Box" from SynGene, the intensity of the bands was quantified using "GeneTools" software from the same manufacturer. The presence of m.3243A>G mutation resulted in *ApaI* digestion of the initial 299 bp PCR-product into two fragments, of 165 bp and 134 bp, respectively. Therefore, the mutation level (heteroplasmy) was calculated, as a ratio of intensity of these bands (165 bp + 134 bp) to that of all bands (299 bp + 165 bp + 134 bp).

Presence of transgenes in tranfected cells was verified by 30 cycles of PCR, using following pairs of primers:

Primer	Annealing t°C (T_{ann})
trk1RTAs: 5' GCCTTGTTGGCGCAATC 3'	45°C
trk1RTBr: 5' TGGAGCCCTGTAGGGGG 3'	
trk2RTAs: 5' TCCTTGTTAGCTCAGTT 3'	39°C
trk2RTBr: 5' TGGCTCCTCATAGGGGG 3'	
trk3RTAs: 5' GAGAATATTGTTTAATGGTAAAAC 3'	42°C
trk3RTBr: 5' TGGTGAGAATAGCTGGAGTTG 3'	

Quantity and quality of DNA preparations was controlled by 30 cycles of PCR amplification (T_{ann} = 55°C) of a 120 bp fragment of 7th chromosome, corresponding to *HUS1* gene, using the following pairs of primers:

hh1As: 5' CCTCCGCATCAGCCCTG 3'

hh1Br: 5' AGTGAGCAGGCCTCTCAG 3'

Presence of G418 (neomycin/kanamycin) resistance gene in cells transfected with pBKE-tRK vectors was verified by 30 cycles of PCR amplification (T_{ann} = 55°C) of its 120 bp fragment, using the following pairs of primers:

nkAs: 5' ATCGGGAGCGGCGATAC 3'

nkBr: 5' CGGCTGGGTGTGGCGG 3'

Amplified PCR products were analysed in native 10% PAAG-TAE.

III.2.16 Isolation of RNA from cultured human cells

For total RNA isolation, we used "TRIzol" reagent (Invitrogen) in proportion 1 ml of reagent per 1×10^6 of cells. We either immediately proceeded with RNA isolation or stored cells in TRIzol at -80°C and unfroze them at room temperature before use. After 5 min incubation at room temperature, we performed RNA extraction. For this, we added 0.2 ml of chloroform per 1 ml of TRIzol, shaked the samples during 15 seconds by inverting the tubes and incubated for 3 min at room temperature. Then, we centrifuged samples (10 000 g, 4°C, 15 min) to separate the phases. RNA from aqueous phase was precipitated by 50% iso-propanol on ice for at least 30 min. Then samples were centrifuged, and RNA pellet was dissolved in water.

RNA from purified mitochondria (see III.2.8) was isolated using TRIzol in proportion 1 ml per 100 μg of mitochondrial protein.

III.2.17 Nothern hybridization

To detect different RNAs we used the following oligonucleotide probes:

Name	Sequence (5' - 3')	T_{ann}
Anti-tRK1(1-34)	GAGTCATACGCGCTACCGATTGCGCCAACAAGGC	60°C
Anti-tRK2(2-32)	GCCGAACGCTCTACCAACTCAGCTAACAAGG	60°C
Anti-tRK3 (1-39)	CTTAAAAGACAACTGTTTTACCATTAAACAAATATTCTC	60°C
Anti-mt-tRNA^{Leu}	GAACCTCTGACTCTAAAG	45°C
Anti-mt-tRNA^{Thr}	CATCTCCGGTTTACAAG	45°C

Anti-mt-tRNA^{Val}	GTTGAAATCTCCTAAGTG	45°C
Anti-cy-RNA^{Lys}	CTTGAACCCTGGACC	45°C
Anti-5.8S rRNA	AAGTGACGCTCAGACAGGCA	45°C

50-100 pmoles of oligonucleotides were labeled for 1h at 37°C in the buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, 100 μCI of [α-^{32}P]-ATP (3000 Ci/mmol) and 10 u of T4 polynucleotidekinase (Promega). Labeled oligonucleotides were purified on DEAE-cellulose (DE-52, Serva) columns, equilibrated with the 0.2 M STE buffer (10 mM Tris-HCl, 1 mM EDTA, 0.2 M NaCl, pH 7.0). Reaction mixture was applied on the column in 1 ml of 0.2M STE, washed with 8 ml of the same buffer, and elution was performed in 4 ml of the 1 M STE (10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, pH 7.0).

RNA preparations were run through 10% PAAG-TBE with 8M urea and transferred to HybondN membrane (GE Healthcare) in 25 mM sodium-phosphate buffer pH 6.5 at 10V/200 mA at 4°C for 6h-16h. After transfer, the membrane was dryed at 25°C for 15 min and RNAs were crosslinked by UV irradiation (0.8 J/cm^2) for 3 min in the Ultraviolet crosslinker (Amersham). After crosslinking, membrane was incubated with 5 ml of the pre-hybridization solution containing 6xSSPE (52.2 g/l NaCl, 8.28 g/l NaH2PO4, 2.22 g/l EDTA, pH 7.4), 10x Denhardt solution (0.2% Ficoll, Mr = 400000; 0.2% polyvinylpyrrolidone, Mr = 400000; 0.2% BSA), 0.2% SDS, at 60°C for 2-3h at constant stirring. After pre-hybridization, [^{32}P]-5'-end-labelled oligonucleotide probe supplemented with an equal volume of pre-hybridization solution was added to the membrane. Hybridization was then performed at the appropriate temperature (T_{ann}) for at least 4h. The membrane was washed 3 times for 15 min with 2xSSPE, 0.1% SDS at 25°C, exposed with a PhosphorImager plate and radioactive signal was visualized and quatified using "Typhoon TRIO" (GE Healthcare) and ImageQuantTL software.

For re-hybridization of the membrane with other oligonucleotide probe, we stripped it 3 times for 15 min with 0.02xSSPE, 0.1% SDS at 80°C.

III.2.18 Analysis of tRNA aminoacylation *in vivo*

Analysis of aminoacylation levels of recombinant tRNAs in cells was performed by PAGE at acid pH and subsequent Nothern-hybridization analysis as described elsewhere

(Varshney et al., 1991). RNA from cells or purified mitochondria (III.2.8) was extracted with TRIzol reagent as described in III.2.16, then precipitated on ice with 50% iso-propanol and dissolved in 10 mM NaAc (pH 4.8), 1mM EDTA. Deacylated controls were prepared by 10 min incubation at 75°C in 0.25 M TrisHCl (pH 8.5-9.0), 0.25M $MgCl_2$. Then both acid and deacylated RNAs were mixed with one volume of the acid loading buffer (0.1 M NaAC pH 5.0), separated by electroporesis in denaturing 6.5% PAAG with 0.1M NaAc (pH 5.0) in a cold room, and analyzed by Northern-hybridization with [^{32}P]-5'-end-labelled oligonucleotide probes as described in III.2.17.

III.2.19 Analysis of import of T7-transcripts by confocal microscopy

(Fluorescent confocal microscopy analysis was performed in colloboration with Sylvie Grosch, IFR 37 en Neurosciences, Strasbourg)

Fluorescent T7-transcripts of tRNAs and 5S rRNA were obtained by *in vitro* T7-transcription in the presence of Alexa Fluor 488-5-UTP from Molecular Probes (Ex/Em 490/520) as described in III.2.5.2. We used 0.5 μg of labeled transcript to transfect 600×10^3 MELAS cybrid cells growing on 35 x 10 mm glass bottom dishes (EMS), following the protocol described in III.2.14.1. Three individual plates with cells were transfected for each T7-transcript, and three plates were left untransfected as controls for each time point. Analysis of transfected cells by fluorescent confocal microscopy (microscope Zeiss LSM 510) was performed 3h, 26h and 76h after transfection. Prior microscopic observation, cells were colored for 15 min with 10nM MitoTracker Red CMXRos from Invitrogen (Ex/Em 579/599). At time points 3h and 26h, we observed living cells on their glass bottomn dishes in PBS. Cells of the last 76h time point were fixed prior observation directly on their glass bottomn dishes by 15 min incubation with 4% PFA, and mounted in Aqua Poly/Mount solution (Polysciences, Inc.). To visualize green signal corresponding to RNA we used argon laser (488 nm, 61%), for red signal, corresponding to mitochondria, we used helium neon laser (543 nm, 81%). The co-localization of two signals within the cell was performed using Zeiss LSM software. The colocalization coefficient represents a relative number of colocalizing pixels in red (Ch1) or green (Ch2) channel, respectively, as compared to the total number of pixels above the threshold.

III.2.20 Western blot

For protein detection by immunoblotting, whole cells were solubilized in a Laemmli's buffer in the way to have SDS: protein ratio ~25-30 (w/w). Samples were sonicated for 5 sec with VibraCell 72408 (Bioblock Scientific) sonicator (20kHz) to fragmentate cellular DNA and incubated for 10 min at 60°C. Then proteins (30 μg per lane) were separated by 12.5% SDS-PAGE (see III.2.10), and subsequently transferred to a nitrocellulose membrane during 2h in Tris-Glycine-SDS buffer with 20% ethanol at 200 mA in a cold room. After transfer, the membrane was briefly washed with TBS buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Tween 20) and incubated overnight with TBS with 3% of non-fat dryed milk in a cold room under gentle shaking in order to reduce unspecific sorbtion of antibodies. Then filters were washed again with TBS and incubated with the appropriate amount of primary antibodies in TBS with 2% BSA for 1h at room temperature. Exceptionally, incubation with antibodies against NDI was performed in TBS with 0.5% of Tween 20 and 3% of non-fat dryed milk. Then, we washed filters 3 times for 15 min with TBS and incubated them with ECL™ horseradish peroxidase conjugated secondary antibodies (1: 10000) (GE Healthcare) in TBS for 1h at room temperature. Filters were then washed 3 times for 15 min with TBS, and immunocomplexes were detected using "ECL Plus Western Blotting Detection Reagent" (GE Healthcare). Visualization was done on "Typhoon TRIO" (GE Healthcare) and quantification was performed by ImageQuantTL software from the same manufacturer.

We used following primary antibodies: polyclonal rabbit antibodies against COXII (1: 1000) and against NDI (1:1000) (a kind gift of Anne Lombès, CRICM, Paris); monoclonal mouse antibodies against COXI (1: 1000) (Invitrogen), against porin (1: 1000) (Calbiochem 529538), and against α-tubulin (1: 20000) (Sigma T6074).

III.2.21 Immunocytochemistry

(Immunocytochemical analysis of mitochondrial proteins was performed in collaboration with Anne Lombès, CRICM, Paris)

For immunofluorescence analysis of COXII subunit, we used primary rabbit polyclonal antibodies (1: 500) and Alexa Fluor 568 secondary goat anti-rabbit antibodies (1: 1000) (Invitrogen). For immunofluorescence analysis of NDI subunit, we used non-purified

rabbit antibodies (1: 500) and CY_3-coupled secondary anti-rabbit antibodies (1: 200) (Jackson ImmunoResearch Laboratories, Inc.). All antibodies were diluted in PBS with 10% of FCS and 0.04% NaN_3.

Cells were grown on 14 mm glass cover slips up to 60% confluence. They were fixed for 20 min in 3% PFA, washed 3 times with PBS, incubated for 10 min in 50 mM NH_4Cl and then permeabilized. For COXII analysis, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and then washed in PBS. For NDI analysis, cells were permeabilised by incubation for 5 min with methanol at -20°C and then washed in PBS.

After fixation and permeabilization, cells on cover slips were incubated for 30 min with primary antibodies, washed with PBS with 0.1% Tween, and incubated for 30 min with secondary antibodies. Afterwards, cover slips were mounted in Mowiol mounting solution (Polysciences, Inc.) with 1 μg/ml DAPI and left at +4°C at least for 8h.

Cells were observed on Zeiss Axiovert 200M fluorescent microscope. More than 200 individual cells were evaluated to determine the fraction of positive ones.

III.2.22 *In vivo* mitochondrial translation analysis

Analysis of mitochondrial protein synthesis was performed as previously described (Kolesnikova et al., 2004) with minor modifications. Briefly, 600×10^3 cells were incubated for 10 min in DMEM without methionine (Sigma) in the presence of 100 μg/ml of emetine to inhibit cytoplasmic translation, followed by 30 min incubation with 200 μCi/ml [^{35}S]-methionine (400 Ci/mmol), and, finally, 10 min chase in the normal growth medium. Afterwards, cells were solubilized in a Laemmli's buffer, sonicated for 5 sec to fragmentate cellular DNA and incubated for 10 min at 37°C. Proteins (100 μg per lane) were then analyzed by 10-20% gradient SDS-PAGE as described in III.2.10. Protein amounts to be loaded were primarily normalized according to porin western blot analysis (see III.2.20). Visualization and quantification were performed using "Typhoon TRIO" and "ImageQuantTL" software from GE Healthcare.

III.2.23 Coloration of cells for COX/SDH activity

Cells were seeded a day prior coloration on glass cover slips. Coloration for COX activity was performed for 4h at 37°C in 50 mM NaP buffer (pH 7.4) with 10% sucrose

containing 4mM diaminobenzidine (DAB), 100μM cytochrome *c*, 10 u/l of catalase and 0.25% DMSO. Then cells were washed with PBS and colored for SDH activity for 1h at 37°C in 50 mM NaP buffer (pH 7.4) with 10% sucrose containing 1.5 mM nitroblue tetrazolium (NBT), 5mM succinate, 0.2 mM phenazine methosulphate and 1 mM NaN_3. After coloration, cells were washed with PBS and dehydrated by subsequent quick exposure of cover slips to 70% EtOH, 95% EtOH, 100% EtOH (two times), and xylene (two times). Then cover slips were dryed for several minutes at 25°C, mounted in DPX Mountant (Fluka) and observed using Leica DM4000B microscope. Pictures were taken using CoolSNAP camera (Photometrics).

III.2.24 Mitochondrial membrane potential (ΔΨ) analysis

(Mitochondrial membrane potential analysis was performed in collaboration with Mikhail Vysokikh, Belozersky Institute of Physico-Chemical Biology, Moscow)

Mitochondrial membrane potential (ΔΨ) was analysed by fluorescent coloration of cells by ΔΨ-dependent TMRM (tetramethyl rhodamine methyl ester). In parallel, we performed coloration by ΔΨ-independent Mitotracker Green FM. Cells were further analysed either by FACS analysis (Cytomics FC500, Beckman Coulter), or by fluorescent confocal microscopy (Zeiss LSM 510).

For fluorescent confocal microscopy analysis, cells were grown on glass bottomn dishes and incubated with 200 nM TMRM and 1 nM of Mitotracker Green FM in DMEM (pH 7.4 was adjusted with 0.5M HEPES-KOH) for 10 min at 37°C.

For FACS analysis cells were colored in suspension with TMRM and Mitotracker Green FM in the same concentrations for 15 min at 37°C.

III.2.25 Measurment of enzymatic activity of mitochondrial complexes

(Measurment of Complex IV enzymatic activity was performed in collaboration with Anne Lombes, CRICM, Paris)

The enzymatic activities of mitochondrial complexes were measured on whole cells by spectrophotometry (Beckman DU 800 for COX/CS, Beckman DU 730 for NOX/CS).

Reaction mixture for Complex IV (cytochrome *c* oxidase, COX) activity measurement contained 50 mM K phosphate buffer (pH 7.0) and 100 μM of reduced cytochrome *c*. Reaction was induced by addition of cell suspension equvivalent to 40 μg of total cell protein, and COX activity (oxidation of cytochrome *c*) was recorded for 5 min at 550 nm and 37°C.

Reaction mixture for citrate synthase (CS) activity measurement contained 100 mM Tris-HCl (pH 8.0), 100 μM DTNB (5,5'-dithiobis 2-nitrobenzoic acid), 300 μM acetyl CoA, 500 μM oxaloacetate, 0.1% Triton X-100 and 40 μg of total cell protein. Reaction was induced by addition of oxaloacetate, and CS activity was recorded for 5 min at 412 nm and 37°C.

To measure NADH oxidase (NOX) activity of Complex I suspension of cells in 1xPBS (0.5×10^6 cells/ml) was sonicated 10 times for 10 sec with 30 sec chilling on ice between sonications. Then, cell suspension was pre-incubated for 5 min at 30°C with or without 1 μM of rotenone. Final reaction mixture in PBS contained 0.15 mM Q_0, 0.1 mM NADH, 1mM KCN, 150 μg of total cell protein and 1 μM rotenone, when Complex I unspecific NOX activity was measured. Decrease of NADH absorbtion at 340 nm was recorded for 5 min at 30°C. To evaluate the specific activity of Complex I, NOX activity in the presence of rotenone was subtracted from NOX activity in the absence of the inhibitor.

III.2.26 Measurement of oxygen consumption

(Measurment of oxygen consumption using Oroboros Oxygraph-2k was performed in collaboration with Mikhail Vysokikh, Belozersky Institute of Physico-Chemical Biology, Moscow)

Measurement of oxygen consumption was performed using either Hansatech Oxygraph, or Oroboros Oxygraph-2k.

The rates of substrate dependent oxygen consumption were measured on $1\text{-}2\times10^6$ cells/ml in a respiration buffer containing 10 mM HEPES-KOH (pH 7.4), 120 mM KCl and 1 mM EGTA. Cells were permeabilized by digitonin. Importantly, the solution of digitonin was prepared the day of experiment by dissolving 10 mg of digitonin powder (Sigma-D141) in 1 ml of water under gentle shaking and heating for 2 min at 100°C, and was stored at 25°C. Digitonin solution was added directly in a chamber with cell suspension in proportion 100 μg of digitonin per 1 mg of total cell protein ($\sim1\times10^6$ cells). The mixture of pyruvate and malate (2M: 0.8M) was used to induce electron transport by respiratory chain from NADH to O_2

through Complex I, while Complex II was inhibited by 5 mM malonic acid. Succinate was used to donate electrons at Complex II level, while Complex I was inhibited by 0.5 μM rotenone. Finally, reduced cytochrome *c* was used to donate electrons directly at Complex IV level, while both Complex I and Complex II were inhibited. Maximal possible respiration rates were measured in the presence of the uncoupler FCCP (50-500 nM) and oligomycin (1 μg/ml). Non-mitochondrial oxygen consumption was measured at the end of each experiment upon addition of 1 mM KCN. Concentrations of substrates, inhibitors and uncouplers used as well as titration volumes are indicated in Table 3.1.

The rates of oxygen consumption of intact (non-permeabilized) cells were measured using 1-2×10^6 cells/ml in PBS in the presence of 5mM glucose. 1 μg/ml of oligomycin, FCCP in the range of 50-500 nM and 1 mM of KCN were sequentially added to measure coupled, uncoupled and non-mitochondrial oxygen consumption, respectively.

Table 3.1.

Substrate	Event	Concentration (solvent)	Storage (°C)	Titration volume (μl)	Final concentration	Syringe (μl)
Glutamate	glu	2M (H_2O)	-20	10	10 mM	25
Malate	mal	0.8M (H_2O)	-20	5	2 mM	25
Pyruvate	pyr	2M (H_2O)	fresh	5	5 mM	25
Succinate	suc	1M (H_2O)	-20	20	10 mM	50
Cyt *c* (red)	cyt	4 mM (H_2O)	-20	5	10 μM	25
Ascorbate	asc	0.8M (H_2O)	-20	5	2 mM	25
TMPD	tmpd	0.2M (H_2O)	-20	5	0.5 mM	25
Uncoupler						
FCCP	sf	0.1 mM (EtOH)	-20	1	50 nM steps	10
Inhibitors						
Rotenone	rot	1 mM (EtOH)	-20	1	0.5 μM	10
Malonic acid	mna	2M (H_2O)	-20	5	5 mM	25
Oligomycin	omy	2 mg/ml (EtOH)	-20	1	1 μg/ml	10
KCN	kcn	1M (H_2O)	fresh	2	1 mM	10
Digitonin	dig	10 mg/ml (H_2O)	fresh		100 μg/1 mg of protein	10

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APPENDIX

Table A.1. Reported Mitochondrial DNA Base Substitution Diseases: rRNA/tRNA mutations (MITOMAP, 2010)

Locus	Disease	Allele	RNA	Homo-plasmy	Hetero-Plasmy	Status
MT-TF	Mitochondrial Myopathy	T582C	tRNA Phe	-	+	Reported
MT-TF	MELAS / MM & EXIT	G583A	tRNA Phe	-	+	Cfrm
MT-TF	Myoglobinuria	A606G	tRNA Phe	+	+	Unclear
MT-TF	Tubulointerstitial nephritis	A608G	tRNA Phe	+	-	Reported
MT-TF	MERRF	G611A	tRNA Phe	-	+	Reported
MT-TF	MM	T618C	tRNA Phe	-	+	Reported
MT-TF	EXIT & Deafness	G622A	tRNA Phe	-	+	Reported
MT-TF	DEAF	A636G	tRNA Phe	+	-	Reported
MT-TF	Ataxia, PEO, deafness	T642C	tRNA Phe	-	+	Reported
MT-RNR1	DEAF	T669C	12S rRNA	+	-	Reported
MT-RNR1	Possibly LVNC-associated	T721C	12S rRNA	+	-	Reported
MT-RNR1	DEAF-associated	A745G	12S rRNA	+	-	Reported
MT-RNR1	SZ-associated	A750A	12S rRNA	+	-	Reported
MT-RNR1	Increased risk of nonsyndromic deafness	C792T	12S rRNA	+	-	Reported
MT-RNR1	DEAF-associated	A801G	12S rRNA	+	-	Reported
MT-RNR1	DEAF	A827G	12S rRNA	+	-	Conflicting reports - B4b'd marker
MT-RNR1	DEAF-associated	A839G	12S rRNA	+	-	Reported
MT-RNR1	Possibly LVNC-associated	T850C	12S rRNA	+	-	Reported
MT-RNR1	LHON helper/AD/DEAF-associated	A856G	12S rRNA	+	-	Reported
MT-RNR1	Possibly LVNC-associated	T921C	12S rRNA	+	-	Reported
MT-RNR1	Possibly DEAF-associated	C960CC	12S rRNA	+	-	Reported
MT-RNR1	Possibly DEAF-associated	C960del	12S rRNA	+	-	Reported
MT-RNR1	DEAF, possibly LVNC-associated	T961C	12S rRNA	+	-	Unclear
MT-RNR1	DEAF/AD-associated	T961delT+/-C(n)ins	12S rRNA	+	+	Unclear
MT-RNR1	Possibly DEAF-associated	T961G	12S rRNA	+	-	Reported
MT-RNR1	DEAF	T961insC	12S rRNA	+	-	Unclear
MT-RNR1	Possible DEAF risk factor	G988A	12S rRNA	.	.	Reported
MT-RNR1	DEAF	T990C	12S rRNA	+	-	Reported
MT-RNR1	DEAF	T1005C	12S rRNA	+	-	Unclear
MT-RNR1	DEAF-associated	A1027G	12S rRNA	+	-	Reported
MT-RNR1	SNHL	T1095C	12S rRNA	+	+	Unclear
MT-RNR1	DEAF	A1116G	12S rRNA	+	-	Reported
MT-RNR1	Possibly DEAF-associated	C1226G	12S rRNA	+	-	Reported

Locus	Disease	Allele	RNA	Homo-plasmy	Hetero-Plasmy	Status
MT-RNR1	Possibly DEAF-associated	T1180G	12S rRNA	+	-	Reported
MT-RNR1	DEAF-associated	C1192A	12S rRNA	+	-	Reported
MT-RNR1	DEAF-associated	C1192T	12S rRNA	+	-	Reported
MT-RNR1	DEAF	T1291C	12S rRNA	+	-	Unclear
MT-RNR1	DEAF-associated	C1310T	12S rRNA	+	-	Reported
MT-RNR1	DEAF-associated	A1331G	12S rRNA	+	-	Reported
MT-RNR1	DEAF-associated	A1374G	12S rRNA	+	-	Reported
MT-RNR1	SZ-associated	A1438A	12S rRNA	+	-	Reported
MT-RNR1	DEAF-associated	T1452C	12S rRNA	+	-	Reported
MT-RNR1	Possible DEAF risk factor	A1453G	12S rRNA	.	.	Reported
MT-RNR1	DEAF	A1491G=A1555G	12S rRNA	.	.	See 1555G
MT-RNR1	DEAF	C1494T	12S rRNA	+	-	Cfrm
MT-RNR1	DEAF	A1517C	12S rRNA	-	+	Reported
MT-RNR1	DEAF	C1537T	12S rRNA	+	-	Reported
MT-RNR1	DEAF	A1555G	12S rRNA	+	-	Cfrm
MT-TV	AMDF	G1606A	tRNA Val	-	+	Cfrm
MT-TV	Leigh Syndrome	C1624T	tRNA Val	+	-	Reported
MT-TV	MELAS	G1642A	tRNA Val	-	+	Reported
MT-TV	Adult Leigh Syndrome	G1644T	tRNA Val	-	+	Reported
MT-TV	Movement Disorder	T1659C	tRNA Val	-	+	Reported
MT-RNR2	Possibly LVNC-associated	T2352C	16S rRNA	+	-	Reported
MT-RNR2	Possibly LVNC-associated	G2361A	16S rRNA	+	-	Reported
MT-RNR2	Possibly LVNC-associated	A2755G	16S rRNA	+	-	Reported
MT-RNR2	Rett Syndrome	C2835T	16S rRNA	-	+	Reported
MT-RNR2	Myopathy	G3090A	16S rRNA	-	+	Reported
MT-RNR2	MELAS	C3093G	16S rRNA	-	+	Reported
MT-RNR2	ADPD	G3196A	16S rRNA	+	+	Reported
MT-TL1	Sporadic bilateral optic neuropathy	A3236G	tRNA Leu (UUR)	.	.	Reported
MT-TL1	MM	G3242A	tRNA Leu (UUR)	+	-	Reported
MT-TL1	MELAS / LS	A3243G	tRNA Leu (UUR)	-	+	Cfrm
MT-TL1	DMDF / MIDD / SNHL / FSGS	A3243G	tRNA Leu (UUR)	-	+	Cfrm
MT-TL1	CPEO / MM	A3243G	tRNA Leu (UUR)	-	+	Cfrm
MT-TL1	MM / MELAS	A3243T	tRNA Leu (UUR)	-	+	Reported
MT-TL1	MELAS	G3244A	tRNA Leu (UUR)	-	+	Reported
MT-TL1	KSS	G3249A	tRNA Leu(UUR)	-	+	Reported
MT-TL1	MM / CPEO	T3250C	tRNA Leu	-	+	Reported

Locus	Disease	Allele	RNA	Homo-plasmy	Hetero-Plasmy	Status
			(UUR)			
MT-TL1	MM	A3251G	tRNA Leu (UUR)	-	+	Reported
MT-TL1	MELAS	A3252G	tRNA Leu (UUR)	-	+	Reported
MT-TL1	Gestational Diabetes (GDM)	C3254A	tRNA Leu (UUR)	-	+	Warrants further study
MT-TL1	MM	C3254G	tRNA Leu (UUR)	-	+	Reported
MT-TL1	CPEO	C3254T	tRNA Leu (UUR)	+	-	Reported
MT-TL1	MERRF/KSS overlap	G3255A	tRNA Leu (UUR)	-	+	Reported
MT-TL1	MELAS	C3256T	tRNA Leu (UUR)	-	+	Cfrm
MT-TL1	MELAS/Myopathy	T3258C	tRNA Leu (UUR)	-	+	Reported
MT-TL1	MMC	A3260G	tRNA Leu (UUR)	-	+	Cfrm
MT-TL1	DM	T3264C	tRNA Leu (UUR)	-	+	Reported
MT-TL1	MELAS	T3271C	tRNA Leu (UUR)	-	+	Cfrm
MT-TL1	DM	T3271C	tRNA Leu (UUR)	-	+	Reported
MT-TL1	PEM	T3271delT	tRNA Leu (UUR)	-	+	Reported
MT-TL1	Ocular myopathy	T3273C	tRNA Leu (UUR)	-	+	Reported
MT-TL1	LHON	C3275A	tRNA Leu (UUR)	+	-	Reported
MT-TL1	Myopathy	A3280G	tRNA Leu (UUR)	-	+	Reported
MT-TL1	Encephalomyopathy	C3287A	tRNA Leu (UUR)	-	+	Reported
MT-TL1	Myopathy	A3288G	tRNA Leu (UUR)	-	+	Reported
MT-TL1	MELAS/Myopathy/Deafness, Cog.Impair.	T3291C	tRNA Leu (UUR)	-	+	Cfrm
MT-TL1	MM	A3302G	tRNA Leu (UUR)	-	+	Cfrm
MT-TL1	MMC	C3303T	tRNA Leu (UUR)	+	+	Cfrm
MT-TI	MM/CPEO	A4267G	tRNA Ile	-	+	Reported
MT-TI	FICP	A4269G	tRNA Ile	-	+	Reported

Locus	Disease	Allele	RNA	Homo-plasmy	Hetero-Plasmy	Status
MT-TI	CPEO/Motor Neuron Disease	T4274C	tRNA Ile	-	+	Reported
MT-TI	Varied familial presentation/spastic paraparesis	G4284A	tRNA Ile	-	+	Reported
MT-TI	CPEO	T4285C	tRNA Ile	-	+	Reported
MT-TI	Progressive Encephalopathy/PEO,myopathy	T4290C	tRNA Ile	+	+	Reported
MT-TI	Hypomagnesemic Metabolic Syndrome	T4291C	tRNA Ile	+	-	Reported
MT-TI	MHCM	A4295G	tRNA Ile	-	+	Reported
MT-TI	CPEO / MS	G4298A	tRNA Ile	-	+	Cfrm
MT-TI	MICM	A4300G	tRNA Ile	+	+	Cfrm
MT-TI	CPEO	A4302G	tRNA Ile	-	+	Reported
MT-TI	CPEO	G4309A	tRNA Ile	-	+	Reported
MT-TI	FICP	A4317G	tRNA Ile	nd	nd	Reported
MT-TI	Mitochondrial Encephalocardiomyopathy	C4320T	tRNA Ile	-	+	Reported
MT-TQ	Encephalopathy / MELAS	G4332A	tRNA Gln	-	+	Cfrm
MT-TQ	ADPD/Hearing Loss &Migraine	T4336C	tRNA Gln	+	+	Unclear
MT-TQ	Possibly associated w DEAF + RP + dev delay	T4363C	tRNA Gln	+	-	Reported
MT-TQ	Myopathy	T4370AT	tRNA Gln	-	+	Reported
MT-TQ	Possibly LVNC-associated	T4373C	tRNA Gln	+	-	Reported
MT-TQ	LHON	A4381G	tRNA Gln	+	-	Reported
MT-NC2	Hypertension	A4401G	NC2 Gln-Met spacer	+	-	Reported
MT-TM	MM	T4409C	tRNA Met	-	+	Reported
MT-TM	EXIT & APS2	A4415G	tRNA Met	-	+	Reported
MT-TM	LHON modulator/Hypertension risk factor	A4435G	tRNA Met	+	-	Reported
MT-TM	Myopathy	G4450A	tRNA Met	-	+	Reported
MT-TM	Possible contributor to mito dysfunction	T4454C	tRNA Met	+	-	Reported
MT-TW	MM	G5521A	tRNA Trp	-	+	Reported
MT-TW	Leigh Syndrome	T5523G	tRNA Trp	-	+	Reported
MT-TW	Gastrointestinal Syndrome	G5532A	tRNA Trp	-	+	Reported
MT-TW	Leigh Syndrome	A5537insT	tRNA Trp	-	+	Cfrm
MT-TW	Encephalomyopathy/DEAF	G5540A	tRNA Trp	-	+	Reported
MT-TW	MM	T5543C	tRNA Trp	-	+	Reported
MT-TW	HCM severe multisystem disorder	C5545T	tRNA Trp	-	+	Reported
MT-TW	DEMCHO	G5549A	tRNA Trp	-	+	Reported
MT-TW	Leigh Syndrome	A5559G	tRNA Trp	-	+	Reported
MT-TW	Myopathy	T5567C	tRNA Trp	-	+	Reported
MT-TW	DEAF	A5568G	tRNA Trp	+	-	Reported

Locus	Disease	Allele	RNA	Homo-plasmy	Hetero-Plasmy	Status
MT-TA	Possible DEAF modifier	T5587C	tRNA Ala	+	-	Reported
MT-TA	Myopathy	G5591A	tRNA Ala	-	+	Reported
MT-TA	CPEO / DEAF enhancer	T5628C	tRNA Ala	-	+	Reported
MT-TA	Myopathy	G5650A	tRNA Ala	-	+	Reported
MT-TA	DEAF enhancer	T5655C	tRNA Ala	+	-	Reported
MT-TN	CPEO / MM	T5692C	tRNA Asn	-	+	Reported
MT-TN	Encephalomyopathy	T5693C	tRNA Asn	+	-	Reported
MT-TN	CPEO/MM	G5698A	tRNA Asn	-	+	Reported
MT-TN	CPEO / MM	G5703A	tRNA Asn	-	+	Cfrm
MT-TN	Multiorgan failure	T5728C	tRNA Asn	-	+	Reported
MT-TC	SNHL	G5780A	tRNA Cys	-	+	Reported
MT-TC	Myopathy deafness	G5783A	tRNA Cys	-	+	Reported
MT-TC	DEAF1555 increased penetrance	T5802C	tRNA Cys	+	-	Reported
MT-TC	Mitochondrial Encephalopathy	T5814C	tRNA Cys	-	+	Also reported as pm, L2b marker
MT-TC	Progressive Dystonia	A5816G	tRNA Cys	+	-	Reported
MT-TC	DEAF helper mut.	G5821A	tRNA Cys	+	-	Reported
MT-TY	FSGS / Mitochondrial Cytopathy	A5843G	tRNA Tyr	+	-	Reported
MT-TY	EXIT	T5874G	tRNA Tyr	-	+	Reported
MT-TS1 precursor?	DEAF	A7445C	tRNA Ser (UCN) precursor	+	-	Reported
MT-TS1 precursor?	SNHL	A7445G	tRNA Ser (UCN) precursor	+	+	Cfrm
MT-TS1 precursor?	SNHL	A7445T	tRNA Ser (UCN) precursor	+	-	Reported
MT-TS1	DEAF	A7456G	tRNA Ser (UCN)	+	-	Unclear
MT-TS1	PEO	G7458A	tRNA Ser (UCN)	-	+	Reported
MT-TS1	PEM/AMDF/Motor neuron disease-like	C7471CC (='7472insC')	tRNA Ser (UCN)	+	+	Cfrm
MT-TS1	MM/DMDF modulator	A7472C	tRNA Ser (UCN)	+	-	Reported
MT-TS1	MM	T7480G	tRNA Ser (UCN)	-	+	Reported
MT-TS1	MM / EXIT	G7497A	tRNA Ser (UCN)	+	+	Cfrm
MT-TS1	Maternally inherited hearing loss	T7505C	tRNA Ser (UCN)	+	-	Reported
MT-TS1	PEO with hearing loss	G7506A	tRNA Ser (UCN)	-	+	Reported

Locus	Disease	Allele	RNA	Homo-plasmy	Hetero-Plasmy	Status
MT-TS1	SNHL	T7510C	tRNA Ser (UCN)	-	+	Reported
MT-TS1	SNHL	T7511C	tRNA Ser (UCN)	+	+	Cfrm
MT-TS1	PEM / MERME	T7512C	tRNA Ser (UCN)	+	+	Reported
MT-TD	Sporadic bilateral optic neuropathy	G7520A	tRNA Asp	.	.	Reported
MT-TD	Mitochondrial Myopaty	A7526G	tRNA Asp	-	+	Reported
MT-TD	MEPR	A7543G	tRNA Asp	-	+	Reported
MT-TK	DMDF/ MERRF/ HCM/ epilepsy	A8296G	tRNA Lys	+	+	Reported
MT-TK	Encephalopathy	A8302T	tRNA Lys	+	-	Unclear
MT-TK	MNGIE	G8313A	tRNA Lys	-	+	Reported
MT-TK	MELAS	T8316C	tRNA Lys	-	+	Reported
MT-TK	Mitochondrial cytopathy	A8326G	tRNA Lys	-	+	Reported
MT-TK	Mitochondrial Encephalopathy	G8328A	tRNA Lys	-	+	Reported
MT-TK	Excercise Intolerance	G8340A	tRNA Lys	.	.	Reported
MT-TK	PEO and Myoclonus	G8342A	tRNA Lys	-	+	Reported
MT-TK	possible PD risk factor	A8343G	tRNA Lys	+	-	Reported
MT-TK	MERRF	A8344G	tRNA Lys	-	+	Cfrm
MT-TK	Depressive mood disorder	A8344G	tRNA Lys	-	+	Reported
MT-TK	Cardiomyopathy	A8348G	tRNA Lys	-	+	Reported
MT-TK	Myopathy	T8355C	tRNA Lys	-	+	Reported
MT-TK	MERRF	T8356C	tRNA Lys	-	+	Cfrm
MT-TK	MERRF	G8361A	tRNA Lys	-	+	Reported
MT-TK	Myopathy	T8362G	tRNA Lys	-	+	Reported
MT-TK	MICM + DEAF/ MERRF/ Autism/ LS/ Ataxia + Lipomas	G8363A	tRNA Lys	-	+	Cfrm
MT-TG	MHCM	T9997C	tRNA Gly	nd	+	Reported
MT-TG	CIPO / Encephalopathy	A10006G	tRNA Gly	+	-	Unclear
MT-TG	PEM	T10010C	tRNA Gly	-	+	Cfrm
MT-TG	Myopathy	G10014A	tRNA Gly	+	-	Unclear
MT-TG	SIDS	A10044G	tRNA Gly	-	+	Unclear
MT-TR	Mitochondrial Myopathy	G10406A	tRNA Arg	-	+	Reported
MT-TR	Progressive Encephalopathy	A10438G	tRNA Arg	-	+	Reported
MT-TR	DEAF helper mut.	T10454C	tRNA Arg	+	-	Reported
MT-TH	MERRF-MELAS / Cerebral edema	G12147A	tRNA His	-	+	Cfrm
MT-TH	RP + DEAF	G12183A	tRNA His	-	+	Reported
MT-TH	MICM	G12192A	tRNA His	+	-	Reported
MT-TS2	Myopathy / Encephalopathy	G12207A	tRNA Ser (AGY)	-	+	Reported
MT-TS2	DEAF helper mut.	C12224T	tRNA Ser (AGY)	+	-	Reported

Locus	Disease	Allele	RNA	Homo-plasmy	Hetero-Plasmy	Status
MT-TS2	DEAF	G12236A	tRNA Ser (AGY)	+	-	Reported
MT-TS2	CIPO	C12246A	tRNA Ser (AGY)	nd	nd	Reported
MT-TS2	DMDF / RP+SNHL	C12258A	tRNA Ser (AGY)	-	+	Reported
MT-TS2	CPEO	G12276A	tRNA Ser (AGY)	-	+	Reported
MT-TL2	CPEO	G12294A	tRNA Leu (CUN)	-	+	Reported
MT-TL2	Dilated Cardiomyopathy	T12297C	tRNA Leu (CUN)	-	+	Reported
MT-TL2	MELAS	A12299C	tRNA Leu (CUN)	-	+	Reported
MT-TL2	3243 suppressor mutant	G12300A	tRNA Leu (CUN)	-	+	Reported
MT-TL2	CPEO / Stroke / CM / Renal & Prostate Cancer Risk/ Altered brain pH	A12308G	tRNA Leu (CUN)	+	+	Haplogroup U marker
MT-TL2	CPEO	T12311C	tRNA Leu (CUN)	+	+	Reported
MT-TL2	FSHD	T12313C	tRNA Leu (CUN)	-	+	Reported
MT-TL2	CPEO/KSS	G12315A	tRNA Leu (CUN)	-	+	Cfrm
MT-TL2	CPEO	G12316A	tRNA Leu (CUN)	-	+	Reported
MT-TL2	MM	A12320G	tRNA Leu (CUN)	-	+	Reported
MT-TE	Reversible COX deficiency myopathy	T14674C	tRNA Glu	+	-	Cfrm
MT-TE	Mitochondrial encephalomyopathy	C14680A	tRNA Glu	-	+	Reported
MT-TE	Mitochondrial myopathy w respiratory failure	A14687G	tRNA Glu	+	-	Reported
MT-TE	LHON helper mut.	A14692G	tRNA Glu	+	-	Reported
MT-TE	MELAS/LHON/DEAF helper	A14693G	tRNA Glu	+	+	Reported
MT-TE	Progressive Encephalopathy	A14696G	tRNA Glu	-	+	Reported
MT-TE	MM+DMDF / Encephalomyopathy	T14709C	tRNA Glu	+	+	Cfrm
MT-TE	Encephalomyopathy+Retinopathy	G14740A	tRNA Glu	-	+	Reported
MT-TE	Mitochondrial leukoencephalopathy	G14724A	tRNA Glu	-	+	Reported
MT-TE	EXIT	G14739A	tRNA Glu	-	+	Reported
MT-TT	DEAF helper mut.	T15908C	tRNA Thr	+	-	Reported

Locus	Disease	Allele	RNA	Homo-plasmy	Hetero-Plasmy	Status
MT-TT	Encephalomyopathy	G15915A	tRNA Thr	-	+	Reported
MT-TT	LIMM	A15923G	tRNA Thr	nd	-	Reported
MT-TT	LIMM	A15924G	tRNA Thr	nd	-	P.M.
MT-TT	Multiple Sclerosis/DEAF1555 increased penetrance	G15927A	tRNA Thr	+	-	P.M./possible helper mutation
MT-TT	Multiple Sclerosis	G15928A	tRNA Thr	+	-	P.M.
MT-TT	Possibly LVNC-associated	T15942C	tRNA Thr	+	-	Reported
MT-TT	Dopaminergic nerve cell deat (PD)	G15950A	tRNA Thr	+	-	Reported
MT-TT	LHON modulator	A15951G	tRNA Thr	+	-	Reported
MT-TP	Dopaminergic nerve cell deat (PD)	A15965G	tRNA Pro	+	-	Reported
MT-TP	Ataxia+RP+deafness	C15975T	tRNA Pro	-	+	Reported
MT-TP	MM	C15990T	tRNA Pro	-	+	Reported
MT-TP	Mitochondrial cytopathy	G15995A	tRNA Pro	-	+	Reported
MT-TP	Mitochondrial cytopathy	T16002C	tRNA Pro	-	+	Reported

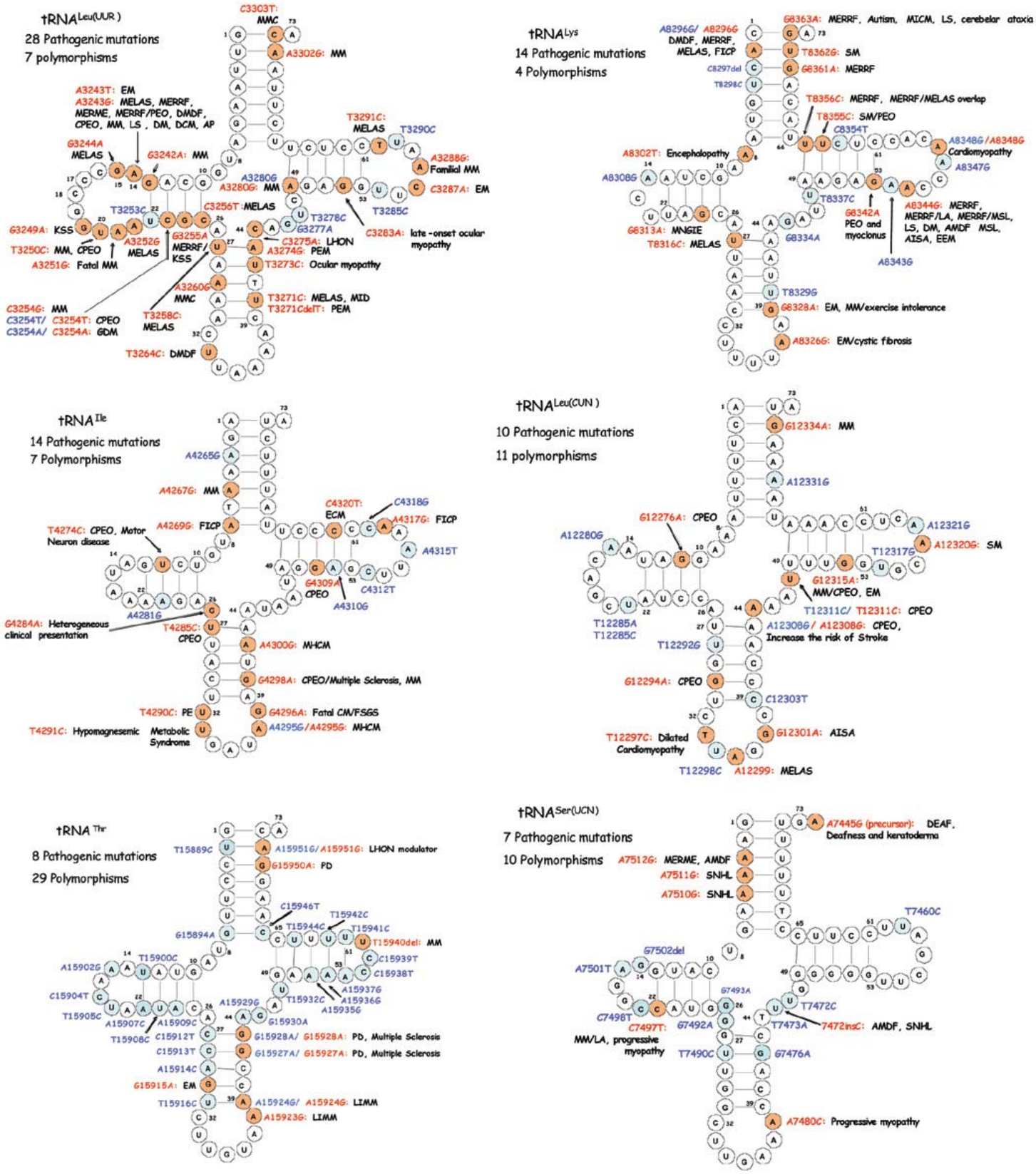
Notes:

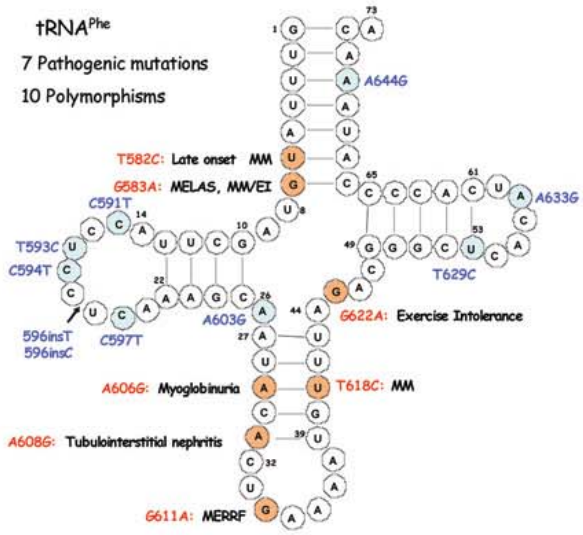
- Homoplasmy = pure mutant mtDNAs.
- Heteroplasmy = mixture of mutant and normal mtDNAs.
- nd = not determined.
- "Reported" status indicates that one or more publications have considered the mutation as possibly pathologic. This is not an assignment of pathogenicity by MITOMAP but is a report of literature. Previously, mutations with this status were termed "Prov" (provisional).
- "Cfrm"(confirmed) status indicates that at least two or more independent laboratories have published reports on the pathogenicity of a specific mutation. These mutations are generally accepted by the mitochondrial research community as being pathogenic. A status of "Cfrm" is not an assignment of pathogenicity by MITOMAP but is a report of published literature. Researchers and clinicians are cautioned that additional data and/or analysis may still be necessary to confirm the pathological significance of some of these mutations.
- "P.M." (point mutation/polymorphism) status indicates that some published reports have determined the mutation to be a non-pathogenic polymorphism.

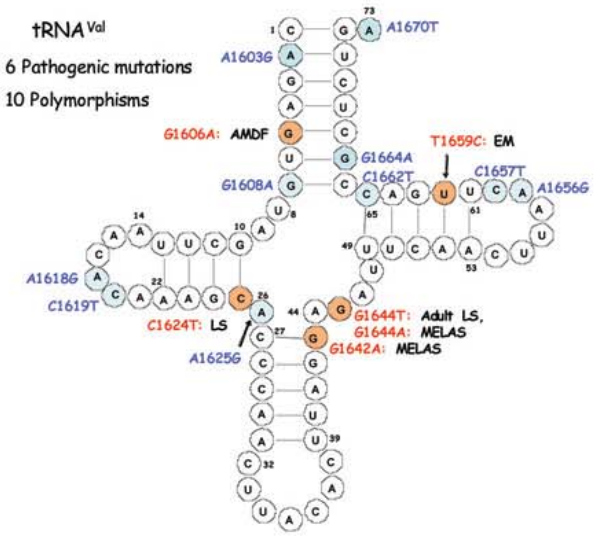
AD	Alzheimer's Disease	**LIMM**	Lethal Infantile Mitochondrial Myopathy
ADPD	Alzheimer's Disease and Parkinson's Disease	**LS**	Leigh Syndrome
AMDF	Ataxia, Mental deterioration, DeaFness	**LVNC**	Left Ventricular NonCompaction
APS2	Autoimmune Polyglandular Syndrome type 2	**MELAS**	Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes
CIPO	Chronic Intestinal Pseudoobstruction with myopathy and Ophthalmoplegia	**MERME**	MERRF/MELAS overlap disease
CM	CardioMyopathy	**MERRF**	Myoclonic Epilepsy and Ragged Red Muscle Fibres
CPEO	Chronic Progressive External Ophthalmoplegia	**MHCM**	Maternally inherited Hypertrophic CardioMyopathy
DEAF	Maternally inherited DEAFness or aminoglycoside-induced DEAFness	**MICM**	Maternal Inherited CardioMyopathy
DEMCHO	DEMentia, CHOrea	**MIDD**	Maternally Inherited Diabetes and Deafness
DM	Diabetes Mellitus	**MM**	Mitochondrial Myopathy
DMDF	Diabetes Mellitus + DeaFness	**MMC**	Maternal Myopathy and Cardiomyopathy
EXIT	EXercise InTolerance	**MNGIE**	Mitochondrial NeuroGastroIntestinal Encephalomyopathy
FICP	Fatal Infantile Cardiomyopathy Plus, a MELAS-associated cardiomyopathy	**NARP**	Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa; alternate phenotype at this locus is reported as Leigh Disease
FSGS	Focal Segmental GlomeruloSclerosis	**PEM**	Progressive EncephaloMyopathy
FSHD	FacioScapuloHumeral Disease	**RP**	Retinitis Pigmentosa
HCM	Hypertrophic CardioMyopathy	**SIDS**	Sudden Infant Death Syndrome
KSS	Kearns Sayre Syndrome	**SNHL**	SensoriNeural Hearing Loss
LDYT	Leber's hereditary optic neuropathy and DYsTonia	**SZ**	SchiZophrenia
LHON	Leber Hereditary Optic Neuropathy		

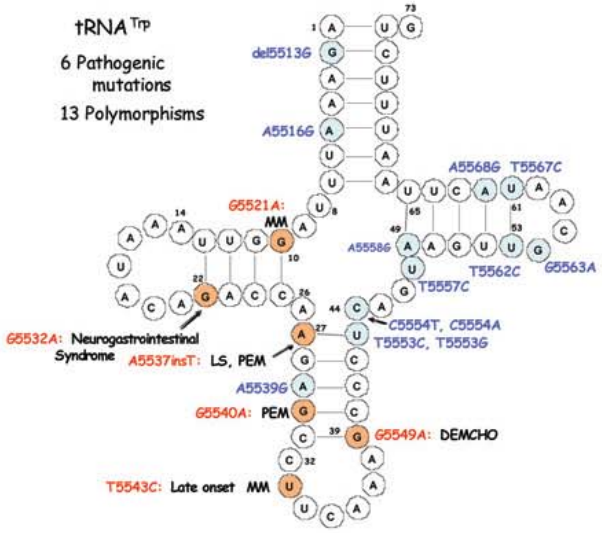
Fig. A.1. Pathogenic and polymorphic mutations in human mitochondrial tRNA genes. (Zifa et al., 2007)

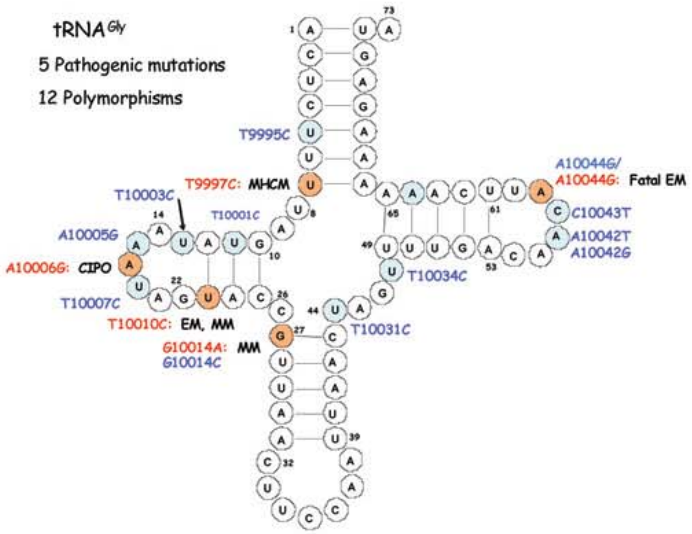
tRNAs are represented in their secondary cloverleaf structures and sorted according to the number of the pathogenic mutations that have been detected (in decreasing order). Pathogenic mutations are indicated in red, and polymorphic in blue. See p. 141 for pathology abbreviations used.

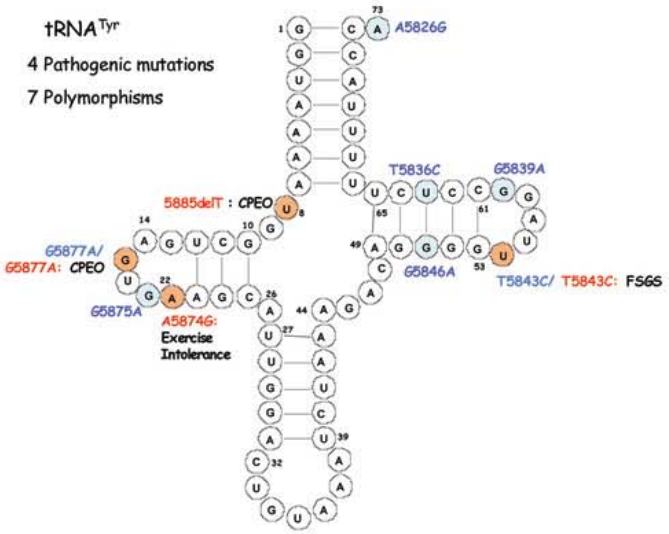


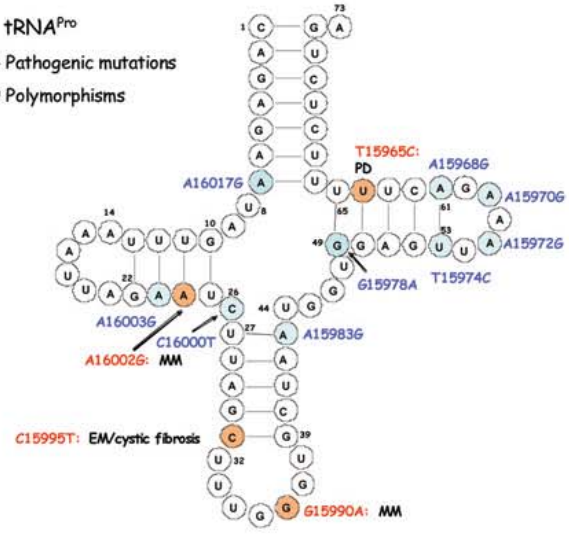

tRNA Phe
7 Pathogenic mutations
10 Polymorphisms
T582C: Late onset MM
G583A: MELAS, MM/EI
A644G
A633G
C591T
T593C
C594T
T629C
596insT
596insC
C597T
A603G
G622A: Exercise Intolerance
A606G: Myoglobinuria
T618C: MM
A608G: Tubulointerstitial nephritis
G611A: MERRF

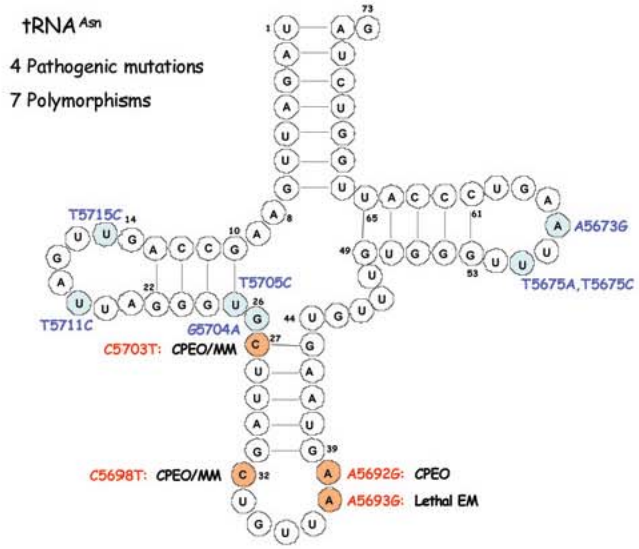

tRNA Val
6 Pathogenic mutations
10 Polymorphisms
A1670T
A1603G
G1606A: AMDF
T1659C: EM
G1664A
G1608A
C1662T
C1657T
A1656G
A1618G
C1619T
C1624T: LS
G1644T: Adult LS,
G1644A: MELAS
G1642A: MELAS
A1625G

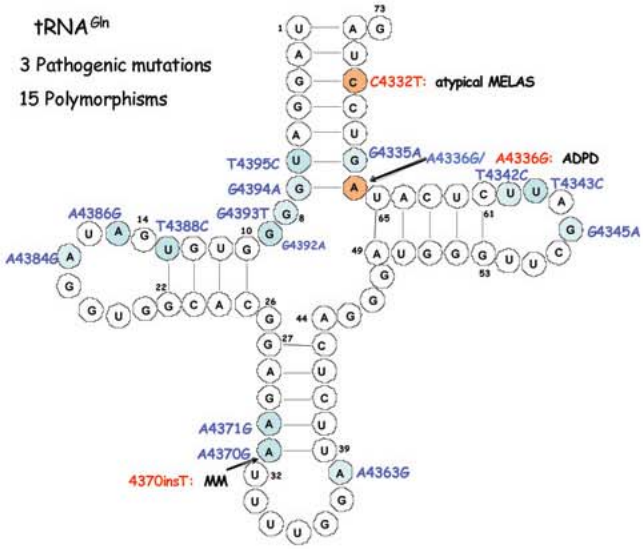

tRNA Trp
6 Pathogenic mutations
13 Polymorphisms
del5513G
A5516G
A5568G T5567C
G5521A: MM
A5558G
T5562C
G5563A
T5557C
G5532A: Neurogastrointestinal Syndrome
A5537insT: LS, PEM
C5554T, C5554A
T5553C, T5553G
A5539G
G5540A: PEM
G5549A: DEMCHO
T5543C: Late onset MM

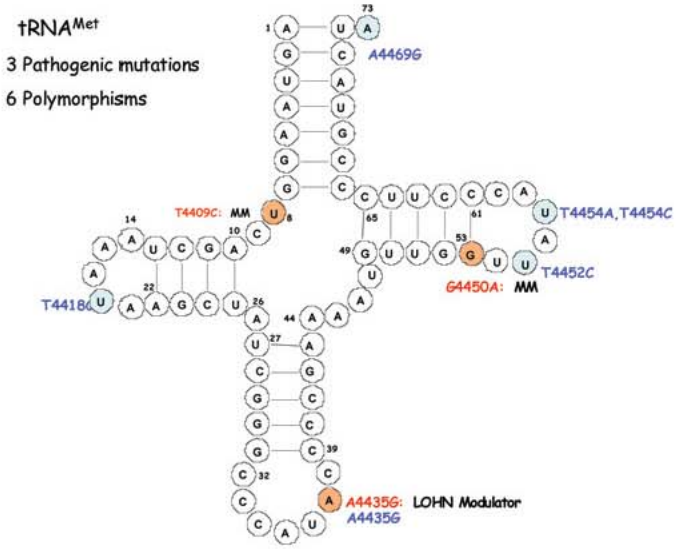

tRNA Gly
5 Pathogenic mutations
12 Polymorphisms
T9995C
T9997C: MHCM
A10044G/
A10044G: Fatal EM
T10003C
T10001C
C10043T
A10042T
A10042G
A10005G
A10006G: CIPO
T10034C
T10007C
T10010C: EM, MM
T10031C
G10014A: MM
G10014C

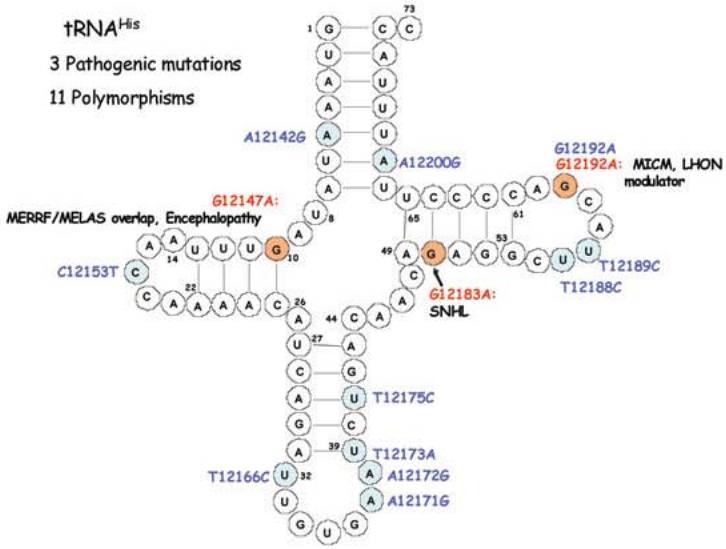

tRNA Tyr
4 Pathogenic mutations
7 Polymorphisms
A5826G
T5836C
G5839A
5885delT : CPEO
G5877A/
G5877A: CPEO
G5846A
T5843C/ T5843C: FSGS
G5875A
A5874G:
Exercise
Intolerance

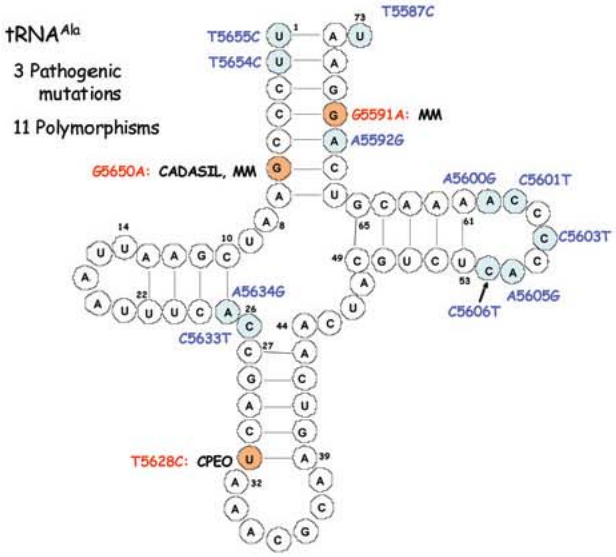

tRNA Pro
4 Pathogenic mutations
9 Polymorphisms
T15965C:
PD
A15968G
A16017G
A15970G
A15972G
G15978A
T15974C
A16003G
C16000T
A15983G
A16002G: MM
C15995T: EM/cystic fibrosis
G15990A: MM

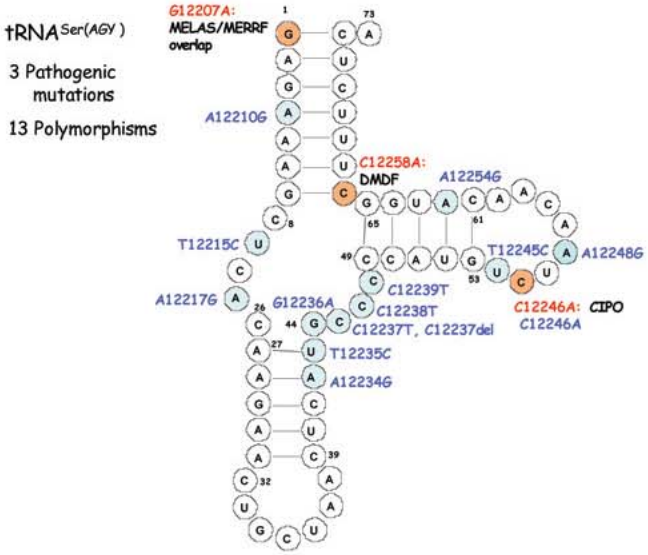

tRNA Asn
4 Pathogenic mutations
7 Polymorphisms
T5715C
A5673G
T5705C
T5675A, T5675C
T5711C
G5704A
C5703T: CPEO/MM
C5698T: CPEO/MM
A5692G: CPEO
A5693G: Lethal EM

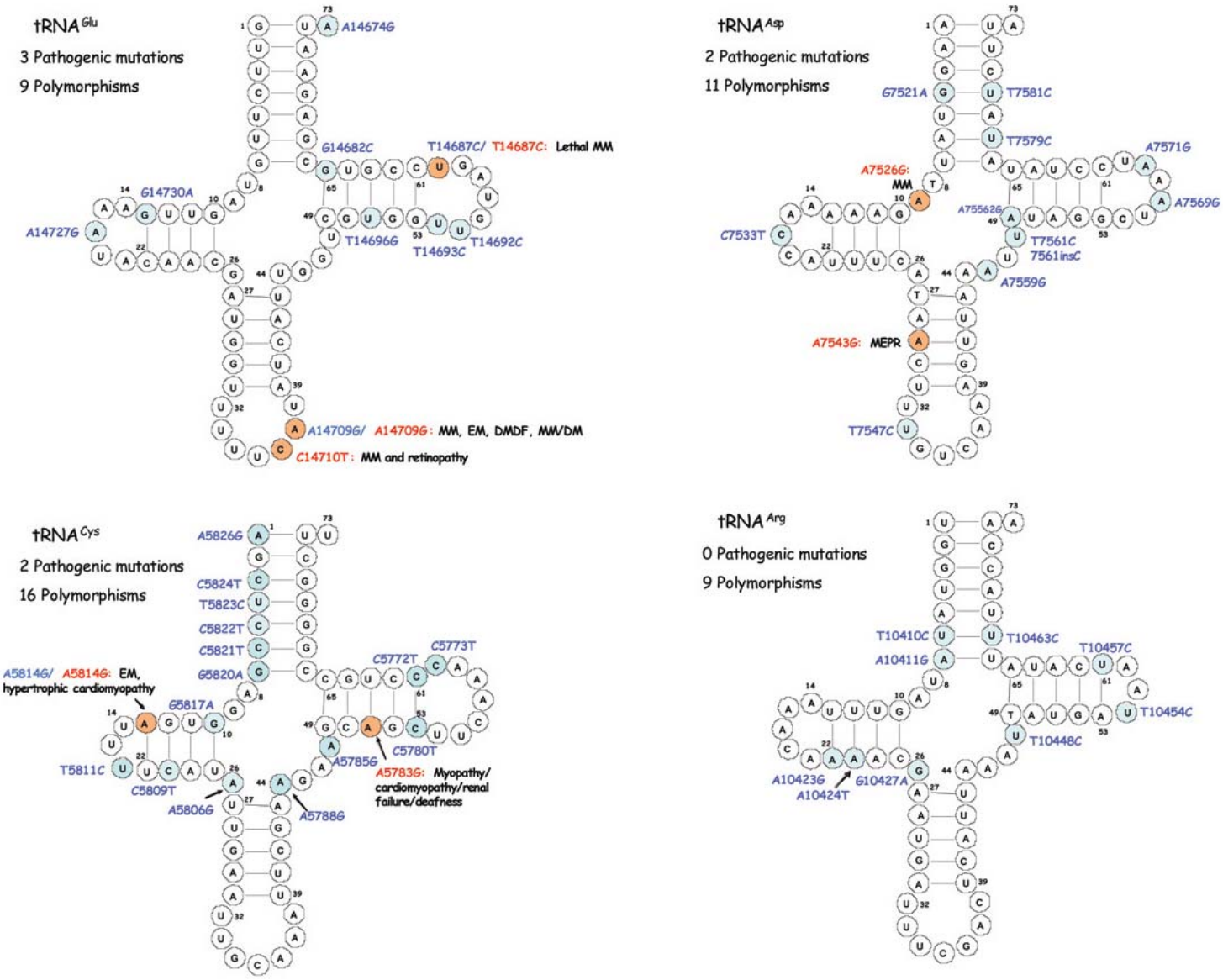

tRNA Gln
3 Pathogenic mutations
15 Polymorphisms
C4332T: atypical MELAS
T4395C
G4335A
A4336G/ A4336G: ADPD
T4342C
G4394A
T4343C
A4386G
T4388C
G4393T
G4345A
A4384G
G4392A
A4371G
A4370G
4370insT: MM
A4363G


tRNA Met
3 Pathogenic mutations
6 Polymorphisms
A4469G
T4409C: MM
T4454A, T4454C
T4452C
G4450A: MM
T4418C
A4435G: LOHN Modulator
A4435G


tRNA His
3 Pathogenic mutations
11 Polymorphisms
A12142G
A12200G
G12192A
G12192A: MICM, LHON modulator
G12147A:
MERRF/MELAS overlap, Encephalopathy
C12153T
T12189C
T12188C
G12183A:
SNHL
T12175C
T12173A
T12166C
A12172G
A12171G


tRNA Ala
3 Pathogenic mutations
11 Polymorphisms
T5587C
T5655C
T5654C
G5591A: MM
A5592G
G5650A: CADASIL, MM
A5600G
C5601T
C5603T
A5634G
A5605G
C5606T
C5633T
T5628C: CPEO


tRNA Ser(AGY)
3 Pathogenic mutations
13 Polymorphisms
G12207A:
MELAS/MERRF overlap
A12210G
C12258A:
DMDF
A12254G
T12215C
T12245C
A12248G
C12239T
A12217G
G12236A
C12238T
C12246A: CIPO
C12237T, C12237del
C12246A
T12235C
A12234G



Pathologies are abbreviated as follows:

A, ataxia;ADPD, Alzeimer's disease and Parkinsons's disease; AISA, acquired idiopatic sideroblastic anemia; AMDF, Ataxia, mental deterioration, deafness; AP, asymptomatic proteinouria; BD, bipolar disorder; CIPO, chronic intestinal pseudo-obstruction with myopathy; CPEO, chronic progressive external ophthalmoplegia; DCM, dilated cardiomyopathy; DEAF, maternally inherited deafness or aminoglycoside-induced deafness; DEMCHO, progressive dementia and chorea; DM, diabetes mellitius; DMDF, diabetes mellitius and deafness; ECM, encephalo-cardio-myopathy; EEM, encephaloenteromyopathy; EI, exercise intolerance; EM, encephalomyopathy; FICP, fatal infantile cardiomyopathy plus a Melas-associated cardiomyopathy; FSGS, focal segment glomerulosclerosis; GDM, gestational diabetes mellitus; KSS, Kearns-Sayre syndrome; LA, lactic acidosis; LHON, Leber hereditary optic neuropathy; LIMM, lethal infantile mitochondrial myopathy; LS, Leigh syndrome; MELAS, mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes; MEPR, myoclonic epilepsy and psychomotor regression; MERRF, myoclonic epilepsy and ragged red muscle fibers; MERME, MERRF/MELAS overlap; MHCM, maternally inherited hypertrophic cardiomyopathy; MICM, maternally inherited diabetes; MIDM, maternal, inherited cardiomyopathy; MM, mitochondrial myopathy; MMC, maternal myopathy and cardiomyopathy; MNGIE, mitochondrial neurogastrointestinal encephalopathy; MS, multiple sclerosis; MSL, multiple symmetric lipomatosis; PE , progressive encephalopathy; PEM, progressive encephalomyopathy; PM, progressive myopathy; PEO, progressive external ophthalmoplegia; SM, skeletal myopathy; SNHL, sensorineural hearing loss.

RÉSUMÉ DÉVELOPPÉ EN FRANÇAIS

Introduction et objectifs

Les mutations de l'ADN mitochondrial (l'ADNmt) sont une cause importante de maladies humaines, qui affectent essentiellement le tissu nerveux et musculaire. Parmi plusieurs mutations et réarrangements répertoriés, plus de 170 sont localisées dans les gènes codant pour les ARN de transfert (l'ARNt). Il est supposé que ces mutations entraînent une altération de fonctions d'ARNt, ce qui cause des déficiences de la synthèse protéique dans l'organite. Les gènes mitochondriaux codant pour l'ARNt-Leu(UUR) et l'ARNt-Lys représentent les vrais «hot-spots» de mutations, parmi les quelles on retrouve celles associées avec les syndromes MELAS (myopathie, encéphalopathie, acidose lactique, accidents vasculaires cérébraux) et MERRF (myoclonie, épilepsie, et fibres rouges loqueteuses), respectivement. La fréquence de ces maladies est assez importante, >1:10,000. Pourtant, à ce jour il n'existe pas de traitement efficace pour ces maladies, ainsi toutes les possibilités sont à envisager, dont la thérapie génique. La livraison du matériel génétique dans la mitochondrie à présent reste un problème non-résolu pour les systèmes *in vivo*. Les autres approches décrites peuvent être regroupées en deux classes: (a) la stratégie "anti-génomique", qui vise à inhiber la réplication de l'ADNmt muté en favorisant celle de génomes normaux (toujours présents dans le contexte hétéroplasmique); (b) l'expression "allotopique" du gène d'intérêt dans le noyau et l'adressage de son produit vers et dans la mitochondrie. Cette dernière approche se base sur le fait que les mitochondries importent des centaines de macromolécules du cytoplasme, dont les plus étudiées sont des protéines. En plus de protéines, plusieurs types de petits ARN non-codant sont également importés par l'organite. Cette dernière voie présente un avantage évident pour modéliser les thérapies géniques, car c'est l'unique voie naturelle connue d'adressage d'acides nucléiques dans les mitochondries. Dans les cellules humaines, l'ARN ribosomal 5S est importé *in vivo*, en plus, les dérivants d'ARNt importés de levure, ainsi que plusieurs petits ARN synthétiques sélectionnés *in vitro* peuvent être importé d'une manière artificielle. Les travaux effectués dans les laboratoires d'accueil ont démontré que la mutation MERRF pouvait être partiellement complémenté par un ARNt spécifiquement construit exprimé dans le noyau et adressé dans les mitochondries ou il substitué l'ARNt-Lys non fonctionnel touché par la mutation. Après le succès de cette première tentative, il était intéressant d'investiguer si cette stratégie pourrait être généralisée. Les premières expériences ont été faites avec des versions d'ARNt-Lys (naturellement importé dans les mitochondries de levure et, artificiellement, dans les mitochondries humaines). Comme les mutations dans l'ARNt-Leu sont de loin les plus fréquentes, il était temps d'investir dans la construction des versions importables ayant cette identité. Ma thèse a donc été logiquement consacré à autre mutation de l'ADNmt humain très fréquente localisée dans ce gène, une substitution A3243G communément associé au syndrome MELAS.

Les objectifs suivants ont été formulés pour mes travaux de thèse :

1. Modéliser et construire des versions d'ARNt importables avec l'identité leucine et de les tester pour import et fonctionnement dans les mitochondries des cellules humaines;

2. Vérifier si la présence des ARNt recombinants dans cellules humaines ayant la mutation MELAS A3243G améliore leur fonctions mitochondriales touchées par la mutation.

Travaux effectuées

1. Construction des d'ARNt recombinants importés dans les mitochondrie et portant l'identité leucine.

Pour avoir un potentiel thérapeutique, c'est-à-dire fonctionnellement substituer l'ARNt-Leu mitochondrial portant la mutation MELAS, les ARNt recombinants devraient pouvoir être aminoacylé par la leucine et importés dans mitochondrie. Puisque les dérivants des trois ARNt-Lys de *Saccharomyces cerevisiae* (tRK1, tRK2, tRK3) se sont révélés importables dans mitochondries humaines *in vitro* ainsi que *in vivo*, ils ont été choisis comme base de toutes les nouvelles versions. Dans les cellules humaines il existe deux leucyl-ARNt-synthétases, une cytoplasmique et l'autre mitochondriale. Pour que cette dernière puisse reconnaître son substrat naturel (l'ARNt-Leu mitochondrial), l'ARNt doit contenir la base discriminatrice A73, l'adénine dans la position 14 de D-loop de l'ARNt et, enfin, l'anticodon leucine (UAA pour l'ARNt-Leu(UUR) mitochondrial). Ces éléments ont été intégrés dans séquences de tRK1, tRK2 et tRK3 par mutagenèse dirigée. Il a été démontré que, outre la baisse de niveau d'aminoacylation de l'ARNt-Leu, la mutation A3243G mène à l'hypomodification d'uridine dans position «wobble » d'anticodon, empêchant à l'ARNt de reconnaître les codons UUG d'ARN messager. Pour palier à ce problème, j'ai construit une autre série d'ARNt recombinants contenant l'anticodon CAA (permettant la reconnaissance de codons UUG sans modification). Finalement j'ai testé si l'ARNt-Leu(UUR) mitochondrial humain peut être importé dans mitochondries du cytoplasme. Plusieurs nucléotides de sa boucle variable ont était substitués par nucléotides correspondants de V-loop de *Macaca mulatta* pour distinguer cet ARNt recombinant de l'ARNt-Leu(UUR) naturellement présent dans cellule. Par conséquent, j'ai obtenu 7 versions différentes des ARNt recombinants : tRK1UAA, tRK1CAA, tRK2UAA, tRK2CAA, tRK3UAA, tRK3CAA et ARNt-Leu(UUR)-V.

2. Analyse d'aminoacylation des ARNt recombinants et de leurs capacités d'import dans mitochondries des cellules humaines.

Pour vérifier la capacité des ARNt recombinants d'être chargé avec leucine, j'ai purifié l'enzyme recombinante de leucyl-ARNt-synthétase à partir d'une culture bactérienne. J'ai démontré que des transcrits synthétiques (obtenus pas l'ARN polymérase T7) des ARNt recombinants peuvent être aminoacylés avec leucine *in vitro* avec l'efficacité proche à celle du transcrit de l'ARNt-Leu(UUR) sauvage.

L'importation des transcrits T7 des ARNt recombinants a été démontrée par la suite *in vitro*, dans mitochondries humaines isolées, ainsi que *in vivo*, dans mitochondries des cellules humaines en culture. L'efficacité d'import de différents ARNt recombinants *in vivo* a été estimée par rapport à l'efficacité d'import des T7-transcrits de tRK1 ou tRK3 sans mutations et variait, en dépendant de la version recombinante, entre 20 et 100%. Les meilleurs versions ont été introduites dans cellules portant la mutation A3243G/MELAS (cellules dites "cybrides") pour tester leur effet sur les fonctions mitochondriales affectées par la mutation dans l'ADNmt.

3. Analyse de l'effet curatif de l'expression transitoire des ARNt recombinants dans les cellules cybrides MELAS

Les premières expériences de transgénèse on été effectuées en utilisant une transfection de cellules cybrides par des ARNt recombinants synthétiques. Pourtant aucun effet sur la synthèse protéique mitochondriale n'a pas été détecté, de plus que l'analyse d'aminoacylation n'a pas démontré de présence significative des formes aminoacylées de ces transcrits. Ce dernier résultat peut être expliqué par l'absence éventuelle de modifications post-transcriptionelles nécessaires pour aminoacylation efficace *in vivo*. Pour palier à ce problème, la stratégie de transfection transitoire avec les plasmides codants pour les différents ARNt recombinants a été adopté. Dans ce système, les ARNt recombinants ont été exprimés à partir d'un promoteur interne ou externe pour ARN polymérase III et ont été détectables dans cellules pendant 2-3 jours ou plus si plusieurs transfections successives on été effectuées. La présence prolongée de certains ARNt recombinants dans cellules cybrides MELAS résultait à une augmentation de la sous unité mitochondriale COX II de complexe IV de la chaîne respiratoire connue d'être affectée par la mutation. L'augmentation maximale a été obtenue avec la version tRK2CAA. Vu le caractère transitoire de transfection, l'effet observé était également transitoire, néanmoins ce système rapide et efficace a permis de tester plusieurs versions des ARNt recombinants avant de passer à la transfection stable.

4. Analyse de l'effet curatif de l'expression stable des ARNt recombinants dans les cellules cybrides MELAS

Plusieurs approches ont été utilisées pour obtenir les lignées des cellules cybrides MELAS exprimant les ARNt recombinants d'une manière stable (différents agents lipidiques et différents plasmides pour délivrer le transgène). Pourtant, même si dans certains cas l'expression a pu être détecté, elle s'avérait instable et disparaissait pendent la procédure du clonage de lignés de cellules. Après plusieurs fiascos successifs, la stratégie d'infection virale en utilisant lentivirus a été adopté. En utilisant le système de tranfection lentiviral, une lignée stable exprimant d'une manière efficace la version tRK1CAA a été obtenue. L'expression stable de tRK1CAA dans cellules cybrides menait à une amélioration significative de leur fonctions mitochondriales. Notamment la restitution partielle de synthèse protéique mitochondriale, l'augmentation de niveau des plusieurs protéines mitochondriales de la chaîne respiratoire comme COXII et NDI et l'amélioration de respiration cellulaire ont été observées dans la lignée trangénique.

Conclusions et perspectives

1. La possibilité de changer la spécificité d'aminoacylation de l'ARNt tout en gardant son habilité d'être importé dans les mitochondries des cellules humaines a été démontrée. Plusieurs versions importables d'ARNt ayant une identité leucine ont été ainsi construites.

2. L'expression de ces ARNt recombinants dans cellules cybrides contenant la mutation A3243G localisé dans le gène d'ARNt-Leu et associé au syndrome MELAS mène à une amélioration de leur fonctions mitochondriales affectées par la mutation.

Les résultats de mon travail de thèse ensemble avec des autres résultats obtenus dans le laboratoire d'accueil démontrent le potentiel important d'utilisation de la voie d'import des ARN dans mitochondries pour le développement de thérapie génique des maladies

mitochondriales. Toutefois les objectifs suivantes restent encore à atteindre : trouver des conditions permettant l'expression plus efficace des gènes codant pour différents ARNt recombinants dans les cellules humaines; obtenir un effet plus prononcé sur la fonction mitochondriale en rendant l'expression, l'aminoacylation et/ou l'importation des ARNt recombinants plus efficace; tester l'effet thérapeutique sur cellules dérivées directement des patients et mises en culture (cellules dites "primaires", *i.e.* fibroblastes ou myoblastes).

Liste de Publications et Communications

Publications

1. Zeharia, A., Shaag, A., Pappo, O., Mager-Heckel, A.M., Saada, A., Beinat, M., Karicheva, O., Mandel, H., Ofek, N., Segel, R., Marom, D., Rotig, A., Tarassov, I., and Elpeleg, O. (2009) Acute infantile liver failure due to mutations in TRMU gene. ***Am J Hum Genet*, 85**, 401-407.

2. Tarassov, I., Kamenski, P., Kolesnikova, O., Karicheva, O., Martin, R.P., Krasheninnikov, I.A. and Entelis, N. (2007) Import of nuclear DNA-encoded RNAs into mitochondria and mitochondrial translation. ***Cell cycle,* 6**, 2473-2477.

3. Olga Z. Karicheva, Olga A. Kolesnikova, Tom Schirtz, Mikhail Y. Vysokikh, Anne-Marie Heckel-Mager, Anne Lombès, Igor A. Krasheninnikov, Nina S. Entelis, Robert Pierre Martin, and Ivan A. Tarassov "Correction of the consequences of mitochondrial 3243A>G mutation in the *MT-TL1* gene causing the MELAS syndrome by tRNA import into mitochondria" *(submitted).*

Communications

1. Karicheva O.Z., Kolesnikova O.A., Schirtz T., Vysokikh M.Y., Heckel-Mager A.-M., Krasheninnikov I.A., Entelis N.S., Martin R.P., and Tarassov I.A **(2009)** tRNA-import into mitochondria: looking for anti-MELAS therapy. Proceedings of 2nd FEBS Advanced Lecture Course "Mitochondria in Life, Death and Disease".

2. Karicheva Olga, Schirtz Tom, Lombés Anne, Entelis Nina, Martin Robert, Krasheninnikov Igor, Tarassov Ivan, Kolesnikova Olga **(2009)** Modélisation de la thérapie génique du syndrome MELAS causé par une mutation de l'ADN mitochondrial. Congrès Meetochondrie, La Grande Motte, France

3. Karicheva O.Z., Kolesnikova O.A., Heckel-Mager A.-M., Krasheninnikov I.A., Entelis N.S., Martin R.P., and Tarassov I.A. **(2007)** Looking for anti-MELAS therapy: tRNA-import into mitochondria. Proceedings of 22nd international tRNA workshop.

4. Karicheva O.Z., Kolesnikova O.A., Heckel-Mager A.-M., Krasheninnikov I.A., Entelis N.S., Martin R.P., and Tarassov I.A. **(2007)** tRNA-import into mitochondria as a tool for gene therapy of MELAS. Proceedings of 5th Scientific Symposium "The new RNA frontiers", Colmar, France.

5. O.Z. Karicheva**,** O.A. Kolesnikova, I.A. Krasheninnikov, I.A. Tarassov **(2006)** tRNA import into mitochondria as a strategy of treatment of MELAS disease. Proceedings of Lomonosov Conference for young Scientists.

6. O. Karicheva, O. Kolesnikova **(2005)** Changing of yeast mitochondrial lysine tRNA anticodon to UAA or UAG does not prevent its import into human mitochondria. Proceedings of Puschino Conference for young Scientists.

7. Tarassov I, Karicheva O, Entelis N, Mager-Hackel AM, Addis V, Martin RP, Kolesnikova O. **(2005)** Directed tRNA import into human mitochondria as a way to complement pathogentic mutations in mtDNA. Proceedings of 21st international tRNA workshop.